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**Morphological and Molecular Systematics of the Genus
Miscanthus Anderss.**

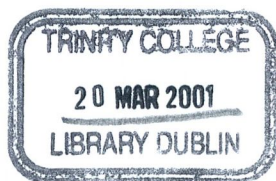
by

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2001 2001

A Thesis submitted to the University of Dublin, Trinity College for
the Degree of Doctor of Philosophy.

January 2001

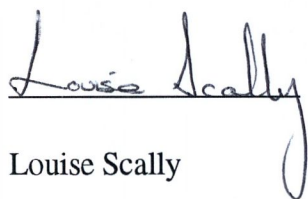


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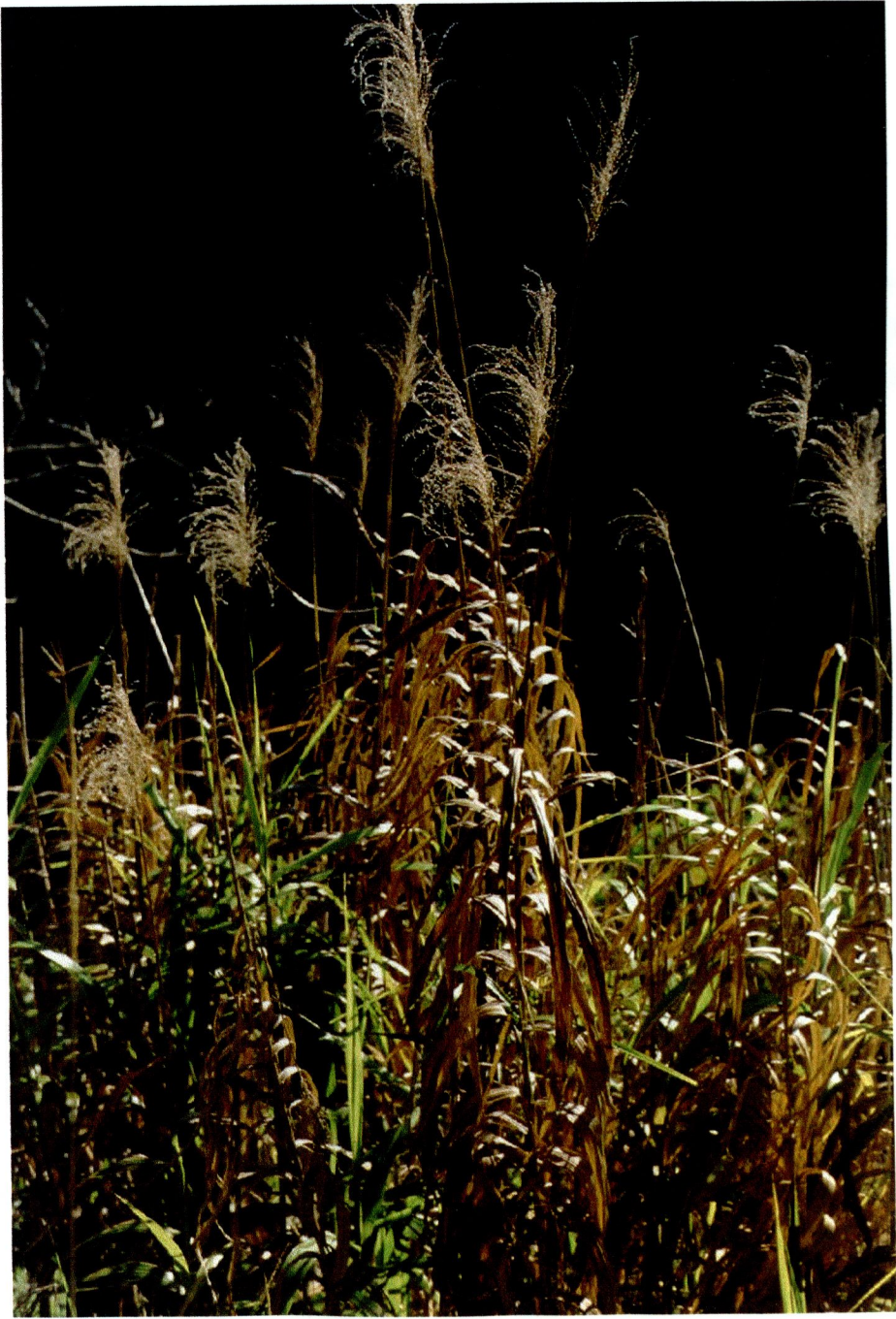
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A handwritten signature in black ink, appearing to read "Louise Scally", written over a horizontal line. The signature is cursive and fluid, with a long, sweeping tail on the final letter.

Louise Scally



Miscanthus floridulus (Labill.) Warb., Mt Aorai, Tahiti. Photo courtesy S. Waldren

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Summary

Increasing fuel costs and a depletion of the earth's natural energy resources have lead to a global search for alternative sources of renewable energy. In response, the "European *Miscanthus* network" funded by the European Agro-Industrial Research programme (AIR) of the European Union's Directorate General (DG) VI was established. The aim of this network was to establish the potential of certain species and cultivars of the genus *Miscanthus* as a potential source of renewable biomass for energy and fibre. During the initial stages of this research it became clear that the classification of the genus *Miscanthus* was confusing and that it was necessary to establish a reliable classification system and a method of identifying individual accessions of the genus.

The aims of this project were, therefore, (1) to characterise a general research collection of *Miscanthus* which had been obtained by the investigating team for research into its potential as a biomass crop. (2) Use a multivariate and phenetic approach with morphological data to investigate inter-relationships between the species of the genus. (3) To compare the utility of DNA fingerprinting methods (Random Amplified Polymorphic DNA and Amplified Fragment Length Polymorphisms) for detecting and describing molecular variation in *Miscanthus*. (4) Compare the ability of morphological and molecular methods to produce useful characters for the identification of the species and cultivars. (5) investigate the parentage of *Miscanthus* x *giganteus*, a sterile hybrid which has demonstrated its potential as a suitable source of Biomass. (6) Establish phylogenetic relationships of the species, particularly of *M. sinensis*, *M. sacchariflorus* and *M. floridulus*.

Current classifications of the genus *Miscanthus* generally state that the *Miscanthus* is comprised of about fourteen species, divided into four sections, *Miscanthus*, *Triarrhena*, *Kariyasua* and *Diandra*. Most of the species are native to south east Asia, China, Japan and Polynesia. However four species are confined to Africa and are sometimes placed in a separate genus, *Miscanthidium*. *Miscanthus* belongs to the tribe Andropogoneae subtribe Saccharinae Griseb. There are twelve other genera within the subtribe Saccharinae. Species of *Miscanthus* are known to hybridise both within itself and with these related genera.

Morphological analysis of the genus carried out on herbarium material demonstrated that although key characters were useful as an aid to the identification of some of the species within the genus, they were unreliable due to the wide range of morphological variation within certain species, especially *M. sinensis*. In addition morphological analysis of the cultivars did not provide any useful characters which would aid in their identification. Previous classifications of the genus were shown to be unworkable as they failed to examine the range of variation between the species.

Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphisms (AFLP) were carried out on DNA extracted by a hot CTAB method from our living collection of *Miscanthus* at Trinity College Botanic Garden. RAPD analysis was carried out using the Polymerase Chain Reaction (PCR) with primers from the Operon Technologies primer kit A followed by agarose gel electrophoresis. The analysis produced highly reproducible results and allowed the identification of specific markers which are extremely useful in the characterisation of the cultivar collection. However, RAPD became too variable at the species level and therefore its utility for species identification within *Miscanthus* was unsatisfactory. AFLP analysis was carried out by amplification of restriction fragments produced following restriction digestion of the DNA with the restriction endonucleases *EcoRI* and *MseI*. These fragments were then visualised by autoradiography following polyacrylamide gel electrophoresis. AFLP proved to be much more successful than RAPD at the species level and proved suitable for differentiating between the various species and cultivars of *Miscanthus*. In addition AFLP was successful in providing support for the proposed infrageneric sections of *Miscanthus*, with the exception of section *Diandra*.

DNA sequencing of the Internal Transcribed Spacer (ITS) region of nuclear ribosomal DNA was used to examine the phylogeny of the genus. *Miscanthus sinensis* was shown to comprise a monophyletic group, distinct from other closely related genera. Within the genus, section *Miscanthus*, which comprises the Asian species of *Miscanthus*, formed a monophyletic group, included with this group are the cultivars of *Miscanthus*. *Miscanthus sacchariflorus*, representing section *Triarrhena* was clearly distinct and supported as a sister group to the core *Miscanthus* group. Section *Kariyasua* formed a monophyletic group, distinct but sister to section *Triarrhena*. Section *Diandra* was not supported, the species of this section being more closely allied to the African *Miscanthidium* species.

This research was successful in resolving the confusion surrounding the classification of *Miscanthus*. It has also provided methods for identifying genotypes being investigated for their biomass potential and provided markers that will be useful to breeders in the future. It has provided further evidence to confirm the hypothesis that *M. x giganteus* is a hybrid involving *M. sinensis* and *M. sacchariflorus*. It has provided a clearer picture of the phylogeny of the genus, especially in relation to *M. floridulus* which was shown to be a distinct species related to but separate from *M. sinensis*.

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CHAPTER 1

GENERAL INTRODUCTION

Biomass energy is increasingly becoming a key element in global plans for increased use of sustainable energy and currently supplies approximately 14% of the world's energy (Hall *et al.*, 1997). It is the fourth largest source of energy in the world, with developing countries deriving as much as 38% of their total energy requirement from biomass. There is a wide range of variation among the areas of biomass use in the world, and Europe is currently one of the lowest utilisers of biomass energy worldwide.

In 1992 the “European *Miscanthus* Network”, funded by the European Agro-Industrial Research programme (AIR) of the European Union's Directorate General (DG) VI, was set up. The aim of this network was to investigate the potential of a particular genus of grasses for their potential as a biomass crop, in an attempt to bridge the gap between biomass production and utilisation in Europe and the rest of the world. The main taxon chosen for this investigation was *Miscanthus x giganteus*, a woody rhizomatous C4 grass with an estimated productive life of 10-15 years, the stems and leaves of this species can be harvested annually.

Limited field trials had already been established in 1989 to investigate the potential of *Miscanthus* as a biomass crop under a European biomass programme (Jones *et al.*, 1994). Based on the results of this investigation intensive field trials of *M. x giganteus* (and a limited number of other *Miscanthus* species) were set up in 1993. These field trials, which were conducted in the British Isles and northern, southern and south-eastern Europe, set out to examine a number of aspects of the growth potential of *Miscanthus* under the various climatic conditions pertaining to Europe. Experiments conducted in tandem with the field trials investigated factors including the response of the *Miscanthus* stands to differing fertiliser regimes and the response of *Miscanthus* leaf expansion to environmental variables. The response of leaf extension rate among different varieties of *Miscanthus* and the level of cold tolerance of *Miscanthus* rhizomes was also investigated (Clifton-Brown, 1997; Jones & Walsh, 2000).

Most of this research targeted only one taxon of *Miscanthus*, *M. x giganteus* Greef et Deu. This taxon was originally collected in Yokohama, Japan by Askel Olson in 1935 and cultivated in Denmark (Nielsen, 1990). By the late 1970's it had been introduced to the rest

of Europe where its potential as a biomass crop was first observed (Greef *et al.*, 1997). However, *M. sinensis*, thought to be one of the parents of *M. x giganteus* had already been investigated as a potential biomass crop in tropical and sub-tropical regions (Ito, 1990). *Miscanthus sinensis* is distributed from southern Hokkaido to Okinawa, and from ancient times has provided a source of fodder and roofing material.

To facilitate much of this research, collections of many species, cultivars and varieties of *Miscanthus* were acquired by one of the investigating teams at the University of Dublin, Trinity College. The collection came from various botanic gardens, universities, private collectors and also from the horticultural community where *Miscanthus* is cultivated as an ornamental. While establishing this collection it became clear that the taxonomy of the genus was confused and an accurate classification of the genus was required. In addition to a clear classification of the species within *Miscanthus* it was also necessary to establish a method of easily and quickly differentiating between the many varieties and cultivars being examined.

One of the main reasons for the interest in *Miscanthus* as a source of biomass, is due to its C4 photosynthetic pathway. Plants assimilate carbon from the atmospheric CO₂ reservoir by one of three photosynthetic pathways. These pathways, referred to as C3, C4 and CAM (Crassulacean Acid Metabolism), represent physiological adaptations to variable climatic and atmospheric conditions (Jacobs *et al.*, 1999). Plants with C3 metabolism dominate terrestrial ecosystems and account for 85% of all plant species. CAM metabolism, on the other hand, is largely confined to succulent plants including Cacti and is a metabolism confined to plants growing in areas with extreme environmental conditions such as high temperature and drought. Plants with C4 photosynthetic systems are generally confined to tropical and sub-tropical regions (Jones, 1985). C4 metabolism is present in a number of monocots and diverse dicots, but the majority of C4 global biomass is represented by graminoids, especially grasses growing in hot, arid habitats (Ehleringer *et al.*, 1997). C4 plants are known to be considerably more efficient at converting intercepted radiation into dry matter (Monteith, 1978) and also make more efficient use of nitrogen (Ozturk *et al.*, 1981), thereby increasing the overall efficiency of photosynthesis. However, C4 species are normally out-competed by C3 plants at mean temperatures below 25°C and in tropical and sub-tropical regions there is a transition from C4 to C3 with increasing altitude between 2000 and 3000 m (Tieszen *et al.* 1979). Plants with C4 metabolism also form a transition gradient in Japan where the forest stage transcends into a *Miscanthus* stage which is followed in turn by an *Imperata* stage (Ito, 1990). *Miscanthus* is more commonly found in tropical and sub-tropical regions, but also naturally occurs in climatic conditions similar to Europe. Despite its C4 metabolism it has also been recorded at altitudes exceeding 3000 m, as indicated by collection information on herbarium specimen labels.

The genus *Miscanthus* is a member of the family Poaceae and of the tribe Andropogoneae, subtribe Saccharinae. Currently it is estimated that *Miscanthus* contains about fourteen species (Hodkinson *et al.*, 1997). *Miscanthus* is considered to belong to the most primitive group in the Andropogoneae (Clayton & Renvoize, 1986). The exact origin of most grasses is obscure, because any link which existed with the rest of the monocotyledons is now extinct (Clayton & Renvoize, 1986). The sister group of grasses is now believed to be the monotypic family Joinvilleaceae (Kellogg & Linder, 1995; Doyle *et al* 1992). The main centre of speciation of *Miscanthus* is south-east Asia (Lee, 1964b; Hayashi *et al.*, 1981) where it forms extensive grassland communities. The genus is currently divided into four sections, *Triarrhena*, *Miscanthus*, *Kariyasua* and *Diandra* (see chapter two for more detail). The current distribution of the genus ranges from south-east Asia, through China, Japan, Siberia and into Polynesia. Section *Miscanthus* is found throughout south-east Asia, China, Japan and Polynesia. Section *Triarrhena* is distributed across China, Japan, Korea and Siberia. Section *Kariyasua* occurs in limited areas of the temperate zones of Japan and Korea. Section *Diandra* is generally confined to the higher altitudes of western China and east India.

An approach to the classification of any biological group can rely on either its evolutionary history or on its phenetic similarities with other members of the group, with no inference to how these similarities evolved. Before Darwin's publication of "*The Origin of species*" in 1859 (Darwin, 1859), species were believed to be fixed *i.e.* they remained morphologically the same and their morphology did not change over time or ecological barriers. Darwin's hypothesis that they were not fixed, but capable of evolving into different species was largely ignored at the time because he was not able to present a satisfactory hypothesis as to how this evolution occurred. Although some of Darwin's ideas had been suggested by earlier scientists, they were fundamentally different. Lamarck for example postulated that species changed over time into new species, but lineages did not branch and extinction did not occur. Neither did he recognise the theory of natural selection, simply believing that some unknown mechanism within the organism caused it to produce slightly different progeny, which may in time result in the formation of a new species. It was not until the 1930s, when Darwin's hypotheses were put together with Mendel's theories on inheritance, that the concept of evolution became accepted. Before this time, classifications were simply based on overall similarities between individuals, the field known as phenetics today. The practice of classifying individuals or groups of individuals based on overall similarity was revolutionised by Carolus Linnaeus when he introduced the binomial system of naming organisms published in *Species Plantarum* in 1753. Debate still surrounds the correct manner of classification of species and the even more fundamental question of, what exactly is a species, has arisen from this. Currently there are five major species concepts considered; the biological (Mayr, 1942), evolutionary (Simpson, 1961), cladistic (Hennig, 1966), recognition (Paterson, 1985) and phylogenetic (Cracraft, 1983) species concepts. More

recently Kornet & McAllister, (1993), have devised the composite species concept which takes elements of both cladistics and phylogenetics. The biological species as defined by Mayr, (1942) states that “species are groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups”. One of the major drawbacks of the biological species concept was that it could only apply to bisexual species and could not be applied to uniparental organisms. The evolutionary concept, on the other hand, states that “a species is a single lineage of ancestor-descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate” (Wiley, 1978). This system addresses some of the short-comings of the biological species concept in that it recognises that interbreeding is a fundamental aspect of bisexual organisms, while at the same time introduces the concept of ancestry being an important aspect of what defines a species.

Following the publication of *Grundzuge einer theorie der phylogetischen systematik* in 1950 (Hennig, 1950) and its subsequent translation into English in 1966 (Hennig, 1966) a third system of classification became popular. The third concept, the cladistic species concept, although related to the evolutionary concept, differs in that it delimits a species based on Hennig’s deviation rule. This rule states that species are delimited by a speciation event where the ancestral species ceases to exist after a split. Thus, after such a speciation event two new sister groups will be formed, one of which may resemble the ancestral species. This system, known as cladistics, is based on the principle that all living entities have evolved from a common ancestor and that any classification ignoring the evolutionary process does not have strong philosophical justification.

The recognition concept deviated from the previous three systems in that it defined a species as “that most inclusive of individual biparental organisms which share a common fertilisation event” (Paterson, 1985). This theory differed in that it suggested genetic isolation was a result of direct selection and not geographic isolation. The phylogenetic species concept differs considerably from the previous four concepts in that it examines the evolutionary process through which an organism has evolved and in this respect defines a species as “the smallest diagnosable cluster of organisms, within which there is a parental pattern of ancestry or descent” (Cracraft, 1983). One of the underlying concepts of the phylogenetic system is that of homology and it is also the concept which has led to much debate. Homology is often defined as “the relation which characterises monophyletic groups” (Patterson, 1982), although in the same paper Patterson also describes four alternative, but in his opinion, incorrect methods of defining homology. Another problem with homology is rooted in the ascertainment of homology. For example, if a character appears to be similar in two separate individuals we must have a method of determining whether or not it is homologous and not just a morphologically similar structure. If non-homologous characters

are inadvertently used in a phylogenetic classification paraphyletic groups can result, producing an incorrect phylogenetic classification. However the advocates of phylogenetics suggest that this problem can be avoided by using the congruence test. Congruence is the most decisive test in both morphological and molecular data sets in that it separates characters useful in systematics from those which are not. Congruent characters are those that specify compatible relationships *i.e.* they are homologous. If the data from morphological or molecular evidence passes the congruence test, one can be reasonably assured of the homology of the characters used (Siebert, 1992).

The advocates of phenetics (Sokal & Sneath, 1963; Sneath & Sokal, 1973) argued that while the phylogenetic basis for classifications was worthwhile it was probably unobtainable. They therefore proposed an extension of Linneaus's original binomial system of classification, whereby large numbers of similarities between organisms, or Operational Taxonomic Units (OTU's), would be scored and that relationships between OTU's would be expressed as an index of similarity. On the other hand, those that support the phylogenetic system of classification believe that genealogical classification is imperative and therefore procedures that permit it must be found (Patterson, 1987). With both of these arguments in mind this project attempted to create a phylogenetic system of classification for *Miscanthus*, and if this proved unobtainable, to create a phenetic system.

Traditional approaches to either phenetic or phylogenetic classifications generally relied on the analysis of morphological characters. In recent years molecular characters have become increasingly popular, especially in phylogenetic classifications. *Miscanthus* has traditionally been classified by morphological descriptive analysis of the whole plant rather than by phenetic or phylogenetic analysis. As our main aim was to produce a classification system which could be utilised by other researchers investigating *Miscanthus* as a biomass crop, a phenetic system of classification may have served this purpose. However, it was also decided to investigate the phylogeny of the genus in the hope that a more meaningful classification would result, as a phylogenetic system also has predictive properties and it is important to identify the gene pool available for breeding purposes.

Traditional approaches to the classification of all species have involved morphological analysis of the taxa of interest, and this approach is still commonly utilised today. The advantages of morphological analyses are that they are relatively inexpensive and require little or no equipment to carry out the analyses. In addition, herbarium material can often be obtained for examination, making expensive and time consuming collecting trips unnecessary. In theory, large data sets can be produced by the scoring of an array of morphological characters. However, in some cases the taxa under consideration do not lend themselves to morphological analysis, and there may not be sufficient morphological

characters suitable for scoring. For these reasons other types of characters are often looked to as an alternative. Until recent years many studies used chemical and cytological data to examine taxonomic relationships where morphological evidence was unsuitable.

DNA sequence data is often considered the optimum source of character information, because it allows direct examination of the basic units of information encoded in organisms. However, the production of sequence information can be costly, and depending on the particular taxonomic questions to be answered, it is not always the most efficient method. There are many other sources of molecular data which can be examined and many of these may provide sufficient information for the analysis of the taxa of interest. Most of these methods are based on a suite of techniques known as DNA fingerprinting. These techniques, which include Restriction Fragment Length Polymorphisms (RFLP), Amplified Fragment Length Polymorphisms (AFLP), Random Amplified Fragment Length Polymorphisms (RAPD) and Microsatellites, among others, allow polymorphisms between different taxa to be observed and variation in these polymorphisms can be used to explain variation between different taxa at different taxonomic levels.

The aims of this thesis were to :

- ...Characterise a general research collection of *Miscanthus*.
- ...Use a multivariate and phenetic approach with morphological data to investigate the inter-relationships between the species
- ...Compare the utility of DNA fingerprinting methods (RAPD and AFLP) for detecting and describing molecular variation in *Miscanthus*
- ...Compare the ability of morphological and molecular methods to provide useful characters to delimit the species and cultivars of *Miscanthus*
- ...Revise the current classification of *Miscanthus* species
- ...Investigate the parentage of *M. x giganteus*
- ...Establish phylogenetic relationships of the species, particularly, *M. sinensis*, *M. sacchariflorus* and *M. floridulus*.

CHAPTER 2

MORPHOLOGICAL ANALYSIS OF THE GENUS *MISCANTHUS*

2.1 Introduction

Modern systems of classification follow one of two schools of thought, the phenetic system (Mayr, 1968) or the phylogenetic system (Hennig, 1966). The former is based on overall similarities between taxa after the examination of as many features as possible. This system does not attempt to ascertain how these similarities actually occurred in evolutionary time. The main aims of numerical taxonomy are to produce a classification system which is repeatable and objective (Sokal & Sneath, 1963). It is believed that in phenetic analysis the greater the number of characters scored the more robust the final analysis will be. If only a small number of characters are scored a biased classification may result, which may be totally altered if a different subset of characters from the same group were measured. Therefore by scoring as many characters as possible any ambiguous characters may be balanced out, giving a clearer picture of the phenotype (Ridley, 1996). Numerical taxonomy uses distance measures and cluster statistics to produce a hierarchical classification. One of the inherent problems with this method is that depending on the statistical method employed a totally different classification may be produced. Any classification will therefore only remain stable as long as the same statistical method is employed throughout.

Phylogenetic classifications group species according to their most recent ancestor. To this end, only shared derived characters are used to produce a classification, all other characters are ignored. Shared derived (synapomorphic) characters are a type of homologous character unique to a group and their most recent ancestor (Patterson, 1982). The main problem with this type of classification is that as new evidence appears phylogenetic trees need to be constantly revised. However, only one true ancestral lineage exists and phylogenetic analysis attempts to reconstruct this true lineage. However, phenetic analysis can provide an infinite number of classifications depending on the characters scored and the statistical method employed (Ridley, 1996).

In practice both methods have their merits and a third school of thought known as evolutionary taxonomy advocated by Mayer (1969) attempted to address this situation by using information from both phenetics and phylogenetics. Evolutionary taxonomists define groups by homologies and some elements of phenetics. It still allows some of the ambiguities of phenetics to remain. In addition, although only one possible classification results, it is not known which groups within the classification are phenetic and which are

phylogenetic (Ridley, 1996). To date the morphological analysis of *Miscanthus* has mainly involved a classical phenetic approach to its classification. This approach has been largely based on descriptive analysis of the whole plant rather than on the use of more modern (multivariate methods) of numerical taxonomy. The aim of this chapter is to produce a classification based on numerical taxonomy. This approach was taken rather than a phylogenetic approach because of the low number of synapomorphic morphological characters within *Miscanthus*. This is the only attempt I am aware of which has taken a multivariate approach to assess variation in the genus by numerical taxonomy.

Sivarajan (1991) claims that in their efforts to produce a meaningful classification system, many taxonomists neglect to make observations of the whole individual, seeking only to find characters within the individual which may assist in establishing differences between taxa. While this idea may hold true when examining relationships between more distantly related taxa, the use of key characters is still required to examine diversity between more closely related species. The result of this approach is evident in the confusion still surrounding the classification of *Miscanthus*, where most of the taxonomic treatments have been based on largely descriptive analysis of the whole plant. The first step in any meaningful assessment by numerical taxonomy lies in the number and choice of characters used. Sokal and Sneath (1963) state that as many characters as possible should be used and estimate that at least one hundred characters are necessary. By assessing such a large number of characters any ambiguous similarities between unrelated taxa should disappear, or become less significant. Obviously this is not always possible, as, depending on the taxa of interest, there may not be this number of measurable characters. Strong characters (e.g. characters not influenced by environmental conditions) are preferable to weak ones and those with a known genetic base are even more desirable. Notwithstanding, it is still important to attempt to score as many characters as possible. The actual choice of characters is generally decided after a wide range of individuals, representing the entire genus, have been examined. A character can be any feature which varies from one organism to another and may also include ratios derived from the characters measured (Sokal & Sneath, 1963). During the final analysis of the characters chosen any which are highly correlated can be eliminated from the analysis, thus reducing a possible form of bias. Obviously a degree of personal judgement is required in assessing whether the degree of correlation is meaningful or not. Current methods of phenetic analysis generally follow the Adansonian practice of equal weighting of characters. Adanson's system attributed equal weight to all characters irrespective of their real or inferred importance (Sivarajan, 1991).

The genus *Miscanthus* belongs to the tribe *Andropogoneae* subtribe *Saccharinae* Griseb. and was first described by N.J. Andersson (Andersson, 1856). There are twelve other genera within the subtribe *Saccharinae* (Clayton & Renvoize, 1986). Basic chromosome numbers of

seven (Church, 1929; Hunter, 1930), eight (Hunter, 1930), nine (Andulov, 1928) and ten (Tateoka, 1954) have been proposed for *Miscanthus*. Li *et al.* (1948) proposed nineteen as the basic number, and this has since become accepted as the basic number, supported by (Takizawa, 1952), Adati & Shiotani (1962) and Hirayoshi *et al.* (1959). Hirayoshi *et al.* (1959) suggested that *Miscanthus* is an amphidiploid resulting from a cross of species with $n = 10$ and $n = 9$ because both of these basic numbers have been found in the Andropogoneae. More recently Adati & Shiotani (1962) provided further evidence in support of this hypothesis.

The following section outlines the current classification of the subtribe Saccharinae and of the genus *Miscanthus*. The main characteristics of the Saccharinae are a terminal inflorescence (except *Eulaliopsis* and *Pogonatherum*) of solitary, digitate or panicle racemes with a tough or fragile rachis and usually slender internodes. Spikelets are paired (except *Polytrias*), one sessile and the other pedicelled but both pedicelled if the rachis is tough (Clayton & Renvoize, 1986). Saccharinae is regarded as the most primitive subtribe and is usually divided into two groups, one centred around *Saccharum* and the other centred around *Eulalia*. *Miscanthus* is a member of the group centred around *Saccharum* which also contains the genera *Spodiopogon*, *Eriochrysis* and *Imperata*. *Saccharum*, is itself often split into a number of genera and one of these, *Erianthus*, is based on the presence of awns. This division according to Clayton and Renvoize (1986) is completely artificial and should therefore be disregarded. The main characteristics of these groups are outlined below.

The subtribe Saccharinae Grieseb. (taken from Clayton and Renvoize, 1986)

Saccharum L.: A genus of stout tufted perennials, with linear leaf blades and a thick midrib. The panicles are large and dense with an elongate axis bearing numerous racemes on its branches. Distributed throughout the tropics and subtropics where it is also cultivated as a sugar crop. There are approximately 35-40 species contained within the genus. *Saccharum* readily hybridises with all other members of the Saccharinae.

Spodiopogon Trin.: *Spodiopogon* is a homogeneous genus characterised by prominent nerves raised into ridges on the lower glume. It is closely related to *Saccharum* but distinguished from it by its nervation. Nine species are included in the genus which has a widespread subtropical to temperate distribution.

Eriochrysis P. Beauv.: Distinguished from *Saccharum* by its compact rufous racemes and slightly dimorphic spikelets. The group contains seven species with an African and tropical American distribution.

Imperata Cyr.: Perennials with long creeping rhizomes and dense panicles with rather short racemes. Closely related to *Miscanthus* but distinguished from it by its contracted panicle and deficient floral parts (the palea is absent). There are eight species within the group, which is common throughout the tropics.

Miscanthus Anderss.: *Miscanthus* is closely related to *Saccharum* with which it readily hybridises. The main characters separating the two are the disposition of the spikelets on the flowering panicle (in *Saccharum* one spikelet is sessile and the other pediceled, both have a pedicel in *Miscanthus*) and the fragility of the rachis, which according to Clayton and Renvoize (1986) are rather weak characters to separate two genera.

Hybridisation between species is common within the genus which adds to the complicated taxonomic status of *Miscanthus*. Since Andersson's first description of *M. sinensis* over seventy different species have been described within the genus but many of these are synonyms (Index Kewensis, CD ROM, Oxford University press 1991). Currently it is estimated that *Miscanthus* contains about fourteen species (Hodkinson *et al.*, 1997). Ten of these species occur in Asia and four in Africa. The first major treatment of the genus was by Honda (1930), who divided the genus into two sections, *Triarrhena* and *Eumiscanthus*. Within these divisions Honda assigned twenty species and ten varieties. This treatment was modified by Ohwi (1956) who reduced the number of species to six and the number of varieties to two. Ohwi also introduced the section *Kariyasua* into the genus. Other work by Keng (1957) established the section *Diandra*. The most recent thorough morphological treatment of the genus was by Lee (1964 a, b & c). Lee's division of the genus consolidated the work of Honda (1930), Ohwi (1956) and Keng (1957). Lee omitted the four African species which are sometimes placed in a different genus, *Miscanthidium*, due to the elongated nature of the axis of the inflorescence. This distinction is rather arbitrary as there are a number of intermediates such as *M. floridulus*, which obscure the distinction (Clayton and Renvoize, 1986). *Miscanthus* may be best treated in the strict sense, excluding the African *Miscanthium* species and the Himalayan *Sclerostachya fuscus*, because although they are morphologically similar, they differ in basic chromosome number. There are ten species in *Miscanthidium* compared to 19 in *Miscanthus*. In addition they have been shown to be clearly separate on the basis of Internal Transcribed Spacer (ITS) sequence data (Hodkinson *et al.*, 1997).

The genus *Miscanthus*

Type specimens of *Miscanthus sacchariflorus* (Max.) Hack. form *purpurescens* and *Miscanthus nudipes* (Griseb.) Hack ssp. *taylorii* (Bor) Y. Lee, comb. nov. at Kew herbarium were examined. Type specimens of all other *Miscanthus* species were unobtainable and therefore the following descriptions of the species are taken from the most recent revision of the genus *Miscanthus* by Lee (1964 a, b and c).

Section *Triarrhena* (Honda)

This section contains only one species, *M. sacchariflorus* (Maxim.) Benth.

Rhizomes elongate; spikelets dull grey-brown, usually awnless; anthers 3; callus hairs 2-4 times as long as the spikelet, silvery white. *M. sacchariflorus* is common in the wet lowlands of Japan and extends into Korea and northern China.

Section *Miscanthus* (Honda)

This section is characterised by short rhizomes, awns present, inflorescence much branched, anthers 3. Three species are included:

M. sinensis Anderss. This variable species contains numerous different varieties and forms. The typical form is described as a tufted stout perennial with thick, short rhizomes. The spikelets are awned and yellowish with a tuft of white or purplish hairs, the panicle is 20-30 cm long, the axis short, less than half as long as the length of the racemes. The racemes are rather numerous and dense. Many of the cultivars of *M. sinensis* are as a result of either natural hybridisation or cultivation and selection by the horticultural industry prompted by the ornamental value of this species. *Miscanthus sinensis* has a wide ranging distribution from lowlands to mountains throughout western Asia and into Japan, China and the Pacific islands of French Polynesia.

M. floridulus (Labill.) Warb. This species is morphologically intermediate between the Asian and African species of *Miscanthus*. It has an elongated inflorescence axis which is one of the characters separating Asian from African groups of *Miscanthus*. Typically *M. floridulus* has awned spikelets, with a tuft of hairs at the base; racemes are numerous, nearly sessile and branched at the base. It has a wide ranging distribution across Japan and the Pacific Islands of French Polynesia.

M. condensatus (Hack) Makino. Possibly a subspecies of *M. sinensis* but stouter with wider leaf blades which are glaucous beneath. The panicles are much less dense than *M. sinensis*. It occurs near seashores but sometimes in mountains. This species is common with a distribution across China, Japan, Indochina and the Pacific islands of French Polynesia.

Section *Kariyasua* (Ohwi)

The species of Section *Kariyasua* are limited to areas of the temperate zone, particularly Japan and Korea. They have a short main axis and very few branches of racemes on the panicle, the length of the anthers is unequal and the midrib contains two major bundles almost enclosed by a sheath. This section is considered to be more advanced than the other sections of *Miscanthus* based on the above characters (Lee, 1964). Other characters which characterise this section are short rhizomes, awns sometimes present and 3 anthers.

M. oligostachyus Stapf. The axis of the panicle is very short and the spikelets are awned, racemes are green or purplish. The callus has a tuft of hairs 3-5 cm long. Occurs in the mountains of Japan.

M. changii Y. Lee. The panicle axis is short with 5-7 racemes. Spikelets are awned and there are long villous hairs on the back of the glumes. Occurs in Korea and Japan.

M. tinctorius (Steud.) Hack. This species is similar to *M. changii* but with much wider leaves. The axis of the panicle is very short with 2-12 racemes. Spikelets have a tuft of brownish hairs at the base. Spikelets awns are absent. It occurs in mountain areas of Honshu, Japan.

Section *Diandra* (Keng)

This section appears to be closely related to section *Kariyasua* (Lee, 1964a). The two sections differ, however in the number of stamens, only two stamens occur in *Diandra*, whereas three occur in all other sections of *Miscanthus*. Like *Kariyasua*, *Diandra* has a very short main axis and few racemes.

M. eulalioides Keng. Culms densely tufted. Axis up to 2/3 the length of inflorescence, spikelets 5-7 mm long, purplish or brownish, callus hairs whitish 1-3 mm long, awn twisted. China and Japan

M. nudipes (Griseb.) Hack. Very short main axis, spikelets up to 5.5 mm long, very short callus hairs, up to 1.5 mm long, awn not twisted as in *M. eulalioides*. Himalayas.

M. brevipilus Hand-Mazz. Differs from *M. eulalioides* in having much smaller spikelets.

M. nepalensis (Trin.) Hack. Short main axis. Spikelets 2-3 mm long. Long golden callus hairs twice as long as the spikelet. India and Burma.

M. szechuanensis Keng. Similar to *M. nudipes* but with smaller spikelets, up to 4 mm long and longer callus hairs, up to 3 mm long. China.

Table 2.0. Main sectional differences of the genus *Miscanthus*

<i>Triarrhena</i>	<i>Miscanthus</i>	<i>Diandra</i>	<i>Kariysua</i>
Height up to 3 M tall	Height up to 1.5 M tall	Height up to 1 M tall	Height up to 1 M tall
Spreading rhizome	Tufted rhizome	Tufted rhizome	Tufted rhizome
Main axis 90-120 mm	Main axis 120-200 mm	Main axis 50-90 mm	Main axis up to 10 mm
Many branches of racemes on the panicle	Many branches of racemes on the panicle	Few branches of racemes on the panicle	few branches of racemes on the panicle
Distributed across China, Siberia, Korea & Japan.	Distributed From SE Asia, China, Japan & Polynesia	High altitudes from West China to east India	Limited to temperate zones of Japan and Korea
Number of stamens = 3	Number of stamens = 3	Number of stamens = 2	Number of stamens = 3
Callus hairs 8-10 mm	Callus hairs 4-6 mm long	Callus hairs 1-10 mm long	Callus hairs 3-8 mm long

There are many problems surrounding the current classification of the genus *Miscanthus* and this thesis aims to address them. Most of the morphological taxonomic treatments of *Miscanthus* have concentrated on descriptive analysis of the species (Honda, 1930; Keng, 1957; Lee, 1964 a, b & c; Ohwi, 1965). There appears to be no extensive numerical taxonomic or phylogenetic treatments based on morphology. In addition, there is very little evidence of any assessment of the many hybrids, varieties and cultivars within the genus. Widespread hybridisation both within the genus itself and with other closely related genera has produced many intermediates which do not fall simply into the above classification.

It is well established that the use of morphological characters is an accepted method of distinguishing between species. However, artificial crossing and extensive cultivation are necessary so that particular characters of value in determining hybrids can be evaluated and this should always precede any diagnosis of natural hybridisation (Baker, 1945). The production of artificial hybrids to study hybridisation and the pattern of inheritance in the wild is further supported by MacHale & Alston (1964) who found that hybrids will generally cross back to the more abundant parent, thereby causing introgression in one direction. The end result will be hybrids that so resemble one of the parent species that they are indistinguishable from it. Scally (1989) found that environmental constraints in *Saxifraga* generally favoured one parent more than the other and that hybridisation and introgression proceeded in the direction of the more abundant parent. This work was supported by artificial hybridisation experiments. Greef *et al.* (1997) attempted a number of such artificial crossing experiments to test the hypothesis that *M. x giganteus* was a hybrid between *M. sinensis* and *M. sacchariflorus*. However these crosses were unsuccessful due to late flowering in *M. sacchariflorus* compared to *M. sinensis*.

The following study evaluates, through a variety of techniques, the morphological classification of the genus *Miscanthus*, as assigned by Lee (1964c). Included in this study are a full range of *Miscanthus* species and many of the cultivars of the species *M. sinensis*. A range of species within the genus *Saccharum* and within the subdivision *Erianthus* are also included as they are closely related to *Miscanthus*. A number of the African *Miscanthus* species, *Miscanthidium*, are also included to examine the hypothesis that these species should be included in *Miscanthus*.

2. 2 Materials and Methods

2.2.1 Morphological analysis of herbarium material

All morphological measurements of materials were made on herbarium specimens. The specimens used were on loan from The Royal Botanic Garden, Kew (**K**); The Natural History Museum, Paris (**P**) and the University of Edinburgh (**E**). Additional material from our own living collection at Trinity College Botanic Garden was dried and mounted and included in the analysis. A list of all of the specimens examined is presented in Appendix 1. It was not possible to obtain material of some of the species belonging to *Miscanthus*. These included *M. eulaliodes*, *M. szechuanensis*, *M. brevipilus* and *M. changii*. Notwithstanding these omissions enough material was obtained to cover all of the sections within *Miscanthus* according to Adati & Shiotani (1962) and Lee (1964c). Specimens from the genus *Saccharum* and a number of individuals labelled *Erianthus* were also included in the analysis to examine the relationship between the genera. Although *Erianthus* is included in *Saccharum* by Clayton and Renvoize (1986) it was treated separately in this analysis as it has been shown to have nuclear ribosomal DNA sequence characteristics that suggest it could be separate to *Miscanthus* (Glaszmann *et al.*, 1990). A list of all of the herbarium material examined is given in Appendix 2.

Choice of characters

The initial choice of characters was based upon an initial survey of the species and is largely subjective. A range of specimens covering all of the sections of *Miscanthus* were examined and a list of potentially useful characters was compiled (Table 2.1). Most of the characters pertained to the flowering panicle. Individual spikelets were removed from the specimen, softened in warm water, blotted dry and placed on a piece of double sided sticky tape on a glass slide. In this way the individual parts of the spikelet could be dissected and measured under a dissecting microscope. All measurements were made directly with a microscope rule graduated into 0.1 mm sections. Most of the characters scored are self explanatory but further clarification is given here for some of them.

Leaf width and leaf mid-vein width: leaf width and mid-vein width are rather meaningless characters on their own. However, when measured as a ratio of one to the other a more meaningful character is obtained.

Hair lengths: In all cases where the length of hairs was measured the length of the longest hair was taken as a certain amount of variability was present in most specimens examined.

Glume, lemma and palea widths: The width of these characters was always taken at the widest point.

Spines per 10 mm: The number of spines on the leaf margin counted across a 10 mm section of the leaf, taken at the mid point of the leaf between the base and the tip.

In cases where the determination on the specimen labels was assessed to be correct these names were used as identifiers in the analysis. In instances where the determination was very obviously wrong, the correct name was given. In some cases where there was ambiguity as to the identity of the specimen the original label was used. It was hoped that the complete morphological analysis would resolve any ambiguities and allow an accurate identification.

Table 2.1 Characters scored for morphological analysis.

No.	Character	No.	Character
1	Awn present/absent	19	Lodicule width
2	Awn length	20	Number of nerves on upper glume
3	Callus hair length	21	Leaf width
4	Pedicel hair length	21	Leaf mid vein width
5	Short pedicel length	23	Abaxial leaf hairs present/absent
6	Long pedicel length	24	Adaxial leaf hairs present/absent
7	Upper glume hair present/absent	25	Inflorescence axis hairs present/absent
8	Upper glume hair present/absent	26	Inflorescence main axis length
9	Upper glume length	27	Inflorescence total length
10	Upper glume width	28	Ratio upper glume length/width
11	Lower glume length	29	Ratio lower glume length/width
12	Lower glume width	30	Ratio Upper lemma length/width
13	Upper lemma length	31	Ratio Lower lemma length/width
14	Upper lemma width	32	Ratio palea length/width
15	Lower lemma length	31	Ratio leaf width/main vein width
16	Lower lemma width	34	Inflorescence total length - axis length
17	Palea length	35	Character 34/axis length
18	Palea width	37	Spines per 10 mm leaf margin
19	Lodicule length		

2.2.2 Statistical analysis

Initially the mean and standard deviation of each character for each species was calculated (Table 2.2), this allowed an examination of the range of variation between characters for the different species to be examined. An array of statistical packages are currently available for the analysis of multivariate data and a number of such packages were applied to the data

matrix. The choice of package was dependent on the final data matrix produced. Some multivariate methods are capable of handling both continuous and bistate data while others can only cope with normally distributed continuous data. As the final data matrix (Appendix 3) produced from this work contained a mixture of both types of characters a number of different methods were used to analyse the data. These methods were chosen so that most of the original data set could be included in at least one form of analysis. As this treatment was largely examining inter and infraspecific variation within a genus the majority of characters scored were continuous and therefore not appropriate to cladistic analysis. For this reason a phenetic approach was taken. Results obtained from one type of analysis could be compared with those from another to examine if the different methods produced similar results. Two basic techniques were used to analyse the data, (1) ordination techniques, which included Principal Components Analysis (PCA), Principal Coordinate Analysis (PCO) and Detrended Correspondence Analysis (DCA) and (2) cluster analysis. A certain amount of statistical liberty was taken in some cases, but only after a strict analysis of the data had been completed. For example, Principal Components Analysis (PCA), which should only be carried out on continuous, normally distributed characters, was also performed on data which included bistate characters (Table 2.3). This data set was standardised before analysis so that all characters were converted to the same scale before analysis. Standardisation of the data was carried out by the method of Parnell and Waldren, (1996), in this way bistate data was recorded on a scale corresponding to the number of characters in the data set. Therefore if 34 characters are present each character could have a value of 1 or 34. The statistical methods used and a brief explanation of their merits is outlined below.

Principal Components Analysis (PCA)

PCA is one of the most commonly used methods of ordination. PCA may be based on the matrix of correlation between taxa. The first axis of the PCA represents the greatest amount of variance with subsequent axes representing less variation. The first axis has the largest eigenvalue which is a measure of the total amount of variation explained by this axis. The individual axes produced by PCA are, however, dependent on the data being normally distributed and any skewness must be removed from the data matrix by normalisation before analysis. In addition only continuous characters may be used in the analysis. However, in some cases bistate data may be used if it is possible to categorise the data. (Parnell & Waldren, 1996). Having examined the data matrix produced for *Miscanthus* it was not possible to use categorical data in this case. The computer program SPSS version 6.1 was used to analyse the final data matrix. This program allows the data to be viewed in its rotated or unrotated forms. Essentially, the data is first analysed by centroid factor analysis (Rohlf & Sokal, 1962), which describes the interrelationships among Operational Taxonomic Units (OTU's) in terms of an uncorrelated system. In this way each new variable added to the analysis is uncorrelated with the others. The first centroid factor accounts for most of the

covariance with subsequent factors representing gradually less. Once all of the factors have been extracted they are transformed by rotation. This means that each of the co-ordinate axes are transformed to a correlated system. This system provides a much simpler and clearer view of the overall variation present in the sample (Sokal & Sneath, 1963).

Table 2.3 Characters used in PCA and PCO.

* indicates those characters included in the full data set which includes bi-state data.

No.	Character	No.	Character
1	Callus hair length	17	Upper lemma width
2	Short pedicel length	18	Ratio upper glume length/width
3	Long pedicel length	19	Ratio lower glume length/width
4	Upper glume length	20	Ratio upper lemma length/width
5	Upper glume width	21	Ratio lower lemma length/width
6	Lower glume length	22	Ratio palea length/width
7	Lower glume width	23	Ratio leaf width/main vein
8	Palea length	24	Ratio Inflorescence total length - axis length
9	Palea width	25	Character 24/Inflorescence axis length
10	Leaf width	26	Number of glume nerves
11	Mid-vein width	27	*Awn present/absent
12	Inflorescence total length	28	*Pedicel hairs present/absent
13	Inflorescence axis length	29	*Upper glume hair present/absent
14	Lower lemma length	30	*Lower glume hairs present/absent
15	Lower lemma width	31	*Inflorescence hairs present/absent
16	Upper lemma length		

Principal Coordinate Analysis (PCO)

Principal Coordinate Analysis (Gower, 1966) is a similar technique to PCA but more general, as other methods of similarity apart from Euclidean distance may be used (Minchin, 1987).The main difference is that it operates on a distance or similarity matrix, rather than a correlation or co-variate matrix as in PCA. In PCO the dissimilarity values are transformed into similarity values before analysis. Presence-absence data may be used and it does not appear to be necessary that the data is normally distributed, although an appropriate distance or similarity matrix should be used. The PCO analysis carried out here was based on all of

the characters listed in Table 2.2. All analyses were carried out using the computer program Syntaxonomy. This program does not supply information on which characters are responsible for the variation explained by each axis. PCO has become the most common ordination technique used in the analysis of molecular data sets. Its inclusion allowed a direct comparison of morphological and molecular data sets to be made.

Correspondence Analysis (CA)

CA is another multivariate ordination technique primarily developed for the analysis of contingency table data. It is however becoming increasingly applied to ecological data to analyse abundance data (TerBraak, 1985). It is based on a method of reciprocal averaging as described by Hill (1973). Detrended Correspondence Analysis (DCA) is an extension of CA whereby the data is detrended (Palmer, 1993). This means that any curvilinearity (sometimes referred to as the horseshoe effect) which sometimes appears in complex data sets and may obscure the dominant gradient of that data is removed (Digby & Kempton, 1987). One of the advantages of CA over PCA is that it is capable of dealing with both continuous and bistate data. In addition it is not as important that the data are normally distributed and therefore characters which display a certain amount of skewness may be included in the analysis. All CA and DCA presented in this chapter were carried out using the Canoco computer program, version 3.11. The characters used in the analysis are those presented in Table 2.2 with the exclusion of those marked with an asterisk. These characters were removed because preliminary analysis showed them to be of little value in delimiting species, or were highly correlated and obviously linked with another character. Highly correlated characters may be eliminated from the data set if, after examination of the characters, it can be reasonably argued that inclusion of both could lead to bias.

Cluster Analysis

Clustering procedures are a suite of methods which encompass a range of clustering algorithms designed to group objects based on their overall similarities or differences. They can be either agglomerative hierarchical methods, whereby clusters are formed by grouping objects into larger and larger clusters until all objects are members of a single cluster, or divisive methods, where the whole set of objects are gradually divided into smaller groups (Hermy, 1988). The data were analysed by a number of clustering procedures (within the SPSS computer program, version 6.1) before a final clustering method was decided on. These included clustering using single linkage, average linkage, Ward's method (Ward, 1963) and Ward's method using Z scores, in which Euclidean distance measures were used. The method which provided the most interpretable results was that which used Ward's method using Z scores and this was therefore chosen to examine the data. This dendrogram was chosen because it provided clearer groupings of the taxa. Standardisation of the data set by converting character scores to a standard scale, taking a mean of zero and a standard

deviation of one was first performed on the data and a squared Euclidean distance matrix was then calculated from this data. Ward's method, which is a hierarchical clustering method, was then performed on this data. Hierarchical clustering methods proceed from individual taxa and progressively combine them in terms of their similarity until all taxa are eventually linked into one group. Ward's method has been found to give the best fit with many other sources of data (archaeological, ecological and morphological, S. Waldren pers comm.) and is therefore recommended for the interpretation of morphological data sets (Scally, 1989; Rushton, 1978; Wishart, 1978).

2.2.3 Breeding Experiments

The Trinity College Dublin accessions of *Miscanthus* were planted outdoors in a section of Trinity College Botanic Garden in spring of 1996. Commencement of breeding experiments was in the spring of 1997. Table 2.3 lists the crosses attempted.

Table 2.4 Artificial crossings of *Miscanthus* individuals carried out.

Donor	Recipient
<i>M. sinensis</i> R36	<i>M. sacchariflorus</i> P66
<i>M. sacchariflorus</i> P66	<i>M. sinensis</i> R36
<i>M. sinensis</i> S11	<i>M. sacchariflorus</i> P91
<i>M. sacchariflorus</i> P91	<i>M. sinensis</i> S11
<i>M. sinensis</i> 'Silverfeather' R14	<i>M. sacchariflorus</i> R15
<i>M. sacchariflorus</i> R15	<i>M. sinensis</i> 'Silverfeather' R14
<i>M. floridulus</i> P17	<i>M. sinensis</i> R36
<i>M. sinensis</i> R36	<i>M. floridulus</i> P17

Two racemes of each of the individuals listed in Table 2.4 were covered by a breathable polythene bag. It was possible to test for selfing by preventing external pollen from reaching the florets of one of the racemes. The second raceme had pollen introduced to it from the donor plant by cutting the inflorescence from the donor and inserting it into the bag with the recipient. The donor raceme had also been bagged prior to it reaching maturity to ensure its purity. Reciprocal crosses of each of the above individuals were also made. The bags were then left in place to allow time for seed set.

2.3 Results

2.3.1 Morphological Analysis

Awn length and awn presence/absence cannot be independent, therefore one of these characters (awn length) was subsequently removed from all further analyses. Awn length was removed rather than awn presence/absence because to include awn length would lead to missing data in the absence of an awn. It was also shown that the mean character scores for lodicule width, lodicule length and the number of spines per 10 mm section of leaf blade did not differ among any of the taxa examined, therefore providing no relevant information and so these characters were removed from further analyses.

Examination of the sample means (Table 2.2) for each character scored between species indicated that callus hair length and the ratio of the leaf width to mid-vein width were important characters in differentiating between *M. sinensis* and *M. sacchariflorus*. Callus hair length was considerably shorter in *M. nudipes* than any other species. *Miscanthus oligostachyus* was shown to have longer glumes than any other species and *M. condensatus* and one individual of *M. sinensis* (labelled *M. formosanus* on the herbarium label) had a short pedicel length which was considerably shorter than any other species. None of the characters examined appeared to be useful in distinguishing *M. floridulus* from the other species. Callus hair length was generally shorter in *Miscanthidium* than *Miscanthus* as was inflorescence axis and total length. The main difference between *Miscanthus* and *Saccharum* was that *Saccharum* had one sessile spikelet and longer inflorescence axis lengths. There was no obvious differences in the sample means for the various characters between the cultivars and *M. sinensis*.

Principal Components Analysis

PCA of the data set which included bi-state data showed that 25.9% of the data variance was explained on axis one, 13.6 % on axis two and 12.3% on axis three of the PCA (Figs. 2.1, 2.2 and 2.3). Axis four is not shown, as it accounted for only 6.8% of the variance and was therefore not easy to interpret. Most of the species, with the exception of *M. sinensis*, were separated on axis one of the PCA. A wide range of spread in variation within *M. sinensis* was demonstrated. In addition all of the cultivars of *Miscanthus* fell within the range of variation of the *M. sinensis* group. These cultivars were originally given different symbols to test their orientation, but were subsequently included as *M. sinensis* for clarity. *Miscanthus nepalensis* formed a compact group on all three axes, but within the range of variation of *M. sinensis*. The six individuals of *Saccharum* also showed a wide range of variation, in some cases overlapping the range of variation of *M. sinensis*. *Erianthus* largely grouped within the

Table 2.2.

Means and standard deviations for each character scored for morphological analysis.

Taxon	No. of Species examined	Callus hair length	Short pedicel length	Long pedicel length	Upper glume length	Lower glume length	Upper glume width	Lower glume width	Ratio upper Glume length/width
<i>M. sinensis (M. formosanus)</i>	1	1.00	0.50	1.40	2.60	2.40	1.00	0.90	0.00
<i>M. sinensis (M. japonicus)</i>	14	5.54±1.24	1.32±0.52	3.28±0.71	3.76±0.62	3.84±0.55	1.04±0.14	1.14±0.19	3.69±1.08
<i>M. purpurescens</i>	5	5.40±1.71	2.18±0.49	4.44±0.52	4.90±0.80	4.98±0.94	1.54±0.38	1.54±0.39	3.25±0.55
<i>M. oligostachyus</i>	3	6.67±0.65	2.00±1.00	4.33±1.53	5.60±0.10	5.57±0.15	1.40±0.17	1.47±0.21	4.04±0.47
<i>M. nepalensis</i>	1	8.00	1.50	2.50	2.20	2.00	0.90	0.90	2.44
<i>M. condensatus</i>	1	5.50	0.50	2.50	4.00	4.20	1.20	1.20	3.33
<i>Miscanthidium ecklonii</i>	1	2.00	3.00	5.50	3.50	2.80	1.00	1.00	3.50
<i>M. floridulus</i>	3	6.43±0.60	2.00±0.29	4.00±0.00	3.13±0.55	2.53±0.90	0.97±0.06	0.77±0.23	3.26±0.67
<i>M. nepalensis</i>	5	8.20±0.27	2.22±0.54	3.90±0.70	2.56±0.34	2.50±0.30	0.82±0.15	0.86±0.09	3.19±0.66
<i>M. nudipes</i>	2	2.35±1.63	1.40±0.57	3.25±0.35	4.60±0.57	4.20±0.00	1.10±0.14	1.10±0.14	4.19±0.02
<i>M. oligostachyus</i>	2	7.50±0.71	2.00±0.00	5.50±0.00	6.95±0.35	6.95±0.35	1.70±0.14	1.70±0.14	4.10±0.13
<i>M. sacchariflorus</i>	14	9.90±1.51	2.6±10.98	5.00±1.19	4.63±0.46	4.74±0.56	1.04±0.18	1.01±0.19	4.61±1.04
<i>M. sinensis</i>	17	5.55±1.22	1.51±0.45	3.79±1.44	4.21±1.00	4.29±0.98	1.15±0.17	1.16±0.22	3.65±0.67
<i>M. sinensis var condensatus</i>	4	6.13±0.75	1.50±1.00	4.23±2.13	4.60±0.59	4.73±0.73	1.35±0.44	1.18±0.24	3.60±0.93
<i>M. sinensis var formosanus</i>	1	6.00	1.00	2.00	3.50	3.50	1.20	1.20	2.92
<i>M. sinensis var variagatus</i>	1	5.50	1.50	3.50	5.40	5.40	1.20	1.30	4.50
<i>M. tinctorius</i>	1	2.00	1.50	2.00	6.00	5.50	1.80	1.60	3.33
<i>Miscanthidium grossweilein</i>	1	2.50	2.00	4.00	4.00	4.00	1.00	1.00	4.00
<i>Miscanthidium junceum</i>	1	2.70	2.50	4.00	5.00	4.80	1.20	1.10	4.17
<i>Miscanthidium tetrefolium</i>	1	3.50	3.00	8.00	5.00	4.80	1.20	1.30	4.17
<i>Saccharum arundinaceum</i>	1	5.00	0.00	6.20	3.20	3.20	0.90	0.90	3.56
<i>Saccharum infirmum</i>	1	8.00	0.00	4.70	3.00	2.90	1.00	0.90	3.00
<i>Saccharum narenga</i>	1	2.50	0.00	3.50	3.00	3.30	1.00	1.10	3.00
<i>Saccharum officinarum</i>	2	9.75±1.77	0.00±0.00	3.65±0.49	3.00±1.27	3.05±1.20	1.10±0.00	1.20±0.00	2.73±1.16
<i>Saccharum spontaneum</i>	1	14.00	0.00	4.60	3.60	3.90	0.90	1.00	4.00
<i>Saccharum sp.</i>	5	8.84±1.07	0.00±0.00	2.90±1.10	3.20±0.45	3.16±0.48	1.02±0.11	1.06±0.15	3.16±0.54
<i>M. x giganteus</i>	3	10.17±1.89	1.33±0.29	3.60±0.17	4.73±0.25	4.67±0.58	1.20±0.20	1.17±0.15	4.04±0.86
<i>M. 'Posidon'</i>	1	7.00	2.00	4.00	5.40	6.00	1.20	1.80	4.50
<i>M. 'Rotsilber'</i>	1	7.80	1.10	3.90	5.20	5.50	1.10	1.80	4.73
<i>M. 'Silberspine'</i>	1	5.00	2.00	5.00	5.00	4.70	1.20	1.40	4.17
<i>M. 'Silverfeather'</i>	2	8.00±0.00	1.05±0.21	3.50±0.71	5.05±0.07	4.90±0.07	1.40±0.14	1.30±0.14	3.63±0.32
<i>M. 'Zebrinus'</i>	1	5.00	1.50	4.00	4.80	5.00	1.20	1.20	4.00

Taxon	Ratio lower glume length/width	Upper lemma length	Upper lemma width	Ratio upper lemma length/width	Lower lemma length	Lower lemma width	Ratio lower lemma length/width	Palea length	Palea width
<i>M. sinensis (M. formosanus)</i>	0.00	1.90	0.40	2.40	2.00	0.50	0.60	0.70	0.60
<i>M. sinensis (M. japonicus)</i>	3.45±0.70	2.760.59	0.69±0.16	4.16±1.12	2.99±0.53	0.79±0.13	3.86±0.80	1.37±0.55	0.38±0.16
<i>M. purpurescens</i>	3.30±0.56	3.64±1.03	0.96±0.15	3.76±0.76	4.24±0.90	1.18±0.57	4.07±1.40	2.46±1.15	0.48±0.13
<i>M. oligostachyus</i>	3.84±0.55	3.83±0.76	0.77±0.06	4.97±0.67	4.10±0.52	0.90±0.17	4.63±0.78	0.87±0.06	0.50±0.10
<i>M. nepalensis</i>	2.22	1.90	0.80	2.38	1.90	0.60	3.17	1.00	0.40
<i>M. condensatus</i>	3.50	3.00	0.70	4.29	3.00	0.60	5.00	2.90	0.30
<i>Miscanthidium ecklonii</i>	2.80	2.50	0.50	5.00	2.30	0.50	4.60	1.80	0.5.
<i>M. floridulus</i>	3.26±0.23	2.77±0.32	0.63±0.32	5.23±2.60	2.57±0.21	0.53±0.06	4.87±0.81	1.20±1.56	0.83±1.01
<i>M. nepalensis</i>	2.92±0.33	2.34±0.26	0.74±0.05	3.18±0.46	2.36±0.27	0.74±0.09	3.25±0.65	1.12±0.18	0.48±0.08
<i>M. nudipes</i>	3.85±0.49	4.20±0.00	0.80±0.28	5.60±1.98	4.30±0.14	0.95±0.07	4.85±0.92	3.25±1.34	0.70±0.00
<i>M. oligostachyus</i>	4.10±0.13	4.35±0.21	0.80±0.00	5.44±0.27	6.65±0.21	1.00±0.00	6.65±0.21	3.60±0.28	0.50±0.00
<i>M. sacchariflorus</i>	4.86±1.04	2.75±0.42	0.46±0.09	6.16±1.20	3.05±0.62	0.54±0.08	5.67±1.10	1.31±0.55	0.29±0.08
<i>M. sinensis</i>	3.69±0.56	3.24±0.90	0.72±0.16	4.69±1.63	3.36±0.82	0.82±0.16	4.32±1.58	1.18±0.58	0.35±0.12
<i>M. sinensis var condensatus</i>	4.11±0.89	3.43±0.43	0.63±0.05	5.47±0.38	3.60±0.42	1.00±0.14	3.63±0.49	1.70±1.01	0.43±0.05
<i>M. sinensis var formosanus</i>	2.92	2.50	0.70	3.57	3.10	0.80	3.88	1.30	0.30
<i>M. sinensis var variagatus</i>	4.15	4.00	0.90	4.44	4.10	1.00	4.10	2.40	0.60
<i>M. tinctorius</i>	3.44	4.70	1.50	3.13	4.40	1.40	3.14	4.00	0.60
<i>Miscanthidium grosswelein</i>	4.00	3.50	0.50	7.00	3.70	0.60	6.17	2.00	0.30
<i>Miscanthidium junceum</i>	4.36	3.50	0.60	5.83	3.50	0.60	5.83	2.50	0.40
<i>Miscanthidium tetrefolium</i>	3.69	4.50	0.60	7.50	4.30	0.50	8.60	1.60	0.30
<i>Saccharum arundinaceum</i>	3.56	2.90	0.80	3.63	2.60	0.90	2.89	1.70	0.50
<i>Saccharum infirmum</i>	3.22	2.80	1.00	2.80	2.70	0.70	2.89	1.30	0.30
<i>Saccharum narenga</i>	3.00	3.00	0.50	6.00	2.60	0.60	4.33	1.60	0.50
<i>Saccharum officinarum</i>	2.541.00	3.20±0.99	0.60±0.28	6.44±4.68	2.650.07	0.65±0.07	3.86±0.00	1.25±0.92	0.20±0.14
<i>Saccharum spontaneum</i>	3.90	2.00	0.20	10.00	2.60	0.80	3.25	0.80	0.40
<i>Saccharum sp.</i>	3.03±0.62	3.30±0.35	0.30±0.28	16.73±8.15	3.32±0.41	0.36±0.26	17.69±15.82	1.88±2.37	0.32±0.16
<i>M. x giganteus</i>	4.01±0.16	2.97±0.45	0.73±0.21	4.18±0.71	3.37±0.15	0.70±0.26	5.21±1.59	1.77±0.25	0.50±0.00
<i>M. 'Posidon'</i>	3.33	3.50	0.80	4.38	4.00	0.90	4.44	1.10	0.40
<i>M. 'Rotsilber'</i>	3.06	3.90	0.80	4.88	3.90	0.80	4.88	1.50	0.40
<i>M. Silberspine'</i>	3.36	3.00	0.80	3.75	4.50	1.30	3.46	2.70	0.40
<i>M. 'Silverfeather'</i>	3.84±0.47	3.20±0.28	1.10±0.14	2.92±0.12	5.000.71	1.00±0.28	5.32±2.21	1.55±0.21	0.55±0.07
<i>M. 'Zebrinus'</i>	4.17	3.80	0.90	4.22	4.00	0.70	5.71	1.00	0.40

Taxon	Ratio palea length/width	Leaf width	Mid vein width	Ratio leaf width/ mid vein width	Inflorescence axis length	Inflorescence axis total length	ratio total length/axis length	Character 24/ axis total length
<i>M. sinensis (M. formosanus)</i>	0.40	17.00	1.20	36.00	280.00	370.00	70.00	0.00
<i>M. sinensis (M. japonicus)</i>	3.66±2.03	13.21±4.67	1.41±0.58	12.63±11.65	225.71±87.53	330.00±89.42	104.29±33.39	0.34±0.15
<i>M. purpurescens</i>	5.07±1.49	10.50±5.79	0.54±0.43	32.60±28.60	30.00±30.82	159.00±70.39	129.00±44.50	0.86±0.14
<i>M. oligostachyus</i>	1.79±0.46	7.17±0.76	0.87±0.60	12.11±8.69	0.00±0.00	135.00±44.44	148.33±41.90	1.00±0.00
<i>M. nepalensis</i>	2.50	6.00	0.50	12.00	20.00	95.00	75.00	0.79
<i>M. condensatus</i>	9.67	12.00	1.00	12.00	80.00	200.00	120.00	0.60
<i>Miscanthidium ecklonii</i>	3.60	7.00	0.60	11.67	40.00	40.00	0.00	0.00
<i>M. floridulus</i>	1.33±0.29	17.67±5.03	1.93±1.01	11.96±9.59	111.67±66.40	283.33±51.32	171.67±116.4	0.57±0.28
<i>M. nepalensis</i>	2.37±0.42	9.20±2.28	0.92±0.11	10.10±2.56	72.00±17.89	198.00±19.24	126.00±18.17	0.64±0.08
<i>M. nudipes</i>	4.65±1.92	4.50±0.71	0.50±0.00	9.00±1.41	70.00±70.71	160.00±98.99	90.00±28.28	0.63±0.21
<i>M. oligostachyus</i>	7.20±0.57	9.00±0.00	0.20±0.00	45.00±0.00	0.00±0.00	117.50±45.96	117.50±45.96	1.00±0.00
<i>M. sacchariflorus</i>	4.82±2.69	12.63±4.60	0.60±0.31	30.59±23.79	71.11±42.03	269.64±62.50	198.54±60.71	0.74±0.15
<i>M. sinensis</i>	3.48±1.44	7.76±3.49	0.95±0.48	10.11±7.39	120.00±99.83	260.88±103.6	140.88±48.45	0.59±0.21
<i>M. sinensis var condensatus</i>	4.13±2.66	9.75±4.92	0.95±0.75	14.00±6.98	63.75±39.02	233.75±54.98	170.00±20.41	0.75±0.12
<i>M. sinensis var formosanus</i>	4.33	7.00	1.80	3.89	60.00	250.00	190.00	0.76
<i>M. sinensis var variagatus</i>	4.00	8.00	2.00	4.00	90.00	230.00	140.00	0.61
<i>M. tinctorius</i>	6.67	10.00	1.00	10.00	0.00	18.00	18.00	1.00
<i>Miscanthidium grossweilein</i>	6.67	12.00	0.50	24.00	60.00	60.00	0.00	0.00
<i>Miscanthidium junceum</i>	6.25	10.00	0.50	20.00	40.00	40.00	0.00	0.00
<i>Miscanthidium tetrefolium</i>	5.33	7.00	1.00	7.00	40.00	40.00	0.00	0.00
<i>Saccharum arundinaceum</i>	3.40	19.00	1.00	19.00	340.00	340.00	0.00	0.00
<i>Saccharum infirmum</i>	4.33	10.00	0.50	20.00	300.00	300.00	0.00	0.00
<i>Saccharum narenga</i>	3.20	6.00	0.50	12.00	350.00	350.00	0.00	0.00
<i>Saccharum officinarum</i>	10.50±12.02	15.00±11.31	0.90±0.14	23.00±0.00	270.50±225.5	340.00±127.28	69.50±98.29	0.28±0.40
<i>Saccharum spontaneum</i>	2.00	6.00	0.50	12.00	440.00	440.00	0.00	0.00
<i>Saccharum sp.</i>	7.84±12.41	11.60±1.67	0.44±0.13	28.93±11.54	198.00±146.0	252.00±74.63	54.00±83.55	0.29±0.44
<i>M. x giganteus</i>	3.53±0.50	6.50±1.50	0.67±0.29	10.33±2.52	70.00±0.00	256.67±35.12	186.67±35.12	0.72±0.04
<i>M. 'Posidon'</i>	2.75	4.00	0.30	13.33	60.00	180.00	120.00	0.67
<i>M. 'Rotsilber'</i>	3.75	7.50	1.50	5.00	180.00	300.00	120.00	0.40
<i>M. 'Silberspine'</i>	6.75	2.50	1.00	2.50	50.00	200.00	150.00	0.75
<i>M. 'Silverfeather'</i>	2.82±0.02	9.00±2.83	1.25±0.35	7.84±4.48	45.00±7.07	225.00±21.21	180.00±14.14	0.80±0.01
<i>M. 'Zebrinus'</i>	2.50	9.00	1.00	9.00	90.00	280.00	190.00	0.68

Fig. 2.1
Principal Component Analysis of *Miscanthus* morphology data, of all characters for axis 1 and 2

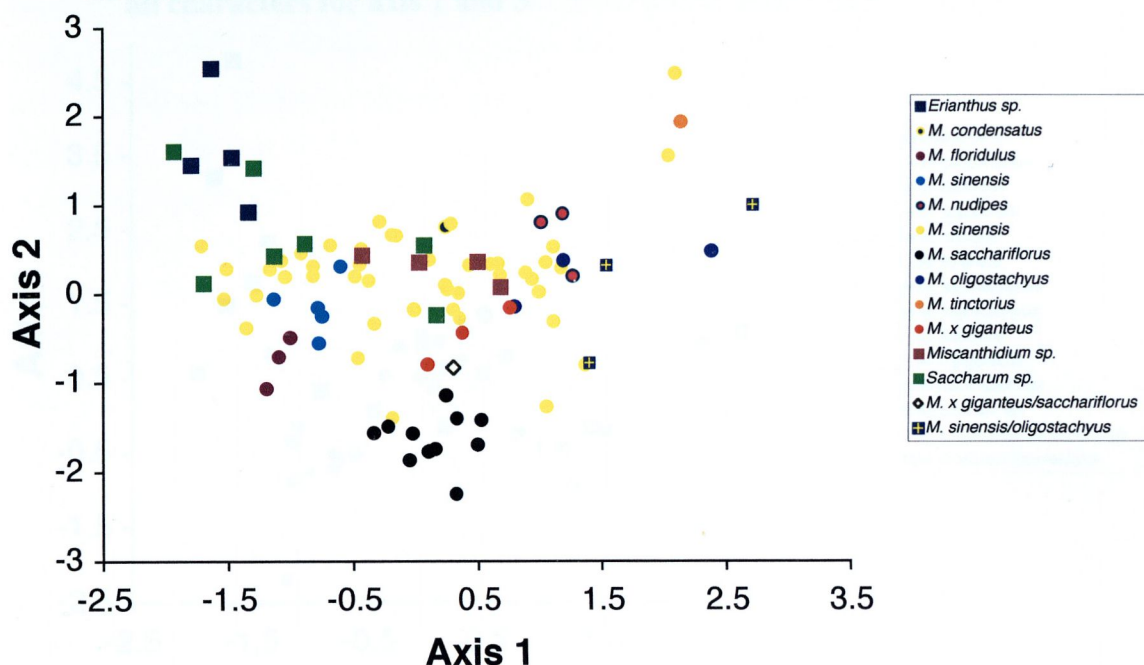


Fig. 2.2
Principal Component Analysis of *Miscanthus* morphology data of all characters for axis 2 and 3

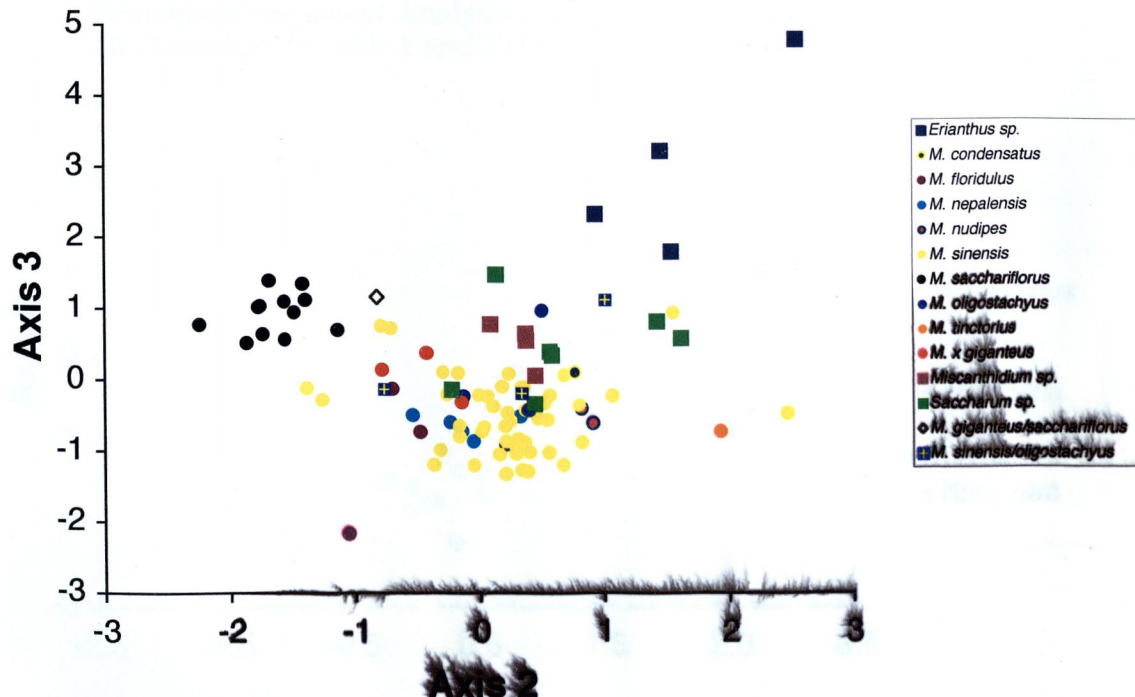


Fig. 2.3
Principal Component Analysis of *Miscanthus* morphology data of all characters for axis 1 and 3

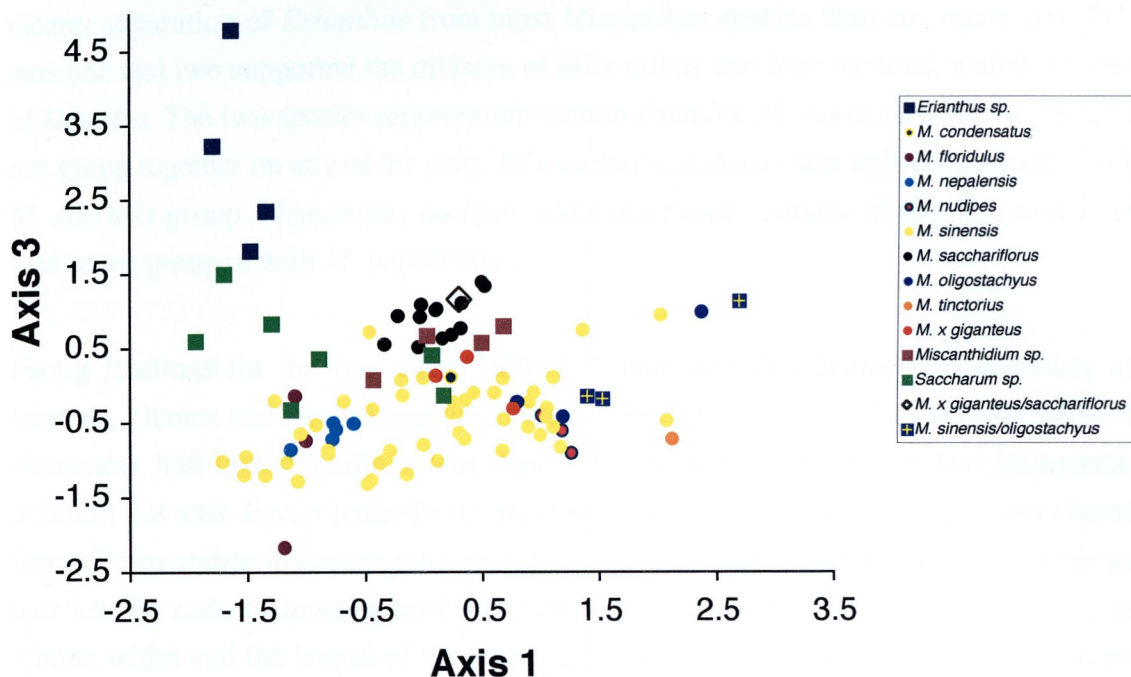
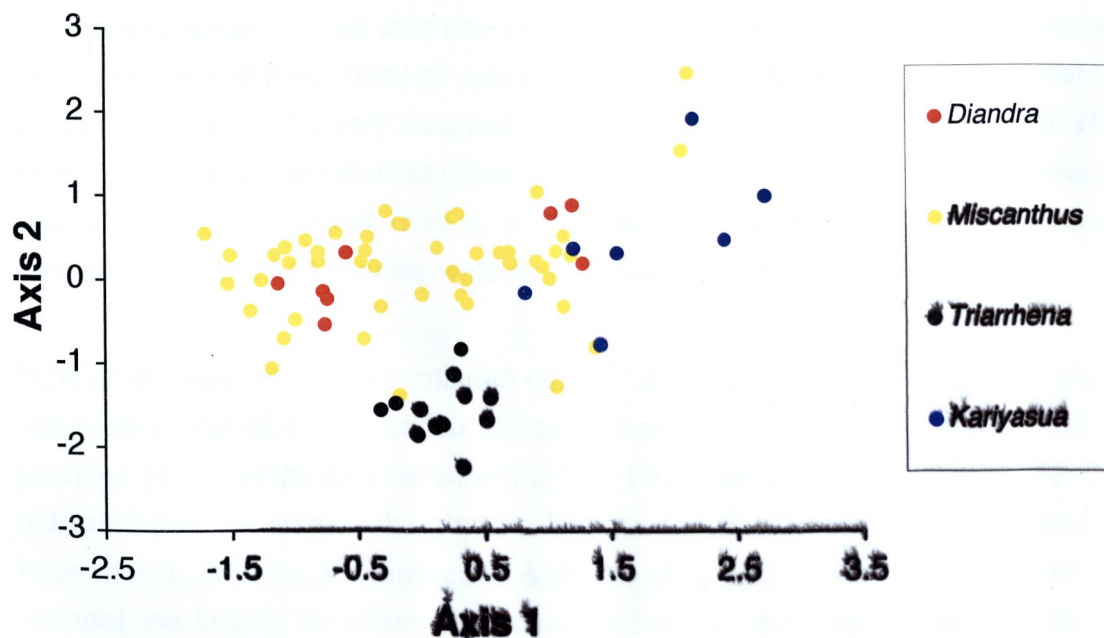


Fig. 2.4
Principal Component Analysis of *Miscanthus* morphology data of all characters for axis 1 and 2 showing the sections of *Miscanthus*.



range of *Saccharum* on axis 1, but were more clearly resolved on axis 3. All of the *Miscanthidium* group fell within the range of *M. sinensis* on axis one and two but were slightly better resolved on axis 3. *Miscanthus x giganteus* had an intermediate position between *M. sinensis* and *M. sacchariflorus* on axis 2. Axis three of the PCA provided a clearer separation of *Erianthus* from most *Miscanthus* species than any other axis. PCA of axis one and two supported the division of *Miscanthus* into four sections, with the exception of *Diandra*. The two species representing section *Diandra*, *M. nepalensis* and *M. nudipes* did not group together on any of the axes. *Miscanthus nepalensis* was indistinguishable from the *M. sinensis* group. *Miscanthus nudipes* while marginally outside of the *M. sinensis* group was never grouped with *M. nepalensis*.

Factor loadings for the first axis (Table 2.5) indicated that characters pertaining to the lemmas, glumes and the inflorescence were important in defining the first axis. Most other characters had factor loadings less than |0.5|, and were therefore of less importance in defining this axis. Factor loadings for axis two of the PCA indicated that far fewer characters were of importance in defining this axis, but those characters that were important were callus hair length, ratio of lower glume length/width, ratio of upper glume length/width, upper lemma width and the length of the longer pedicel. Axis three (Fig. 2.3), which represented only 12.2% of the data variance had only two characters with factor loadings scoring greater than 0.5, indicating that fewer characters were of importance in defining this axis. Axis three was rather difficult to interpret but showed a clear separation of *Miscanthidium* from all other species and genera which was obscured on axis one and two.

PCA was generally successful in distinguishing the sections of *Miscanthus*. Examination of the sections plotted against axis one and two of the PCA (Fig. 2.4) indicated that section *Triarrhena* was distinct from all other sections. Section *Kariyasua* was reasonably well separated but showed a very marginal overlap with section *Miscanthus*. Section *Diandra* formed two separate and distinct clusters within the limits of the overall *Miscanthus* section and they were unresolvable from it. Section *Miscanthus* itself had a very wide spread of variation, largely reflected in the wide range of variation found in *M. sinensis*.

PCA of the data set which contained only continuous characters (all bi-state characters removed) produced a very similar picture to that provided by the full data set. Axis one provided 25.9% of the data variance (Fig 2.5). Factor loadings (Table 2.5) for the first axis indicated that the size of the glumes, lemmas and the inflorescence axis length were important in defining the first axis. Axis two (Fig 2.6) which provided 13.6% of the variance was largely determined by callus hair length, upper lemma width and the ratio of upper and lower glume length/widths. Only two characters, the ratio of the upper glume length/width and the ratio of the palea length/width, were important in determining axis three

Fig. 2.5
Principal Component Analysis of *Miscanthus* morphology data,
excluding bi-state characters, for axis 1 and 2

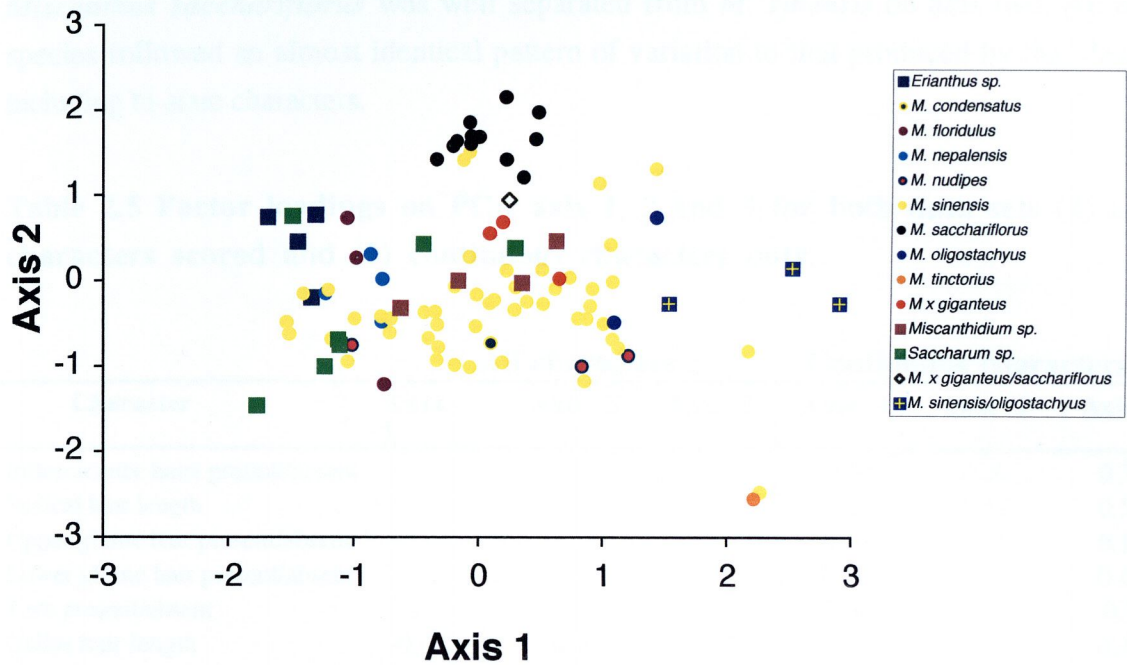
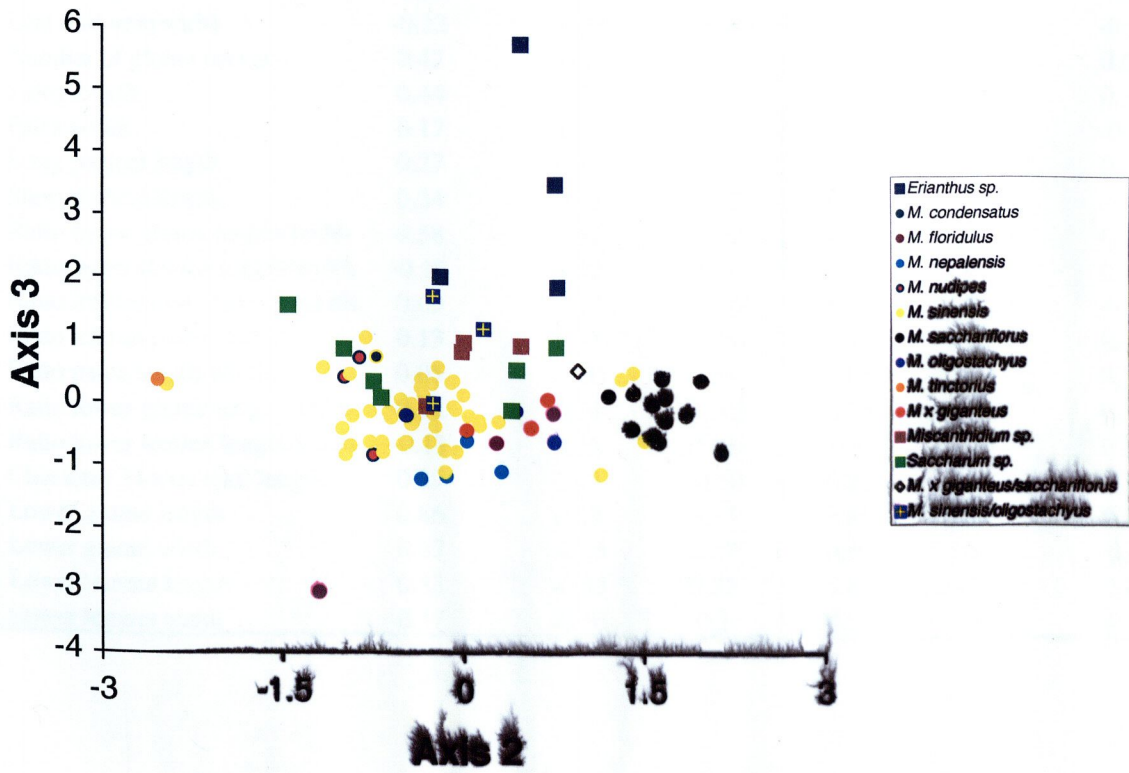


Fig. 2.6
Principal component Analysis of *Miscanthus* morphology data,
excluding bi-state characters, for axis 2 and 3



(Fig. 2.7) which explained 12.3% of the variance. The separation of genera and species produced from this analysis was practically identical to that produced by the full data set on axis one, two and three despite the fact that presence absence/characters were not included in this data set. *Miscanthus sinensis* again showed a wide range in variation on axis one. *Miscanthus sacchariflorus* was well separated from *M. sinensis* on axis two. All other species followed an almost identical pattern of variation to that produced by the data set including bi-state characters.

Table 2.5 Factor loadings on PCA axis 1, 2 and 3 for both data sets (1) all characters scored and (2) continuous characters only.

Character	All characters			Continuous characters only		
	Axis 1	Axis 2	Axis 3	Axis 1	Axis 2	Axis 3
Inflorescence hairs present/absent				-0.40	0.26	0.32
Pedicel hair length				-0.25	0.47	0.53
Upper glume hair present/absent				0.69	-0.09	0.16
Lower glume hair present/absent				0.50	-0.08	0.44
Awn present/absent				0.06	0.45	-0.38
Callus hair length	-0.08	0.60	0.07	-0.10	-0.45	0.41
Upper glume length	0.90	0.21	0.09	0.90	-0.12	0.16
Upper glume width	0.72	-0.38	0.18	0.69	0.42	-0.05
Upper lemma length	0.69	-0.04	0.36	0.67	0.27	0.25
Upper lemma width	0.49	-0.54	-0.36	0.50	0.26	-0.58
Inflorescence axis length	-0.68	-0.14	0.31	-0.71	0.24	0.13
Inflorescence total length	-0.55	0.14	-0.01	-0.58	-0.18	0.01
Leaf width	-0.33	-0.01	-0.08	-0.33	-0.12	-0.05
Leaf mid-vein width	-0.22	-0.23	-0.44	-0.25	-0.07	-0.55
Number of glume nerves	0.42	-0.15	0.14	0.42	0.20	0.05
Palea length	0.44	-0.29	0.42	0.44	0.48	0.32
Palea width	0.17	-0.36	-0.36	0.44	0.30	-0.22
Long pedicel length	0.27	0.51	-0.13	0.26	-0.47	0.18
Short pedicel length	0.34	0.43	-0.42	0.35	-0.59	-0.07
Ratio upper glume length/width	0.38	0.67	-0.09	0.36	-0.60	0.24
Ratio upper lemma length/width	-0.19	0.32	0.67	-0.22	0.15	0.75
Ratio inflorescence axis/total lth.	0.69	0.17	-0.36	0.69	-0.29	-0.17
Ratio leaf/mid-vein width	0.13	0.17	0.25	0.16	-0.05	0.40
Ratio palea length/width	0.05	-0.03	0.63	0.01	0.32	0.50
Ratio lower glume length/width	0.38	0.74	-0.12	0.35	-0.70	0.26
Ratio lower lemma length/width	-0.13	0.25	0.07	-0.16	0.18	0.70
Character 24/axis total length	0.29	0.43	-0.50	0.28	-0.64	-0.19
Lower glume length	0.86	0.23	0.13	0.80	0.24	0.20
Lower glume width	0.67	-0.35	0.27	0.67	0.49	-0.01
Lower lemma length	0.83	-0.05	0.32	0.85	-0.12	0.18
Lower lemma width	0.57	-0.56	-0.22	0.56	0.38	-0.45

Fig. 2.7
Principal Component Analysis of *Miscanthus* morphology data, excluding bi-state characters, for axis 1 and 3

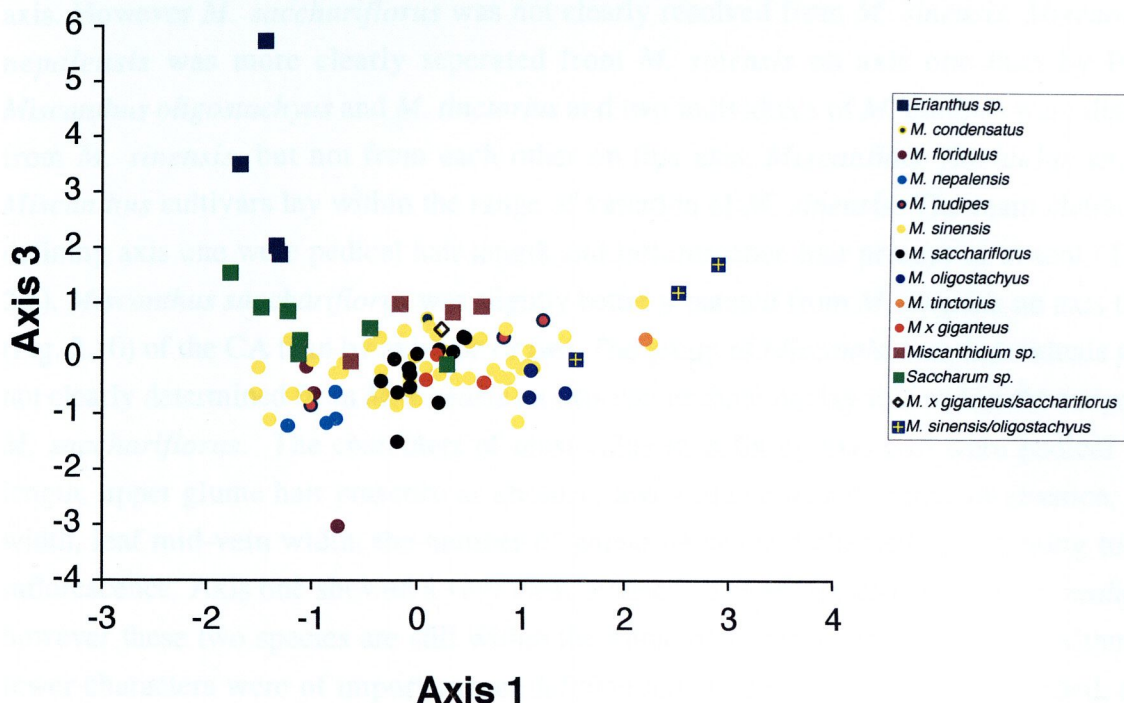
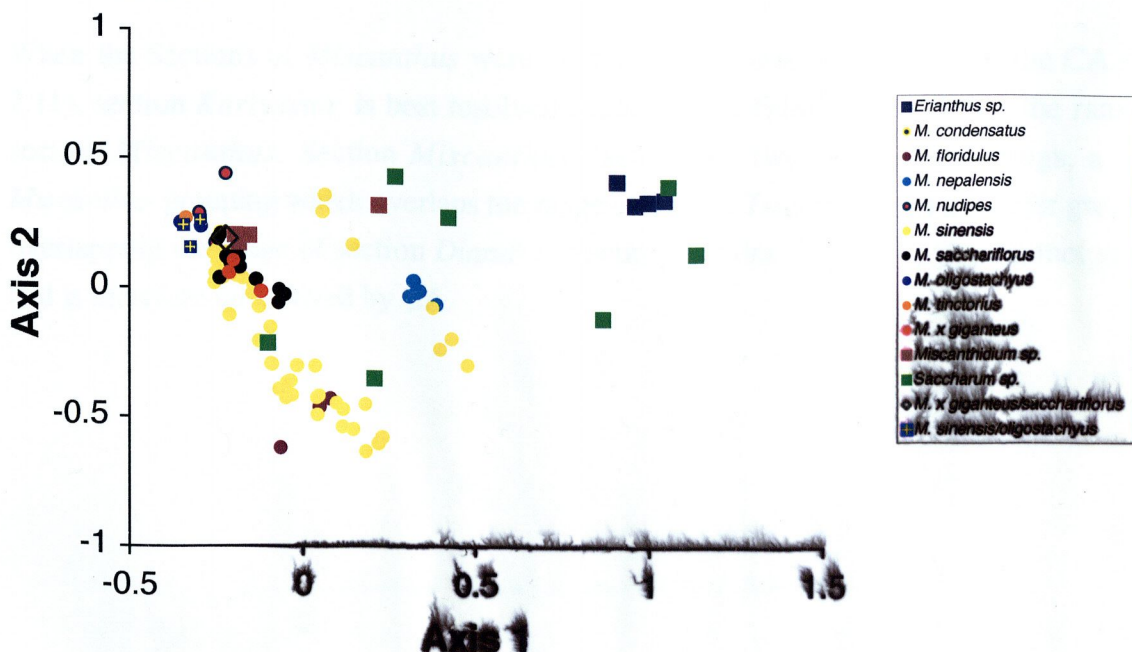


Fig. 2.8
Correspondence Analysis of *Miscanthus* morphology data for axis 1 and 2



Correspondence Analysis (CA)

Correspondence Analysis of character scores, for those characters shown in Table 2.3, indicated a slightly better resolution of some species on axis one (Fig. 2.8) than that observed on the PCA. *Miscanthus sinensis* showed a narrower spread of variation on this axis. However *M. sacchariflorus* was not clearly resolved from *M. sinensis*. *Miscanthus nepalensis* was more clearly separated from *M. sinensis* on axis one than by PCA. *Miscanthus oligostachyus* and *M. tinctorius* and two individuals of *M. nudipes* were distinct from *M. sinensis*, but not from each other on this axis. *Miscanthus floridulus* and all *Miscanthus* cultivars lay within the range of variation of *M. sinensis*. The main characters defining axis one were pedicel hair length and inflorescence hair present or absent (Table 2.6). *Miscanthus sacchariflorus* was slightly better separated from *M. sinensis* on axis three (Fig. 2.10) of the CA than by axis one or two. The group of *Miscanthidium* individuals were not clearly determined from *M. sinensis* on axis one or three but lay just within the range for *M. sacchariflorus*. The characters of most value in defining axis two were pedicel hair length, upper glume hair presence or absence, lower glume hair presence or absence, leaf width, leaf mid-vein width, the number of glume nerves and characters pertaining to the inflorescence. Axis one showed a very clear distinction of *M. nepalensis* from *M. nudipes*, however these two species are still within the range of variation of *M. sinensis*. Although fewer characters were of importance in defining axis three those that were included, awn presence or absence, pedicel hair length, the number of glume nerves, the ratio of the palea length to width, ratio of leaf width to mid vein width and inflorescence axis length. *Erianthus* was always well defined from *Miscanthus* on each of the three axes and generally grouped closer to *Saccharum*.

When the Sections of *Miscanthus* were plotted against axis one and two of the CA (Fig. 2.11), section *Kariyasua* is best resolved while section *Triarrhena* overlaps the range of section *Miscanthus*. Section *Miscanthus* itself forms two separate groupings, a main *Miscanthus* grouping which overlaps the range of section *Triarrhena* and a smaller grouping overlapping the range of section *Diandra*. Section *Diandra* is split into two distinct groups and is therefore unresolved by CA.

Fig. 2.9
Correspondence Analysis of *Miscanthus* morphology data for axis 2 and 3

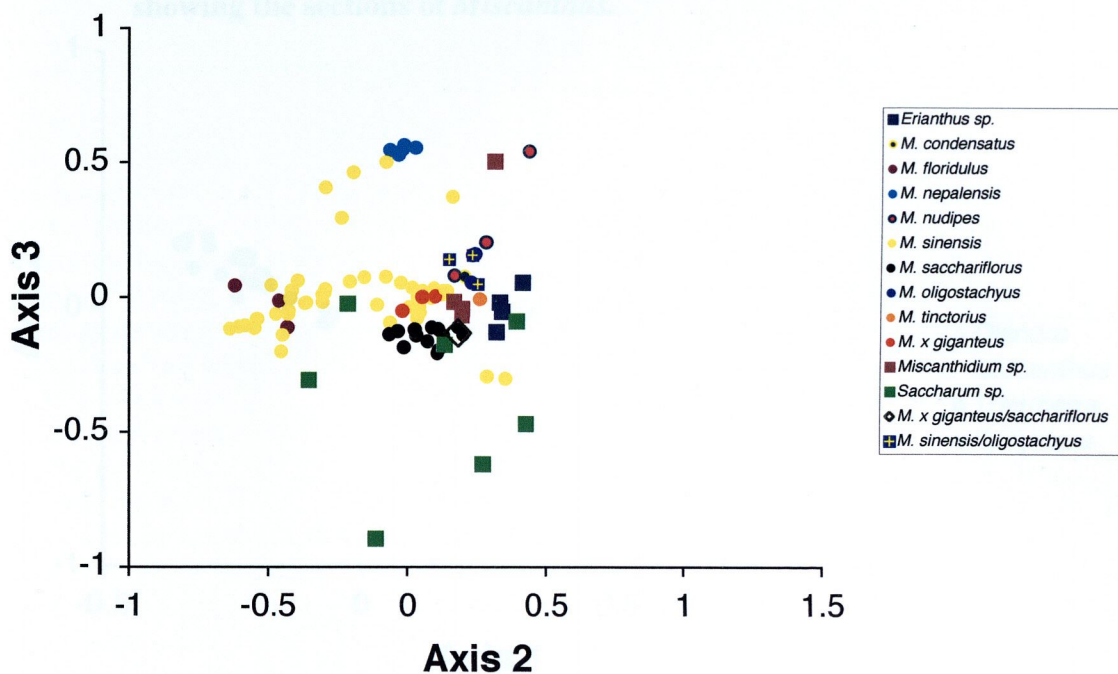


Fig. 2.10
Correspondence Analysis of *Miscanthus* morphology data for axis 1 and 3

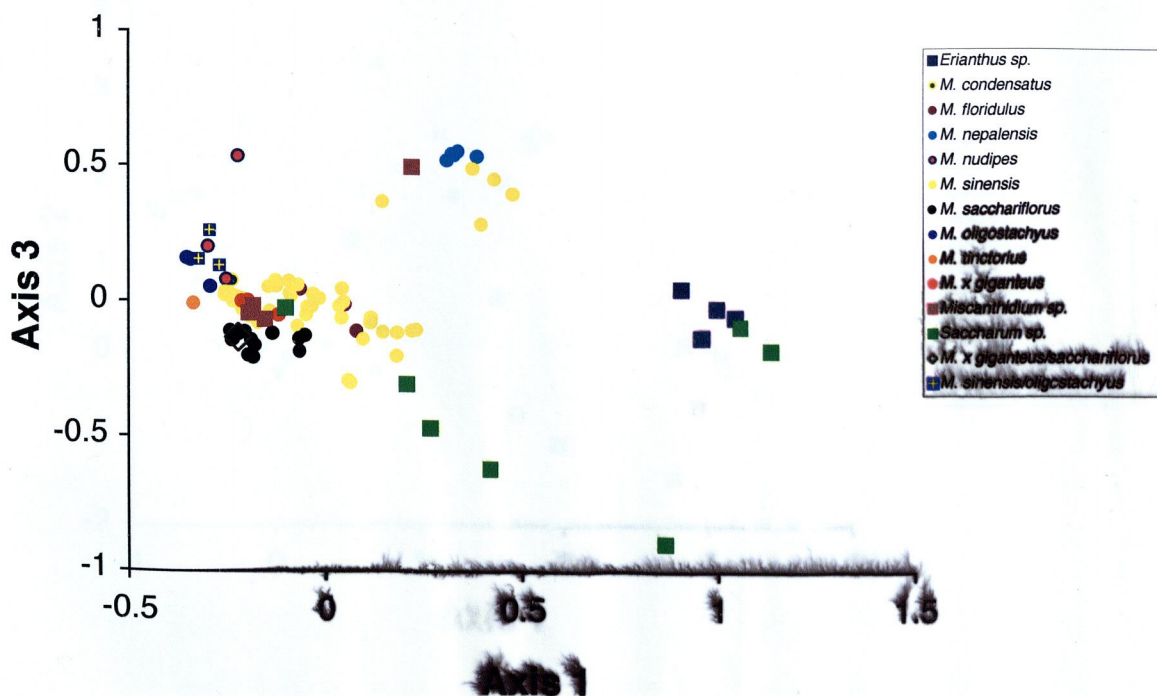


Fig. 2.11
Correspondence Analysis of morphology data for axis 1 and 2
showing the sections of *Miscanthus*.

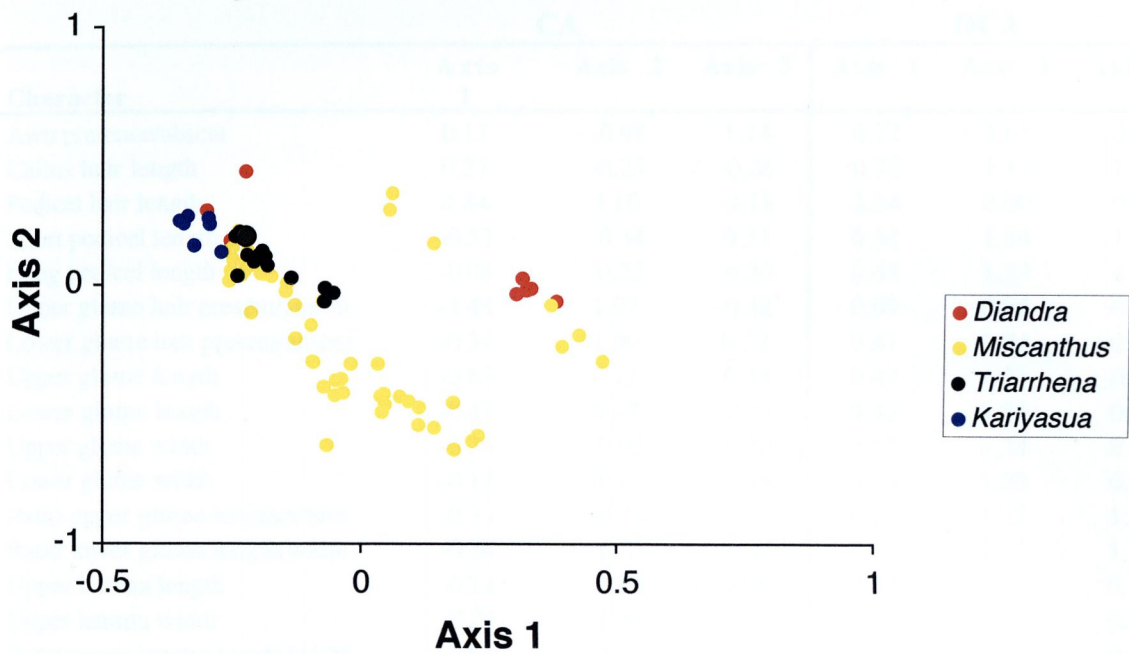


Fig. 2.12
Detrended Correspondence Analysis of *Miscanthus* morphology
data for axis 1 and 2

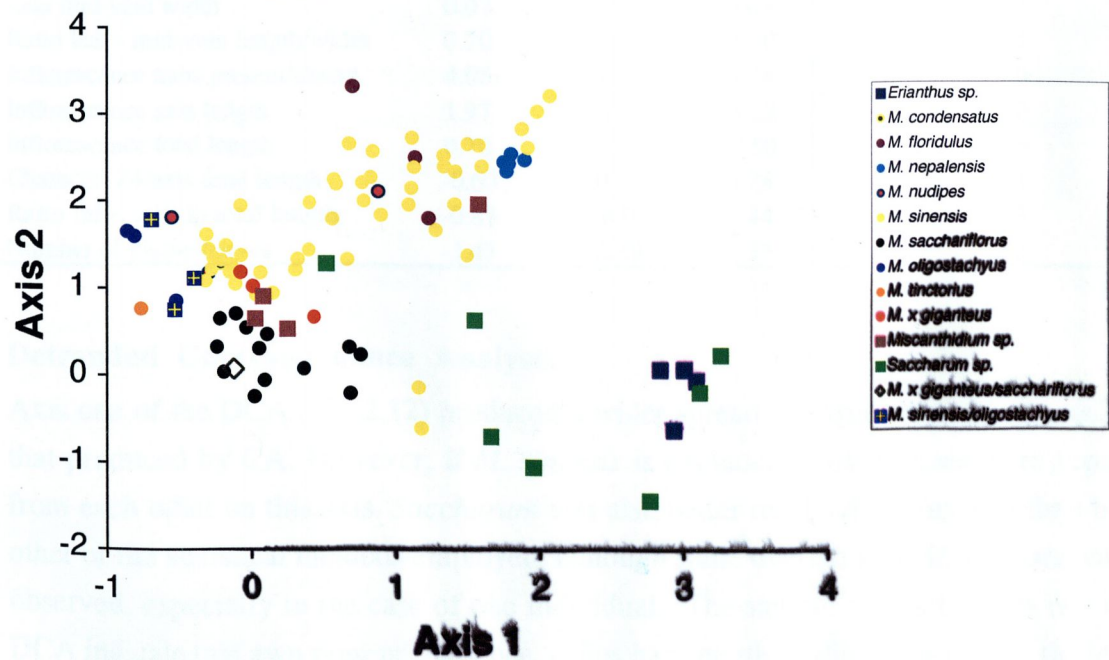


Table 2.6 Sample scores for axis one, two and three of Correspondence and Detrended Correspondence Analyses.

Character	CA			DCA		
	Axis 1	Axis 2	Axis 3	Axis 1	Axis 2	Axis 3
Awn presence/absent	0.11	-0.98	1.14	0.72	1.65	0.76
Callus hair length	0.27	-0.23	-0.26	0.72	1.17	1.18
Pedicle hair length	4.84	3.10	-4.18	2.04	0.00	0.50
Short pedicle length	-0.53	-0.34	0.33	0.51	1.34	1.24
Long pedicle length	-0.08	-0.22	-0.20	0.63	1.23	1.19
Upper glume hair present/absent	-1.43	1.97	-0.48	0.09	0.95	0.87
Lower glume hair present/absent	-0.31	1.80	0.22	0.47	0.94	0.93
Upper glume length	-0.63	0.22	-0.14	0.43	1.21	0.89
Lower glume length	-0.47	0.07	-0.25	0.48	1.21	0.91
Upper glume width	-0.24	-0.02	-0.28	0.57	1.24	0.78
Lower glume width	-0.12	-0.05	-0.18	0.60	1.25	0.78
Ratio upper glume length/width	-0.33	-0.14	0.00	0.54	1.27	1.12
Ratio lower glume length/width	-0.34	-0.21	-0.25	0.54	1.23	1.16
Upper lemma length	-0.14	0.29	0.08	0.58	1.24	0.78
Upper lemma width	-0.29	-0.59	0.03	0.58	1.39	0.89
Ratio upper lemma length/width	1.47	0.85	-0.15	1.05	1.00	0.91
Lower lemma length	-0.44	0.38	-0.03	0.48	1.21	0.70
Lower lemma width	-0.16	-0.35	0.00	0.62	1.34	0.86
Ratio lower lemma length/width	1.28	0.60	-0.35	0.99	1.00	0.81
Palea length	0.03	0.64	-0.16	0.65	1.10	0.62
Palea width	-0.06	0.01	0.49	0.65	1.34	0.82
Ratio palea length/width	0.60	0.30	-1.02	0.81	0.98	0.76
Leaf width	0.35	-1.21	-1.18	0.81	1.22	1.19
Leaf mid-vein width	0.07	-1.97	0.05	0.74	1.66	1.15
Ratio leaf - mid-vein length/width	0.20	0.38	-1.30	0.73	0.84	1.00
Inflorescence hairs present/absent	4.08	1.27	4.94	1.93	1.60	0.83
Inflorescence axis length	1.97	-1.61	-1.22	1.32	1.30	1.07
Inflorescence total length	0.70	-1.26	-0.50	0.91	1.36	1.16
Character 24/axis total length	-0.60	0.77	0.28	0.48	1.41	1.21
Ratio Inflr. axis to total length	-0.83	-0.07	0.44	0.38	1.33	0.99
Number of glume nerves	-1.47	2.39	2.33	0.00	1.43	0.00

Detrended Correspondence Analysis

Axis one of the DCA (Fig 2.12) produced a wider spread of variation in *M. sinensis* than that produced by CA. However, if *M. sinensis* is excluded, most species were separated from each other on this axis. *Saccharum* was also better resolved on this axis than by any other of the statistical methods employed, although some overlap with *M. sinensis* was still observed, especially in the case of one individual. The sample scores for axis one of the DCA indicate that awn presence/absence, callus hair length, pedicle hair length, the lengths of the pedicels and characters pertaining to the inflorescence (hairs and axis length) were important in defining this axis. This is the only analysis which demonstrated the importance

of characters other than glume or lemma features as being of importance in defining the variation of axis one. Axis two of the DCA (Fig 2.12 & 2.13) was successful in separating *M. sinensis* from *M. sacchariflorus*. Practically all characters scored highly on this axis (Table 2.4) but awn presence/absence scores higher than any other character on axis two. DCA was also capable of distinguishing *M. tinctorius* and *M. oligostachyus* as being distinct from all other individuals on axis one and two. Axis three of the DCA (Fig. 2.14) was again successful in separating *M. sinensis* from *M. sacchariflorus* and *M. nepalensis* was intermediate between the two.

The plot of the sections of *Miscanthus* against axis one and two of the DCA (Fig. 2.15) clearly separates sections *Miscanthus*, *Triarrhena* and *Kariyasua* but as with all previous ordinations section *Diandra* is subdivided but within the range of section *Miscanthus*.

Principal Coordinate Analysis

PCO analysis produced an extremely wide spread of variation within *M. sinensis* (Fig 2.16) on axis one, which explained 30.8% of the data variance. The only *Miscanthus* species outside of this range were *M. tinctorius*, two individuals of *M. oligostachyus* and two individuals of *Saccharum*. Unfortunately the PCO analysis carried out did not provide information on which characters were important in defining the various axis so the cause for this variation cannot be explained. Axis two of the PCO (Fig. 2.16 & 2.17) which explained 14.6% of the variance separated *Erianthus* and six out of seven individuals of *Saccharum* from all other species. It also showed a clear separation of *M. sacchariflorus* and *M. nepalensis* from *M. sinensis* but not from each other. Axis three of the PCO (Fig 2.18) accounting for 12.0% of the data variance was successful in differentiating between *M. sacchariflorus*, *M. sinensis*. *Miscanthium* was better resolved from *M. sinensis* on this axis by PCO than with the other methods employed. *Erianthus* and *Saccharum* were clearly separate from each other on this axis.

As with all other forms of analysis used sections *Miscanthus*, *Kariyasua* and *Triarrhena* were supported by PCO, while Section *Diandra* was obscured. As in all previous ordinations of the sections of *Miscanthus*, section *Diandra* is split into two groups, one containing members of the species *M. nepalensis* and the other *M. nudipes* (Fig. 2.19).

Cluster Analysis

Cluster analysis of all characters scored using Wards method transformed to Z scores (Fig 2.20) produced similar results to those obtained by ordination techniques. i.e. *M. sinensis* produced a wide spread of variation which overlapped the range of variation of most other species. However, the separation of the species was less than with any of the other

Fig. 2.13
Detrended Correspondence Analysis of *Miscanthus* morphology
data for axis 2 and 3

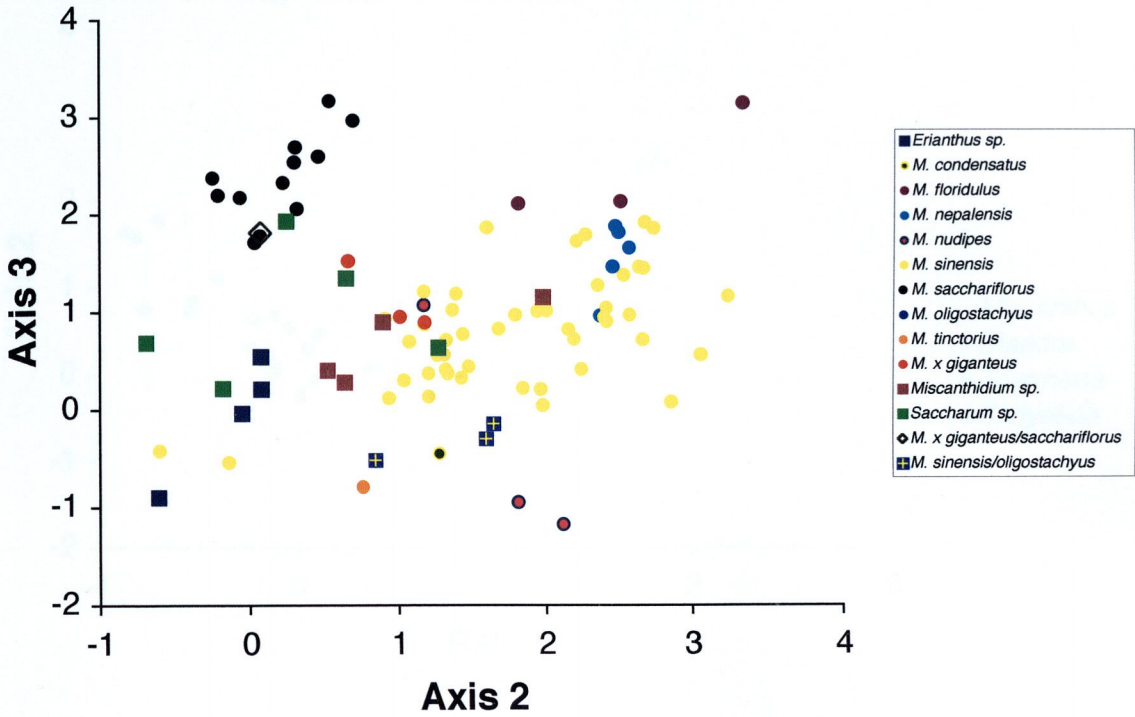


Fig. 2.14
Detrended Correspondence Analysis of *Miscanthus* morphology
data for axis 1 and 3

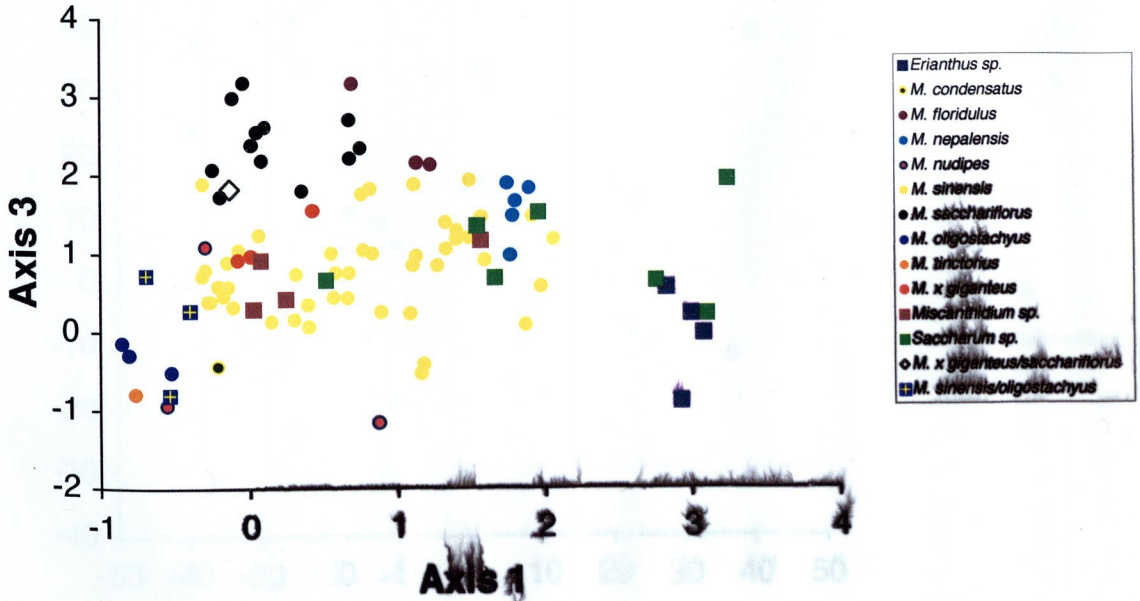


Fig. 2.15

Detrended Correspondence Analysis of morphology data for axis 1 and 2 showing *Miscanthus* sections.

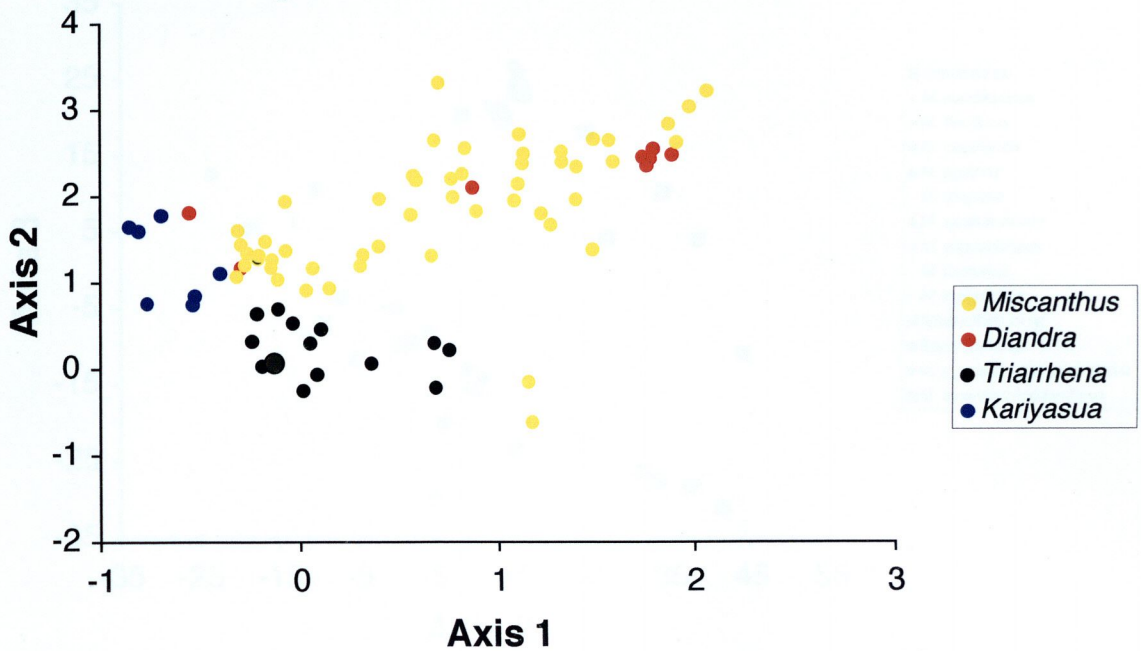


Fig. 2.16

Principal Coordinate Analysis of *Miscanthus* morphology data for axis 1 and 2

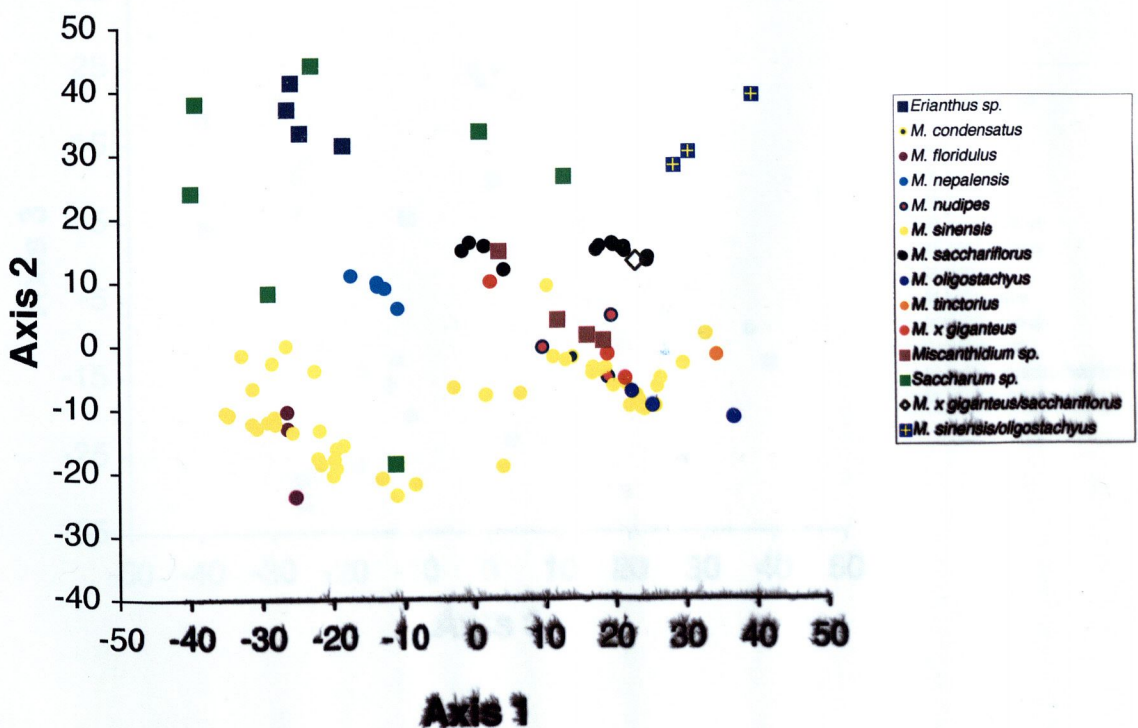


Fig. 2.17
Principal Coordinate Analysis of *Miscanthus* morphology data for axis 2 and 3

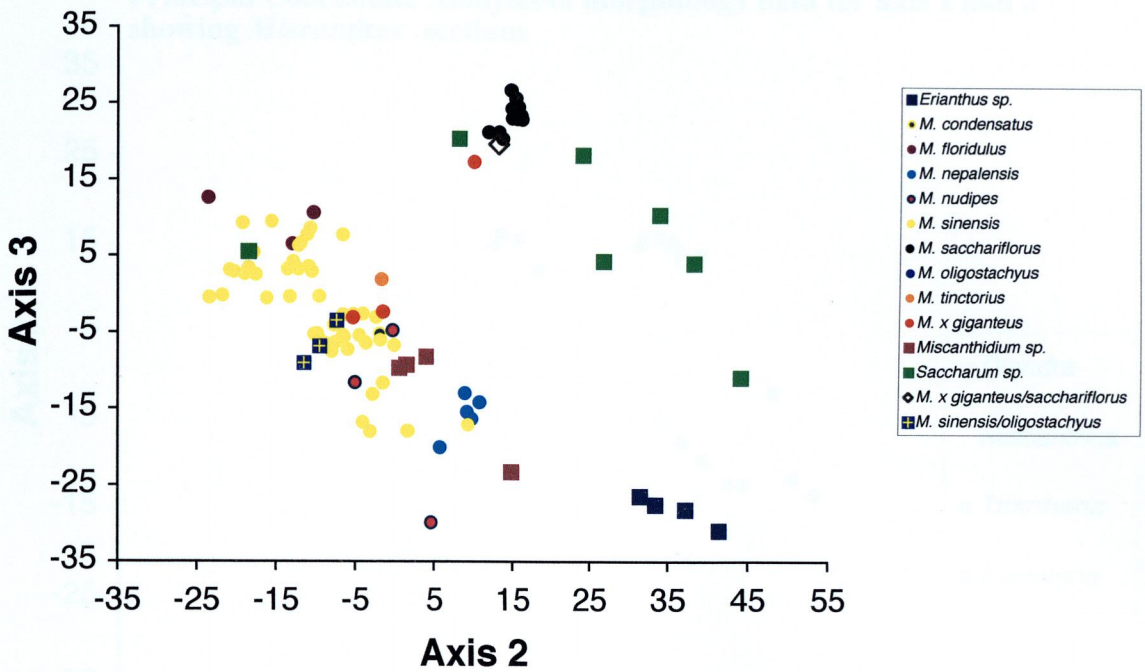


Fig. 2.18
Principal Coordinate Analysis of *Miscanthus* morphology data for axis 1 and 3

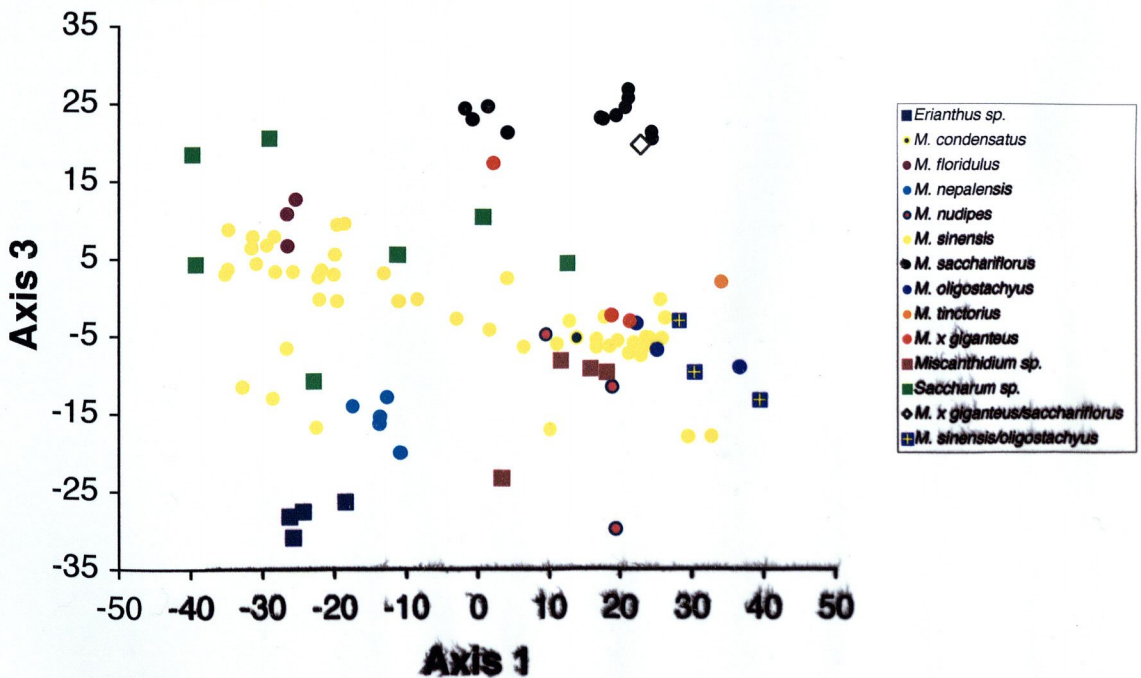


Fig. 2.19
Principal Coordinate Analysis of morphology data for axis 1 and 2
showing *Miscanthus* sections

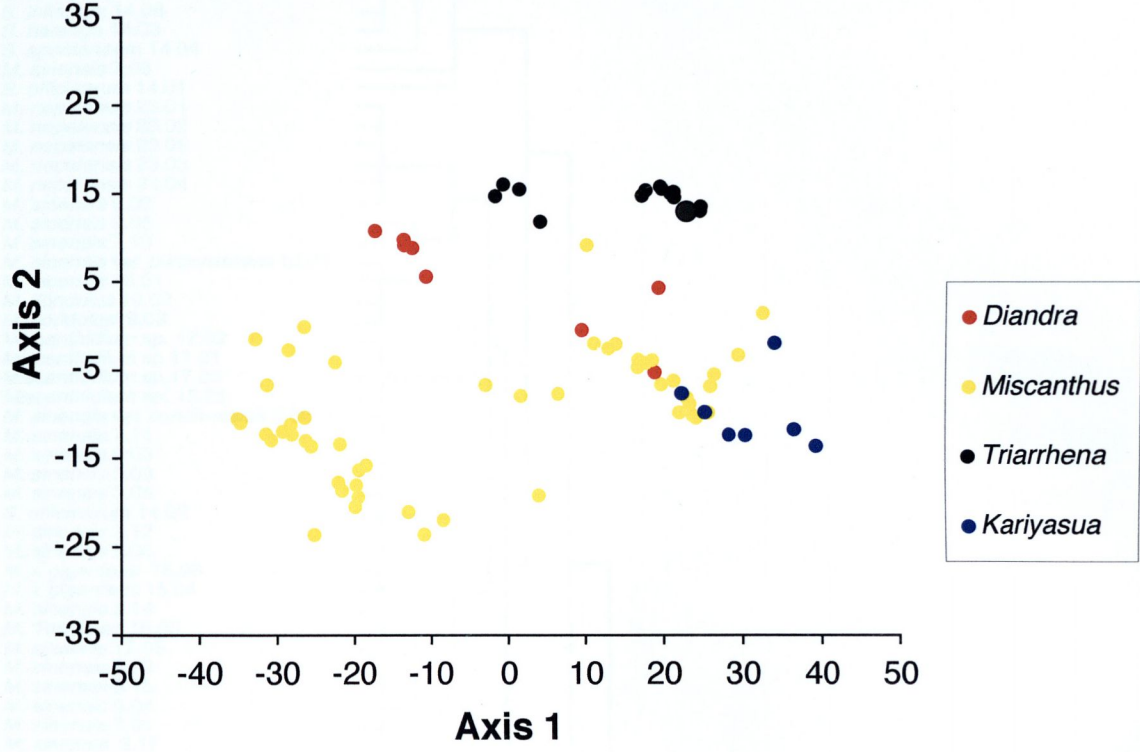


Fig 2.20 Cluster analysis of *Miscanthus* morphology data using Wards method, transformed to Z-scores. Figures after species names refer to herbarium sample examined and listed in appendix 2.

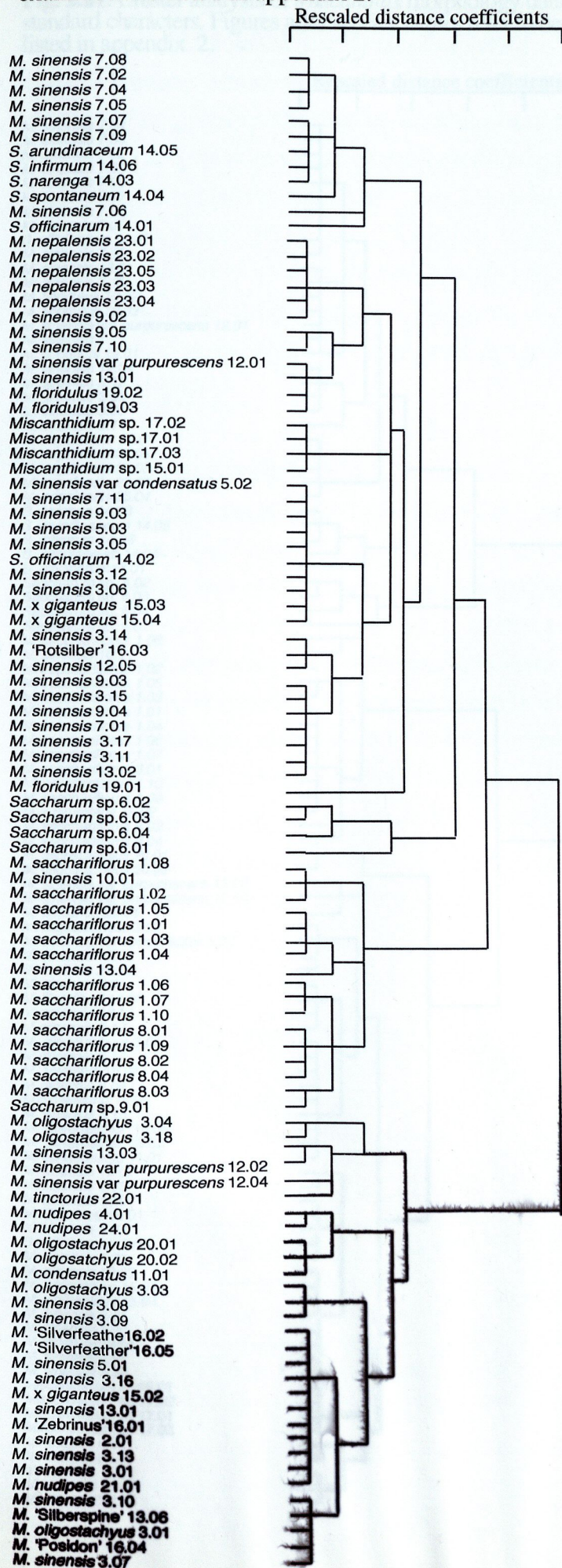
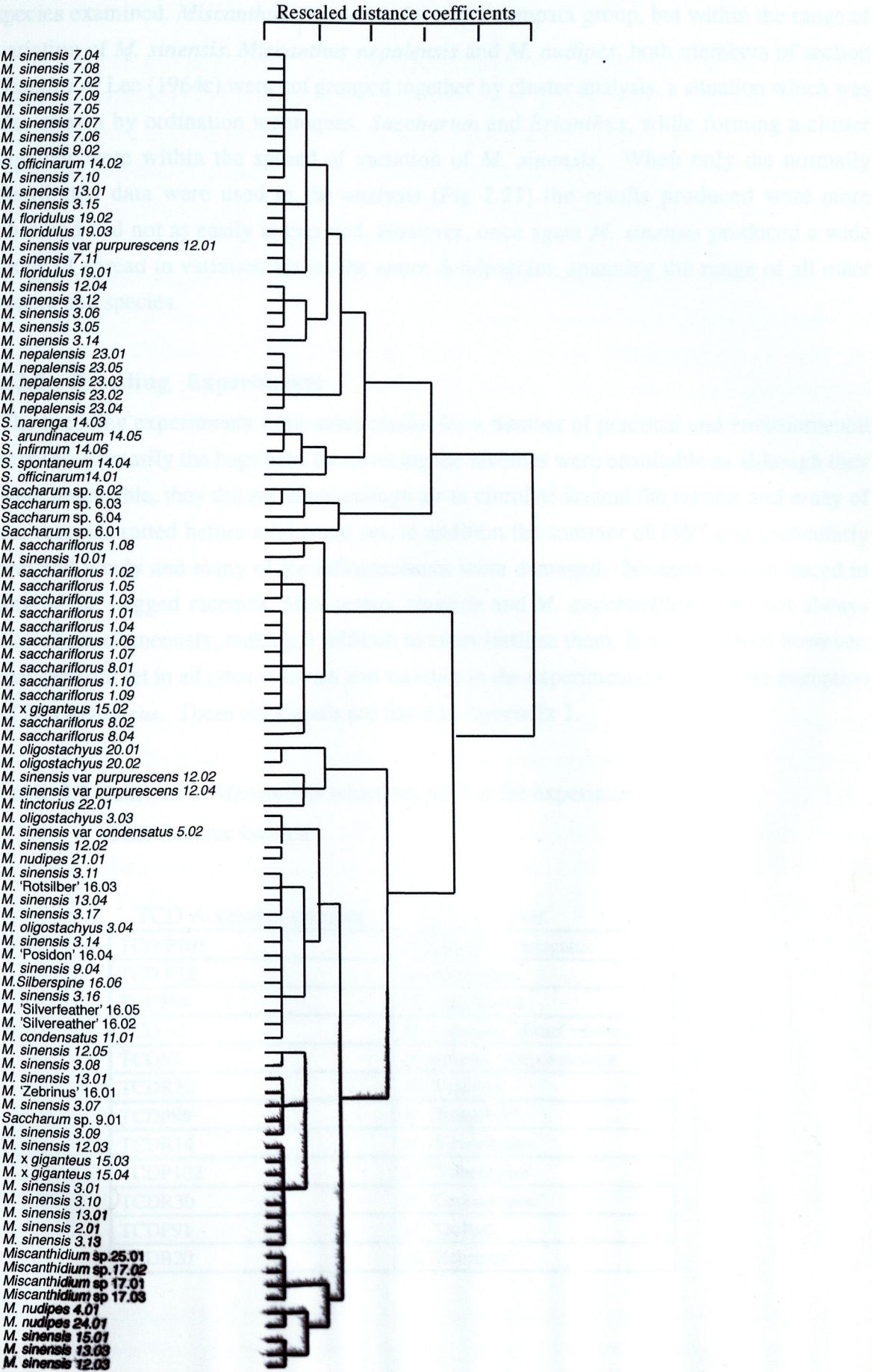


Fig. 2.21. Cluster analysis of *Miscanthus* morphology data using Wards method of all standard characters. Figures after species names refer to herbarium sample examined and listed in appendix 2.



techniques used. Again the main separation was between *M. sinensis* and *M. sacchariflorus*, while *M. sinensis* produced a wide range of variation across the entire range of genera and species examined. *Miscanthus nepalensis* formed a compact group, but within the range of variation of *M. sinensis*. *Miscanthus nepalensis* and *M. nudipes*, both members of section *Diandra* of Lee (1964c) were not grouped together by cluster analysis, a situation which was also shown by ordination techniques. *Saccharum* and *Erianthus*, while forming a cluster together were within the spread of variation of *M. sinensis*. When only the normally distributed data were used in the analysis (Fig 2.21) the results produced were more obscured and not as easily interpreted. However, once again *M. sinensis* produced a wide range of spread in variation across the entire dendrogram, spanning the range of all other genera and species.

2.3.2. Breeding Experiments

The breeding experiments were unsuccessful for a number of practical and environmental reasons. Primarily the bags used for covering the racemes were unsuitable as although they were breathable, they did not allow enough air to circulate around the raceme and many of the racemes rotted before seed could set. In addition the summer of 1997 was particularly wet and windy and many of the inflorescences were damaged. No seed was produced in any of the bagged racemes. *Miscanthus sinensis* and *M. sacchariflorus* did not always flower simultaneously, making it difficult to cross fertilise them. It was observed however, that seed did set in all other cultivars and varieties in the experimental plot with the exception of *M x giganteus*. These individuals are listed in Appendix 1.

Table 2.7 Cultivars of *Miscanthus* which set seed in the experimental plot at TCD Botanic Garden.

TCD Accession number	Cultivar
TCD P103	<i>M. sinensis</i> 'variagatus'
TCD P18	<i>M. gracillimus</i>
TCDP94	<i>M. condensatus</i>
TCD S1	<i>M. x sinensis</i> (dwarf variety)
TCDS2	<i>M. sinensis</i> 'purpurecence'
TCDR32	<i>M.</i> 'Posidon'
TCDP99	<i>M.</i> 'Rotsilber'
TCDR14	<i>M.</i> 'Silverfeather'
TCDP102	<i>M.</i> 'Silberspine'
TCDR30	<i>M.</i> 'Grossefontae'
TCDP91	<i>M.</i> 'Goliath'
TCDB20	<i>M.</i> 'Zebrinus'

2. 4 Discussion

At the beginning of this work it was hoped that the data set produced from the morphological analysis would permit a cladistic approach to be taken in addition to a phenetic approach. This, however, did not prove possible as only a very limited number of discrete characters could be recorded. It would thus appear that it is not possible to produce a well resolved and supported phylogeny for *Miscanthus* based on morphological analysis alone. To the advocates of cladistics, this in itself is quite meaningful, as it could be argued that if it is not possible to find discrete characters, within the limits of the species as currently defined, they may not be true species. However, finding discrete characters is one of the inherent problems of cladistics. Many characters which phenetists would call continuous are categorised by cladists to force them into being discrete. This leads to the questioning of whether they are cladistically significant or not (Scotland, 1992). Notwithstanding the possibility that the lack of discrete characters may result in the inability to define the current species, all of the characters scored were subjected to the techniques of numerical taxonomy. The aim of analysing the data in this manner was to examine if these techniques could provide an estimate of the range of variation within the constituent species of *Miscanthus* and related genera.

The aim of all ordination techniques is to arrange entities in a continuous way in multivariate space. It is not a statistical test, simply a way in which data sets can be simplified so that the spread of data can be examined. The summarising of data in this manner allows hypotheses to be made about the underlying causes of patterns in the data (Hermý, 1988). For these reasons multivariate methods are often exploratory methods and contrast with cluster methods, which attempt to structure the data into groups of similar entities with the aim of producing a classification. In the examination of the data collected from the morphological analysis of *Miscanthus* a number of ordination techniques were employed in order to explore the spread of variation within *Miscanthus* rather than produce a classification. In general most of these techniques provided a roughly similar, but not identical result. This is to be expected as, depending on the type of data, not all techniques will give a similar result. Many of the causes of this variation are outlined under the materials and methods section of this chapter and are also explained by Minchin (1987), who examined the robustness of PCA, PCO and DCA in relation to each other. Many of Minchin's assumptions, are however, based on the use of ecological data sets and not all of the problems encountered with ecological data will apply to morphological characters.

The general consensus of the ordination techniques used here is that while the majority of the individuals within a species did group together, especially in two dimensional space (axis

one and two), there was considerable overlap between some of them. *Miscanthus sinensis* has such a wide spread of variation that it encompassed all other species within the genus, with the exception of *M. sacchariflorus*, *M. tinctorius* and *M. oligostachyus* which appear to be marginally outside of the range of *M. sinensis*. Unfortunately only one individual of *M. tinctorius* was available for analysis and so the variation within this species could not be examined. It is possible that if more individuals were examined the distinction of *M. tinctorius* could be more clearly resolved. The two individuals of *M. oligostachyus* and the three specimens labelled as *M. sinensis*/*M. oligostachyus* in the analysis appeared to fall into a separate group apart from all other species with the exception of *M. tinctorius*. It appears clear now that the specimens labelled *M. sinensis*/*M. oligostachyus* can be determined as *M. oligostachyus*. *Miscanthus nepalensis* generally formed a group with a relatively narrow range of variation, but always within the range of variation of *M. sinensis*. The broad range of variation in *M. sinensis* has been observed by others (Ohwi, 1965). This variation is not surprising due to the geographic range of the species and its ability to freely hybridise with *Saccharum*. This species in particular has also been cultivated within the horticultural community giving rise to a large number of ornamental varieties. All of the cultivars of *M. sinensis* examined in this analysis demonstrated their close relationship to the parental species. *Miscanthus x giganteus*, considered to be a cross between *M. sacchariflorus* and *M. sinensis* by Greef & Deuter (1993), generally appeared morphologically closer to *M. sacchariflorus* when analysed by CA, but overlapped the *M. sinensis* group by all other ordination methods used. As part of the aim of this project was to examine the taxonomy of the various cultivars of *Miscanthus* it can now be stated that based on multivariate techniques all of these cultivars appear to be closely related to *M. sinensis*, and should be included within it. Their relationship cannot however be fully resolved by morphological techniques due to the broad range of variation within *M. sinensis*.

When the characters responsible for the variation on the various axes of all of the statistical methods employed are considered together, those characters of most importance appear to be those pertaining to the lemmas, glumes, glume hairs, inflorescence axis length and hair presence and pedicel hair presence. Lee (1964c) considered the main axis of the inflorescence, nerves of glume, dorsal hairs on glume, awns and callus hairs as being useful in delimiting both species and sections within *Miscanthus*. The results of this analysis would only agree with the main axis of the inflorescence and glume hairs of being of importance. Awns and callus hairs only scored highly on axis one of the DCA and glume nerves did not score highly on any axis. While various keys based on a few key characters have been produced by others (Clayton & Renvoize, 1986; Hodgkinson *et al.*, 1997; Lee, 1964c) and are successful in differentiating between species, a multivariate approach obscures these differences. Therefore, while it is usually possible to differentiate species by key characters, one has to make a decision as to where to delimit species when using a multivariate

approach. In the case of the genus *Miscanthus* the only species which are quite well separated are *M. sinensis*, *M. sacchariflorus*, *M. tinctorius* and *M. oligostachyus*.

It is interesting to note that DCA was the only form of analysis which considered presence/absence characters to be of importance in explaining the variation on the individual axis. Even with these characters the overall result of the analysis was similar to those techniques which either did not include these characters or calculated them as being not significant.

The ordination techniques used provided a clear assessment of the relationships between the sections of *Miscanthus* with the exception of section *Diandra*. Section *Diandra* is acknowledged by Lee (1964c) as being very similar to section *Kariyasua*, the only distinction being the number of stamens in *Diandra* being two rather than three. However, this analysis would place the two members of section *Diandra* examined (*M. nepalensis* and *M. nudipes*) within section *Miscanthus*. Section *Triarrhena*, which contains only *M. sacchariflorus* is clearly resolved and therefore well supported. Section *Kariyasua* which contains *M. tinctorius* and *M. oligostachyus* is also supported, with the realisation that the third member of this group, *M. changii* has not been examined.

When the relationship between the genera is examined the distinction between *Miscanthus* and *Saccharum* is unresolved by any of the statistical methods employed. The distinction between these two genera is currently based on the sessile nature of one of the spikelets and the fragility of the rachis in *Saccharum*, characters which according to Clayton & Renvoize (1986) are rather weak characters on which to split a genus. When one of these characters, the sessile nature of the spikelet, is included in a multivariate data set, where all characters are given equal weighting, its value is diminished to the point that it is no longer of importance in explaining any of the variation. When factor scores for the various characters are examined, in any of the statistical methods used, this character never produced a high factor score. The genus *Erianthus* which consistently occurred outside of the range of variation of most other species and of the genus *Saccharum* is in fact considered to be a sub-genus of *Saccharum* by Clayton and Renvoize (1986). The morphological distinction between the two being based on the presence of an awn in *Erianthus*. However, even when the data were analysed by PCA without the inclusions of this character, *Erianthus* was still more clearly resolved than *Saccharum*. When it is considered that *Miscanthus* also has awned and unawned species within it, it appears that to split a closely related genus on this character is inconsistent and rather superficial, a view that is also supported by Bentham (1882). If *Erianthus* is included within *Saccharum* then the distinction between *Miscanthus* and *Saccharum* appears somewhat clearer.

Miscanthidium, which includes the four African species of *Miscanthus* (*Miscanthidium ecklonii*, *M. violaceus*, *M. junceus* and *M. sorghum*) always fell within the range of variation of *Miscanthus*. These four species are separated from the genus *Miscanthus* by their elongated inflorescence axis and short racemes. However this distinction is blurred by intermediates such as *M. floridulus* which does not warrant placing them in a separate genus (Clayton & Renvoize, 1986).

The clustering methods used are designed to produce a classification from the data matrix. The two classifications produced from Wards method using Z scores (Fig. 2.20) and Wards method using continuous characters only (Fig. 2.21) largely agreed with each other. Both techniques showed *M. sacchariflorus* to be distinct and separate group from all other species. *Miscanthus sacchariflorus* can be identified by its lack of awns and profusion of long callus hairs. *Miscanthus sinensis* showed a wide spread of variation across all other species, as was also indicated by the ordination techniques. The distinction among all of the other species was not clearly resolved, *M. nepalensis* was unresolvable from the *M. sinensis* group and removed from *M. nudipes* which belongs to the same section within the genus. *Miscanthus oligostachyus* and *M. tinctorius* clustered together forming the section *Kariyasua*. The classification produced would not agree with the current classification of *Miscanthus* based on key characters. The African species of *Miscanthus* referred to as *Miscanthidium* are not classified separately to *Miscanthus* and are in fact grouped closely to *M. floridulus*, reflecting the views of Clayton & Renvoize (1986), that it should not be placed in a separate genus. The position of *Saccharum* in relation to *Miscanthus* shows *Saccharum* to have been subdivided into two distinct groups both covered by the range of variation within *Miscanthus*. It does not appear to be distinct based on the morphological characters used in this analysis. However as only a limited number of species were examined the range of variation within *Saccharum* cannot be determined.

This is the first known multivariate assessment of morphology which examines the relationship of the cultivars in relation to the other species. The results demonstrated that all of the cultivars show a close relationship to *M. sinensis*, indicating that they are taxonomically closer to this species than any other within the genus. The three individuals of *M. x giganteus* appear to be intermediate between *M. sinensis* and *M. sacchariflorus* when analysed by ordination techniques, which would support the theory that this is a hybrid between these two species. However, the position of *M. x giganteus*, when analysed by cluster analysis is more difficult to interpret, with two of the individuals clustering with *M. sinensis* and the third with *M. sacchariflorus*.

The general consensus of the morphological assessment of *Miscanthus* is that the wide spread of variation within *M. sinensis* obscures the definition of *M. floridulus*, *M.*

nepalensis and *M. nudipes*. It also indicates that the section *Diandra* of *Miscanthus* is unresolvable by multivariate techniques. However the constituent species of *Diandra* were included in the “*Saccharum* complex”, an informal group of *Saccharum* species involved in the origin of commercial sugar cane Daniels & Roach (1987). Further investigation of these species by other methods is warranted before a firm decision on their status is taken. However, it must be noted that Daniels & Roach (1987) considered *Diandra* to be the most taxonomically confused section within *Miscanthus*. This they attribute to the revision of the species of *Diandra* by (Grassl, 1971), a revision which they believe to be largely invalid. The results of multivariate analysis further suggests that the African *Miscanthus* species are not sufficiently distinct from the Asian species to warrant their placement in a separate genus. *Saccharum* is also not separated by ordination techniques in relation to *Miscanthus*. With this inability to fully elucidate the classification of *Miscanthus* by multivariate morphological techniques, a molecular approach was taken. It has to be concluded here from morphological analyses that although the sections of *Miscanthus* (Lee, 1964) are taxonomically useful, they are not distinct when numerous characters are considered. Such findings are supported in subsequent chapters of the thesis that utilise molecular characters and a phylogenetic approach.

CHAPTER 3

RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS

3.1 INTRODUCTION

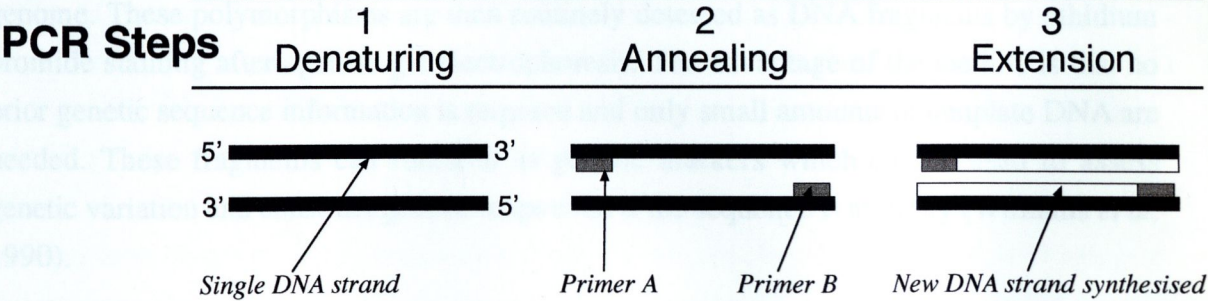
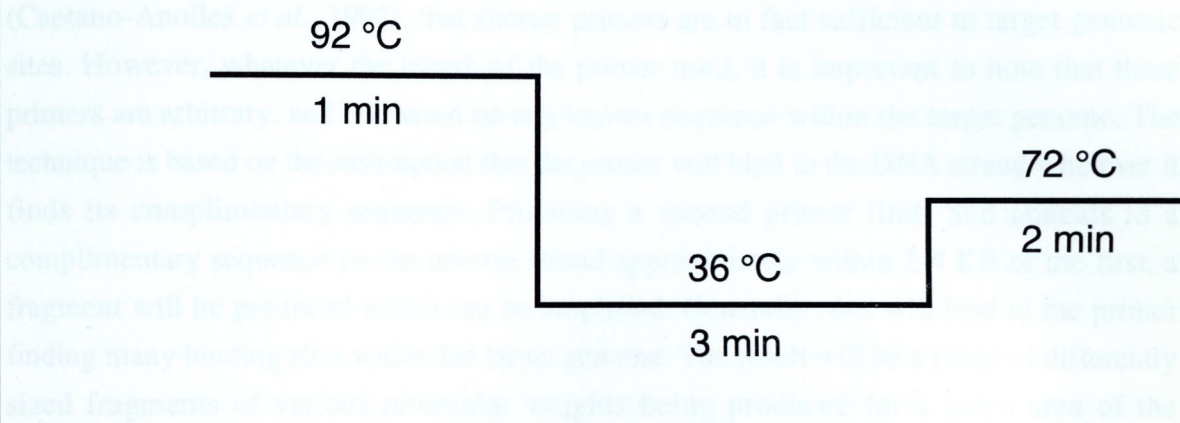
The Polymerase Chain Reaction (PCR) was first described by Keppe *et al.* (1971), although credit for its development is attributed to Mullins & Faloona (1987), at the Cetus Corporation. The development of the PCR technique by the Cetus Corporation opened up a vast new area in molecular biology whereby minute pieces of DNA could be amplified to amounts which could be handled and worked with in a wide range of applications. As the PCR is the precursor to all of the molecular techniques described in this thesis the principal of the technique is outlined in the following section.

The basic PCR cycle is relatively simple and consists of three basic steps (Fig. 3.1). In the first step double stranded DNA is denatured to produce its constituent two strands. This is necessary as single stranded template is required for the PCR. The second step involves annealing single stranded oligonucleotide primers to the single stranded DNA. By lowering the reaction temperature to approximately 45-50°C the primers will anneal to the single stranded DNA wherever they find their complimentary sequence. In the third step *Taq* DNA polymerase (a heat-stable DNA polymerase, isolated from the thermophilic bacterium, *Thermus aquaticus*, which was first discovered in hot spring systems) is added to the reaction. This enzyme is particularly important in the PCR reaction as it is able to withstand the high denaturing temperatures used in the reaction. The polymerase adds nucleotides (which have been added to the reaction mixture to provide a substrate) to the 3' end of the primer sequence annealed to the template DNA. The primers are necessary for the initiation of the reaction and the template DNA serves as reference for the polymerase. The complimentary strands are then allowed to reassociate by cooling. The primers are incorporated into each new strand of DNA produced, thus providing new priming sites in all subsequent PCR cycles. During each cycle, the target DNA is replicated by a factor of two, thus within twenty five cycles a single strand of DNA can be amplified 2^{24} times.

When the PCR technique was first devised, temperatures required for the three steps were achieved by manually moving reaction tubes between water baths or hot blocks at the correct temperature. The development of automated thermal cycling instruments which allows the required temperature for each step to be achieved rapidly and precisely and repeated many times has revolutionised the PCR technique. The PCR was a major development in the analysis of DNA because it simplified existing technology and allowed the development of

fast, simple and relatively inexpensive DNA analysis methods which would not have been previously possible.

The original RAPD (Randomly Amplified Polymorphic DNA) technique was developed by Williams *et al.* (1990) and Walsh & McClelland (1990). The technique is arguably one of the simplest and cheapest molecular techniques by which DNA fingerprints can be produced. RAPD is a technique whereby DNA polymorphisms are produced using



As RAPD can produce many polymorphisms from a single genomic region, it is particularly useful for the analysis of genetic variation. However, as the technique is based on the analysis of random DNA fragments, the method is only useful for the detection of gross genomic differences (Williams *et al.* 1993). With this in mind, the advantages of the technique are that DNA fingerprints could easily be compared with those of other individuals. The optimisation of the PCR technique has been a major area of research, with considerably reduced cycle times (e.g. 30 cycles) and the use of miniaturised processing (Yu & Paulsen 1993). However, the use of PCR in molecular ecology has been reviewed by (e.g. 1993).

fast, simple and relatively inexpensive DNA analysis methods which would not have been previously possible.

The acronym RAPD (Random Amplified Polymorphic DNA) was first coined by Williams *et al.* (1990), although the actual technique to which the name is applied was simultaneously developed by Williams *et al.* (1990) and Walsh & McClelland (1990). The technique is arguably one of the simplest and cheapest molecular techniques by which DNA fingerprints can be produced. RAPD is a technique whereby DNA polymorphisms are produced using single, typically ten base pair long, primers and the PCR. It has been suggested by others (Caetano-Anolles *et al.*, 1992), that shorter primers are in fact sufficient to target genomic sites. However, whatever the length of the primer used, it is important to note that these primers are arbitrary, and not based on any known sequence within the target genome. The technique is based on the assumption that the primer will bind to the DNA strand wherever it finds its complementary sequence. Providing a second primer finds and anneals to a complementary sequence on the reverse strand approximately within 2-4 KB of the first, a fragment will be produced which can be amplified. Generally, this will lead to the primer finding many binding sites within the target genome. The result will be a range of differently sized fragments of various molecular weights being produced for a given area of the genome. These polymorphisms are then routinely detected as DNA fragments by ethidium bromide staining after agarose gel electrophoresis. The advantage of the method is that no prior genetic sequence information is required and only small amounts of template DNA are needed. These fragments can function as genetic markers which can be used to assess genetic variation and construct genetic maps even if the sequence is arbitrary (Williams *et al.* 1990).

As RAPD can potentially detect numerous loci in the genome, the method is particularly useful for the analysis of genetic distance. However, it is important to realise that RAPD is based on the analysis of anonymous bands of DNA and the fact that any fingerprinting method is only useful if the variation in banding patterns reflects true allelic segregation at independent loci. This should not be confused with PCR artefacts (Clark and Lanigan, 1993). With this borne in mind, the obvious advantages of the technique are that DNA fingerprints could quickly and rapidly be established. Within a relatively short period of time optimisation of the PCR technique for RAPD was developed, allowing run times to be considerably reduced and therefore increasing the numbers of samples that could be processed (Yu & Pauls, 1992). Reiter *et al.* (1992), showed that genetic linkage maps could be constructed in a fraction of the time taken to produce a similar map by Restriction Fragment Length Polymorphism (RFLP) analysis. These factors have led to wealth of publications utilising the technique for various studies. The use of RAPD analysis in molecular ecology was the subject of a mini review by Hadrys *et al.*, (1992). In this review

the authors illustrate several potential applications of RAPD, including determination of taxonomic identities, detection of interspecific gene flow, assessment of kinship relationships, analysis of mixed genome samples and production of specific probes. All of these areas have since been explored and proved possible by the RAPD technique. RAPD have produced species-specific markers for Irises (Arnold *et al.*, 1991), cultivar specific markers, (Graham *et al.*, 1994) and population specific markers (Reiter *et al.*, 1992). They have also been successfully used to assess geographic migration patterns in *Juniperus* (Adams *et al.*, 1993) and for resolving interspecific and intraspecific evolutionary pathways in the grass genus *Brachypodium* (Catalan *et al.*, 1995).

Although RAPD initially showed a great deal of promise as a new and innovative DNA fingerprinting method, other researchers were quick to point out the disadvantages of the method and in many cases these outweighed the advantages. The primary disadvantage of RAPD is that the homology of co-migrating bands cannot be ascertained without excising the band from the gel and characterising it further by for example reprobing the product by Southern analysis (Southern, 1975) or sequencing the amplification product (Hadrys *et al.*, 1992). This rather negates the simplicity of the technique, although it has been claimed by some authors that when sequenced, co-migrating bands were homologous (Wilkie *et al.*, 1993). However, other workers (Catalan *et al.*, 1995) have shown that while co-migrating bands were homologous between closely related species (within *Brachypodium*) and may be useful at the specific and infraspecific levels, they can be non-homologous in more distantly related species and were in fact unreliable at the tribal level in grasses (Catalan *et al.*, 1995). (Xu *et al.*, 1995) have shown that bands which appeared absent after ethidium bromide staining were detected after hybridisation. They further concluded that while RAPD analysis did produce fingerprints for the analysis of grape rootstocks, they were unreliable due to inconsistencies in the amplification reactions. Xu *et al.*, (1995) postulated that standardisation of the technique would be difficult to achieve within a laboratory let alone between different laboratories. This problem was further tested by a group of European laboratories who also found difficulties in reproducing results for the same samples tested in different laboratories, even though they all followed a standardised protocol (Jones *et al.*, 1997).

Nearly all RAPD markers are dominant, i.e. the presence of a band is dominant over the absence of a band and heterozygotes cannot therefore be detected (Williams *et al.* 1990; Carlson *et al.* 1991), which makes them less convenient genetic markers than co-dominantly inherited markers such as Restriction Fragment Length Polymorphisms (RFLP). Because the analysis of population genetic structure is hindered by the dominance of inherited bands, Lynch and Milligan (1994) have estimated that two to ten times more individuals per locus need to be sampled when dominant markers are being scored. Clark & Lanigan (1993) have

shown that dominance will result in an underestimate of nucleotide diversity and that this must be corrected for in any analysis using RAPD data to estimate diversity. Although RAPD data has been used to distinguish individuals, cultivars or accessions, the difficulty of achieving fully reliable profiles makes their validity for 'typing' questionable (Karp *et al.*, 1996). Due to the dominant nature of bands RAPD should not be used for phylogeny reconstruction. However, there are some cases where the dominance effect becomes insignificant. Van Heusden & Bachmann (1992) showed the utility of RAPD markers for detecting monophyletic strains in populations of *Microseris elegans* where a virtual absence of heterozygosity exists due to the inbreeding pattern of reproduction within these particular populations, these populations had previously been determined to represent a clonal population.

The reproducibility of the technique has also been called into question by many authors (Graham *et al* 1994; Marshall *et al.*, 1995; Skroch and Nienhuis, 1995). In all cases it has been noted that even a slight variation within the technique (e.g. altering the amount of template DNA or primer concentrations) can produce additional bands. However, it has also been shown by these authors that standardising the conditions of the reaction can remove most of this error. Skroch & Nienhuis (1995) have also shown that the actual scoring of bands can be a problem and have suggested scoring errors of 2%, while Weedon *et al.* (1992) have demonstrated scoring errors of up to 8%. Scoring errors are largely attributed to human error whereby weak bands, which may be artefacts, are scored as present or bands are scored as absent when they are actually present but obscured due to the presence of a strong wide band. Scoring errors are more problematic when high levels of polymorphism occur. As the sensitivity of the technique can be increased by using different types of gels (e.g. acrylamide) and different staining systems (e.g. silver staining), it is recommended that these techniques are only used when low levels of polymorphism are present (Bachmann, 1994).

Another source of error arises from the amplification of unrelated DNA. This may include impurities and parasites on the material from which the DNA has been extracted (Bachmann, 1994). This may be a particular problem when extracting DNA from herbarium specimens which are often infected by fungal parasites. For this reason strict quality controls should be implemented when extracting DNA, especially from small amounts of material. It must be borne in mind at all times that the bands are essentially anonymous fragments of DNA and that standardisation of the RAPD technique, running of positive and negative controls and sufficient replication of samples are essential to limiting the number of unreliable bands.

Even with a strict quality control system in operation unreliable bands cannot be completely eliminated and it is safer to disregard them (Bachmann, 1994).

3.2.1 DNA extraction

Other techniques, known as Inter simple sequence repeats PCR (InterSSR PCR) and interSSR-anchored PCR amplification have evolved out of RAPD analysis. These techniques rely on the fact that simple sequences are repeated many times throughout a plant's genome. These units which are often less than four nucleotides in length are highly interspersed throughout the eukaryotic genome (Thomas & Scott, 1993). These repeat regions, also known as microsatellites have been used as primers to which either a purine or a pyrimidine have been anchored to the 3' end of the microsatellite. This anchoring avoids the problem of priming from within a repeat and provides specificity that reduces the number of targeted regions matching the 3' terminal residues (Zietkiewicz *et al.*, 1994). The areas between the regions can then be amplified by PCR. The various size fragments produced can be analysed in exactly the same manner as conventional RAPD analysis. The benefits of using this technique is that there is some knowledge of the area that is being amplified. In addition higher annealing temperatures can be used with these primers. This creates more stringent PCR conditions which improves the reproducibility of the technique over RAPD. Moreover, it has been suggested that because these tandem repeats occur with such frequency within the genome they should detect a higher level of polymorphism to that produced by RAPD.

Notwithstanding the problems associated with RAPD, the technique can still provide useful and reliable information provided all of its constraints are realised and taken into account. When the technique is being used as a tool in conjunction with a number of other molecular techniques it can be of benefit in helping to confirm or disprove hypotheses. For this reason RAPDs were chosen as the first molecular method used to screen several different species and genotypes of *Miscanthus* in this project. InterSSR PCR was also investigated as a method of screening genotype variation to supplement the RAPD data and to assess a different part of the genome *i.e.* the area between microsatellites.

3.2 MATERIALS AND METHODS

3.2.1 DNA extraction

A number of different DNA extraction procedures were investigated in the early stages of this project to determine which was best suited to yielding sufficient quantities of high molecular weight DNA. Initial extractions focused on phenol/ chloroform extraction after grinding in liquid nitrogen. Although phenol/chloroform extraction procedures were relatively successful, a large amount of smearing was observed when the extracted DNA was examined after gel electrophoresis with ethidium bromide staining. This was attributed to the possibility of RNA contamination in the extracted DNA. The addition of RNase to the extracted DNA was successful in eliminating most of this smearing by removing the RNA. The method which consistently provided the highest quality DNA was the method of (Hillis *et al.*, 1990). and is outlined below with the following exception:

β - mercapthoethanol was replaced with Dithiothreitol (DTT). Dithiothreitol acts to protect the sulphydral groups of DNA in the same manner as other thiols such as β - mercapthoethanol. However, DTT is more efficient at achieving this as the reaction occurs more rapidly.

Note: all of the solutions used in this section are marked with an asterisk (*) and the protocol for preparing them outlined at the end of this section.

1. Leaf material for DNA extraction was collected from living specimens by cutting newly emerging leaves from the plant and placing them directly into a plastic bag which was immediately plunged into a container of liquid nitrogen. In this way leaf material could be transported from the field site to the laboratory with the minimum amount of degradation to the samples.
2. Approximately 1.0 g (frozen weight) of leaf material was then placed in a mortar containing liquid nitrogen and ground to a paste with a pestle. The tough midrib of each leaf was removed before grinding.
3. The ground leaf material was divided between six 1.5 ml tubes and 1.25 ml CTAB/DTT solution*, which had been previously heated to 65°C, was added to each tube.
4. The sample tubes were then incubated in a water bath at 65°C for 45 minutes with occasional inversion.
5. 400 μ l of chloroform/isoamyl alcohol (24:1) were added to each tube and the tubes were shaken on a mechanical shaker for 15 minutes at room temperature.
6. The tubes were then spun in a microcentrifuge at 10,000 rpm for 5 minutes.

7. The upper aqueous layer was removed from each tube and placed in a fresh tube. Another aliquot of 400 μ l of chloroform/isoamyl alcohol was added to each tube and they were shaken for a further 5 minutes.
8. The tubes were spun again at 10,000 rpm for 5 minutes.
9. The upper aqueous layer was again removed and transferred to a fresh tube.
10. An equal volume of isopropyl alcohol was added to each tube and they were placed in a freezer at -20°C overnight to precipitate the DNA.
11. The tubes were then spun at 10,000 rpm for 5 minutes to pellet the DNA.
12. The isopropyl alcohol was poured off and 500 μ l of 95% ethanol was added to each tube. The tubes were spun again at 10,000 rpm for 5 minutes.
13. The DNA pellet was given a final wash in 70% alcohol.
14. After the alcohol had been poured off the tubes were left to dry at room temperature for about one hour to ensure all the ethanol had evaporated off.
15. 50 μ l of TE * was then added to each tube to resuspend the DNA.
16. 3 μ l RNase was added to each 50 μ l volume of resuspended DNA and the solution was incubated at 37°C for 20 minutes.
17. The DNA was then either frozen at -20°C for immediate use or at -80 °C for long term storage.
18. The extracted DNA was examined for quantity and quality by running it out on a 1.0% agarose gel with ethidium bromide incorporated at 4 μ l (10 mgml⁻¹) per 100ml of agarose. Lambda DNA diluted serially was used to estimate the quantity of DNA and the quality of the DNA was examined by viewing the DNA under ultra-violet light.

In addition to the DNA extracted from our collection at Trinity College Botanic Garden DNA samples extracted by Dr. T. Hodgkinson from material at the Royal Botanic Gardens, Kew were also used in this study. A list of the samples from which DNA was extracted and subsequently analysed by RAPD is given in Table 3.1.

Table 3.1 *Miscanthus* accessions analysed by RAPD.

Species	Accession number	Extracted by:
<i>M. sinensis</i>	TCD40	L. Scally
<i>M. sinensis</i>	TCD11	L. Scally
<i>M. sinensis</i>	TCD12	L. Scally
<i>M. sinensis</i>	TCD56	L. Scally
<i>M. sacchariflorus</i>	TCD91	L. Scally
<i>M. sacchariflorus</i>	TCD67	L. Scally
<i>M. sacchariflorus</i>	TCD53	L. Scally
<i>M. transmorrissonensis</i>	TCD106	L. Scally
<i>M. transmorrissonensis</i>	Kew 1990-2748	T. Hodkinson
<i>M. oligostachyus</i>	TCD 16	L. Scally
<i>M. oligostachyus</i>	Kew 1995-1864 (161)	T. Hodkinson
<i>M. floridulus</i>	TCD 96	L. Scally
<i>M. floridulus</i>	TCD97	L. Scally
<i>M. floridulus</i>	TCD100	L. Scally
<i>M. condensatus</i>	TCD94	L. Scally
<i>M. nudipes</i>	KEW 2007 (herbarium) (109)	T. Hodkinson
<i>M. nepalensis</i>	KEW 1992-1293	T. Hodkinson
<i>M. nepalensis</i>	KEW25	T. Hodkinson
<i>Saccharum officiarum</i>	TCD89	L. Scally
<i>Saccharum spontaneum</i>	KEW 26672 (herbarium)	T. Hodkinson
<i>M. x giganteus</i>	TCD37	L. Scally
<i>M. x giganteus</i>	TCD39	L. Scally
<i>M. sinensis</i> cv <i>variagatus</i>	TCD103	L. Scally
<i>M. sinensis</i> cv <i>gracillimus</i>	TCD18	L. Scally
<i>M. sinensis</i> cv <i>purpurescens</i>	TCD19	L. Scally
<i>M. 'Silberspine'</i>	TCD102	L. Scally
<i>M. 'Rotsilber'</i>	TCD99	L. Scally
<i>M. 'Goliath'</i>	TCD92	L. Scally
<i>M. 'Posidon'</i>	TCD32	L. Scally
<i>M. 'Roland'</i>	KEW PN95/16	T. Hodkinson
<i>M. 'Silverfeather'</i>	TCD95	L. Scally
<i>M. 'Silverfeather'</i>	TCD89	L. Scally
<i>M. 'Kleinefontane'</i>	KEW PN/95/08	T. Hodkinson
<i>M. 'Grossefontane'</i>	TCD30	L. Scally
<i>M. 'Gracillimus'</i>	TCD77	L. Scally
<i>M. 'Undine'</i>	TCD100	L. Scally

3.2.2 Analysis of samples by RAPD

PCR conditions:

All reactions were carried out on a Perkin Elmer 480 Thermal Cycler, without a heated lid. The same instrument was used for each run. 50 µl reactions were carried out in thin walled 0.5 ml microfuge tubes and the same tube type was used for all reactions. Each reaction was overlaid with two drops of silicon oil to prevent evaporation of the sample. The PCR cycling conditions were as follows:

- Denaturing step: 92°C for 1 minute, 45 cycles
- Annealing step: 35°C for 3 minutes, 45 cycles
- Extension step: 72°C for 2 minutes, 45 cycles
- Final extension: 1 cycle of 72°C for 5 minutes
- Soak temperature: 4°C

50 µl reactions were carried out. The following reaction concentrations were used:

	Volume	Concentration in 50 µl
DNA (100ngµl ⁻¹)	1 µl	100 ng
Buffer (10mM)	5µl	1mM
MgCl ₂ (25 mM)	6µl	1.5 mM
*Primer (100ngµl ⁻¹)	5µl	500 ng
dNTP (2 mM)	5µl	200 µM
Taq polymerase (6 unitsµl ⁻¹)	0.25µl	1.5 unit
Water	27.75 µl	

The choice of primer used for RAPD analysis is wholly empirical. In this study primers from Operon Technologies, Inc. were used throughout. Twenty primers from Operon primer kit A (Appendix 4c) were tested for their potential in detecting DNA polymorphisms in *Miscanthus*. Primers OPA-16, OPA-17 and OPA-20 proved most suitable, as they showed the highest degree of polymorphism between samples, and were therefore used for analysis of all samples. The sequences of these primers are as follows:

TBE buffer 5 x stock solution

Trizma base, 54 g

Boric acid, 27.5 g

EDTA-Na, 4.6 g

Made up to 1 litre with distilled water

Primer	5'	3'
OPA-16	AGCCAGCGAA	
OPA-17	GACCGCTTGT	
OPA-20	GTTGCGATCC	

3.2.3 Sample analysis by agarose gel electrophoresis

10 μ l aliquots of PCR product were mixed with 3 μ l of loading dye (0.25% bromophenolblue and 40% sucrose in 5 x TBE). The solution was loaded onto a 1.5% agarose gel containing ethidium bromide and run at 40 volts for 2 hours in 0.5 x TBE running buffer. The gel rig used allowed a gel of dimensions 25 cm x 20 cm to be poured. Two rows of combs were placed on the gel allowing a total of 40 samples per gel to be run with a run distance of 12 cm per sample. A 1kb DNA ladder (GIBCO BRL) at a concentration of 0.5 μ g μ l⁻¹ was run at each end of a row of samples.

The gels were visualised by viewing over an ultra-violet light box. A record of the results was obtained by photographing the gel on the light box using a Polaroid camera with Polaroid 667 film. A Kodak Wratten orange 23A gelatin filter was inserted in front of the camera lens to allow photography over ultra-violet light.

Protocols for preparation of Solutions used.

CTAB/DTT Extraction solution (200 ml)

CTAB (Hexadecyltrimethylammonium) 3 g

5 M NaCl, 42 ml

0.5 M EDTA-Na, 6 ml

2 M Tris-HCl pH 8.0, 30 ml

DTT (Dithiothreitol) to a final concentration of 10 mM (added to the CTAB solution immediately before use.

Water, 119 ml

TE buffer 10 x stock solution

0.01 M Tris-HCl (pH 7.5) and 0.001 M EDTA-Na

TBE buffer 5 x stock solution

Trizma base, 54 g,

Boric acid, 27.5 g,

EDTA-Na, 4.6 g

Made up to 1 litre with distilled water.

Scoring of gels

Gels were scored manually by comparing the fragments produced on each gel with each other and with a 1KB DNA ladder. The molecular weight of each band can be estimated by simple regression analysis. The distance each band on the gel has travelled from the loading well was measured. Regression analysis of this distance was calculated for each band against a standard ladder of known molecular weights. A band matrix was then produced based on the presence or absence of a particular band. Very weak or inconsistent bands were not included in the analysis.

3.2.4 Reproducibility and quality control.

Initially one DNA sample (derived from a single *M. sinensis* genotype) was analysed with the primer OPA-16. The amount of DNA used was varied from 50ng μl^{-1} to 200 ng μl^{-1} and the annealing temperature was varied from 34°C to 39°C. All thermal cycling time schedules remained unchanged. Varying of the amount of DNA allowed the optimal amount of DNA for PCR to be estimated. Variation of annealing temperatures allowed an estimation of the maximum annealing temperature which could produce a banding pattern to be established. Higher annealing temperatures help to reduce the possibility of PCR artefacts by reducing the amount of primer mis-match. Every individual DNA sample was replicated four times for PCR. However this proved to be costly and unnecessary and sample replication was reduced to two individual PCR reactions per sample. Products from both of these PCR reactions were then run out side by side on the gel. This allowed comparisons to be made in the case of weak bands. Where a weak band only appeared in one of two replicates this band was ignored. If a strong band only appeared in one of two samples the sample was re analysed. Two blank controls were run with every batch of samples analysed. Blank controls contained all components of the PCR reaction except for the DNA. A positive control was also analysed with each batch of samples run. The positive control consisted of DNA that consistently worked well, extracted from one *Miscanthus sinensis* individual. The RAPD profile for this individual sample was tested with each of the primers used and then the sample was re-run with every batch of samples subjected to PCR.

3.2.5 Sample analysis for Inter Simple Sequence Repeat - Anchored PCR

PCR conditions:

All reactions were carried out on a Perkin Elmer 480 Thermal Cycler. The same instrument was used for each run. 50 µl reactions were carried out in 0.5ml tubes and the exact same tube type was used for all reactions. The PCR cycling conditions were as follows:

Denaturing step: 93°C for 20seconds, 40 cycles

Annealing step: 45°C for 1 minutes, 40 cycles

Extension step: 72°C for 1 minutes, 40 cycles

Final extension: 72°C for 7 minutes, 1 cycle

Soak temperature: 4°C

50 µl reactions were carried out. The following reaction concentrations were used:

	Volume	Concentration
DNA (100ngµl ⁻¹)	1 µl	100 ng
Buffer (10 mM)	5µl	0.5 mM
MgCl ₂ (25 mM)	4µl	1.25 mM
*Primer (100ngµl ⁻¹)	5µl	500 ng
dNTP (10 mM)	1µl	0.1 mM
Taq polymersae (6 unitsµl ⁻¹)	0.25µl	1.5 units
Water	38.25 µl	

* Two primers were tested for their suitability, these were (CA)₈RG and (CA)₈RY

All other running conditions and gel analysis were exactly the same as those used for RAPD analysis (see section 3.2.3).

3.2.6 Statistical analysis of RAPD band matrix

Three statistical methods were employed to analyse the data. Principal Co-ordinate analysis (PCO), described in Chapter two, section 2.2.2 of this thesis, was used to provide a multivariate ordination of the data. Unweighted Pair-group method with Arithmetic Mean

(UPGMA) and Neighbor-Joining are two clustering methods which use distance measures to describe the data and are described here. Both UPGMA and Neighbor-Joining methods were subjected to bootstrap analysis to test the robustness of the results obtained. Both UPGMA and Neighbor-Joining methods were performed using the PAUP computer program version 4.0b2 for Macintosh (Swofford, 1999).

Unweighted Pair-Group method with Arithmetic Mean (UPGMA)

This technique is a hierarchical clustering method whereby a sequential clustering algorithm is used. Local topological relationships are inferred in order of decreasing similarity, in this way the tree is constructed in a stepwise manner (Li, 1997). Firstly the algorithm identifies the two taxa which are most similar to each other, this pair is then formed into a single cluster. The next step is to identify from among the new clusters the pair with the highest similarity and these are joined to the existing clusters. The process is thus continued until only two clusters are left. This method relies on the data being ultrametric. This means that the pairwise distance values measured fit a rooted tree with a constant molecular clock (Hillis *et al.* 1990). If this is not the case some error may be introduced into the final tree.

Neighbor-Joining

Neighbor-Joining (NJ), (Saitou & Nei, 1987), is based on the additive tree technique. Additive distances can be fitted to an unrooted tree so that all distances are equal to the sum of the branch lengths. In this way the lengths of the branches between any two taxa can infer a quantity. Neighbor-Joining, in contrast to UPGMA, does not require the data to be ultrametric, it does however assume them to be additive or close to additive. To overcome errors which might arise if the data is not additive a normalisation algorithm is first applied to the original data matrix to produce a modified distance matrix in which each pair of nodes on the tree are adjusted based on their average distance from all other nodes. This is possible with the NJ technique because unlike UPGMA the tree is constructed by the addition of most similar nodes on the tree rather than the addition of most similar taxa Swofford & Olsen (1990). Both NJ and UPGMA data are illustrated as phenograms in this thesis, in this way branch lengths representing relative genetic distance can be seen even where bootstrap support is low.

Bootstrap analysis

Bootstrap analysis is a computational technique for estimating statistics or parameters when the distribution is difficult to derive analytically. Its application to phylogeny reconstruction and systematics in general was first described by Felsenstein (1985). Essentially bootstrapping is a method of numerical resampling which allows variances to be estimated and confidence intervals to be constructed. The resampling technique in the bootstrap works by randomly sampling data points from the original data matrix and replacing them with

other random points until a new data set containing the original number of observations is obtained. The technique works by re-running a particular analysis many times and estimating the number of occasions that the analysis produces the same result. In this way a confidence estimate can be constructed. If, for example, an analysis is run 100 times and on each occasion the same result is obtained it can be said that the cluster is supported 100%. In all the analysis carried out for the RAPD data in this chapter 1000 reanalyses were carried out. Generally bootstrap values below 50% indicate that any clusters within this grouping are not significant and the position of the individual taxa within the group could equally be swapped for other positions within the cluster. High bootstrap values represent a high level of confidence for the clusters they represent.

Distance indices

Species and cultivar distances were calculated by pairwise distances based on the number of shared bands according to the method of Nei & Li (1979). This method allows the degree of polymorphism between species and between cultivars to be expressed. The method was originally devised to examine nucleotide divergence within populations using a measure the authors refer to as “nucleotide diversity”. This method has since become widely used as a method of measuring genetic diversity in many types of molecular data. By using the distance index the genetic dissimilarity between all cultivars and *M. sinensis* and *M. sacchariflorus* and between all of the species alone was estimated according to the formula:

$$1 - F = 1 - [2n_{xy}/(n_x + n_y)]$$

where F is the ratio of shared bands between x and y, n_{xy} is the number of shared bands, and n_x and n_y are the number of bands observed in individual x and in individual y, respectively.

3.3 RESULTS

Fig. 3.2. Principal Co-ordinate analysis of RAPD data for all species

The RAPD technique was successful in producing individual profiles for all of the species and cultivars analysed, examples of these are given in appendix 4. The most common reason for failed PCR reactions was due to the quality of the DNA used. Generally, if a reaction failed and was rerun using a smaller amount of DNA it was successful. This may be a result of lowering the amounts of other impurities in the sample by using a smaller volume of DNA. The reproducibility of the technique was excellent. Identical banding patterns were obtained for duplicate PCR reactions for the same individual. If a band appeared to be weak in one of two duplicate PCR reactions the reaction was rerun to test the consistency of the band in question. If a band was consistently weak in one of the replicates it was initially scored as weak but not included in the final analysis. An example of this situation is shown in Appendix 4. In this example lane 8 and 9 represent the RAPD profile of *M. 'Zebrinus'*. Lane 8 can be seen to have four bands, 2 weak upper bands and two strong lower bands. Lane 9 shows only 2 weak lower bands. A sample such as this would have been rerun to check if the upper two bands were actual RAPD bands and not artefacts. While the rerun would also establish the consistence of the lower two bands. If the repeat run had shown the upper two bands to remain weak in one sample and non-existent in the second these bands would not have been scored as present.

In general it was found that the technique, once standardised, was easily reproduced. Initial problems with contamination were overcome by aliquoting all reagents used into small volume tubes which were autoclaved before use. Variations in the amount of DNA did not appear to affect the result of the banding pattern. Some DNA samples consistently failed amplification by PCR, but these samples were always of poor quality (degraded) DNA and did not successfully amplify by any of the other molecular techniques investigated.

3.3.1 Statistical analysis of RAPD data.

A total of 44 bands were scored for all of the individuals analysed, including *Saccharum*. Of these, thirty five were polymorphic between all species and thirty nine were polymorphic between cultivars and *M. sinensis* and *M. sacchariflorus*. *Saccharum* contained all of the bands observed in *Miscanthus* plus an additional three bands.

RAPD band matrix analysis by Principal Co-ordinate Analysis (PCO)

In the Principal Co-ordinate Analysis (PCO) of the RAPD data 30.8% of the data variance was explained by axis one (Fig. 3.2.a), 14.5% by axis two (Fig. 3.2.b), 12% by axis three (Fig. 3.2.c), and 7.4% on axis four (not shown as pattern of variation was difficult to interpret). The results of the analysis are rather difficult to interpret for any axis. There

Fig. 3.2. Principal Coordinate analysis of RAPD data for all *Miscanthus* species with primers OPA 16/17 & 20. (a) axis 1 & 2 (b) axis 2 & 3.

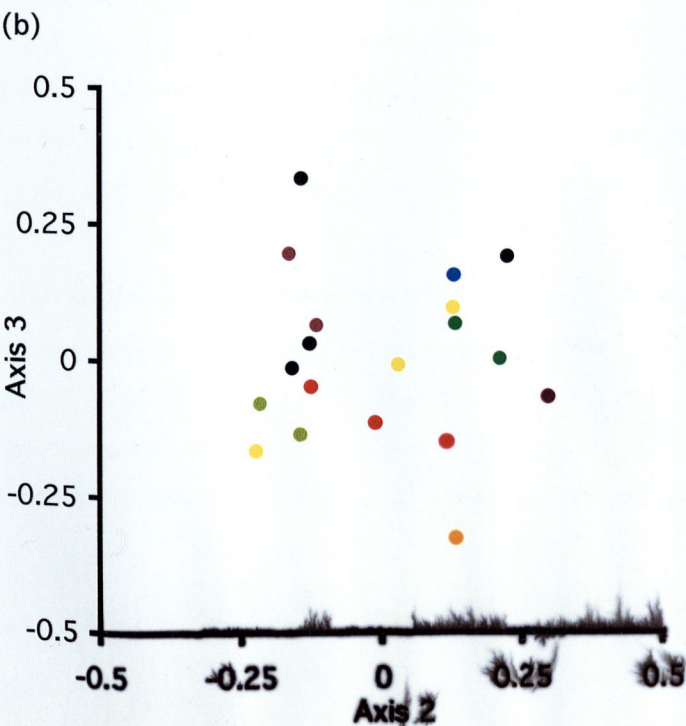
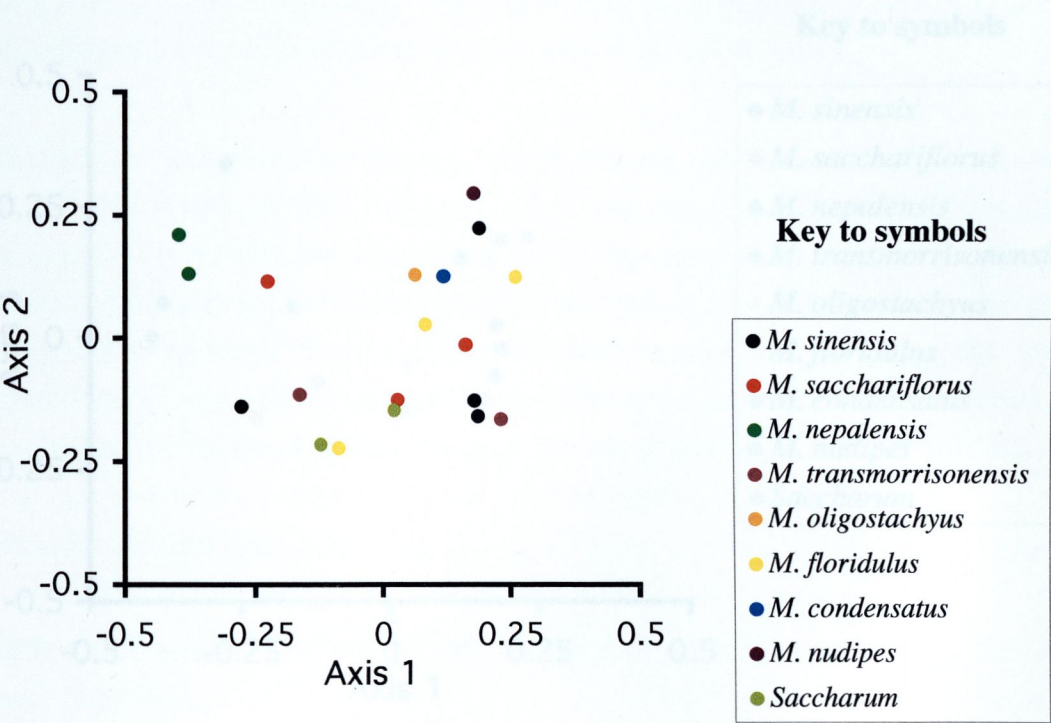
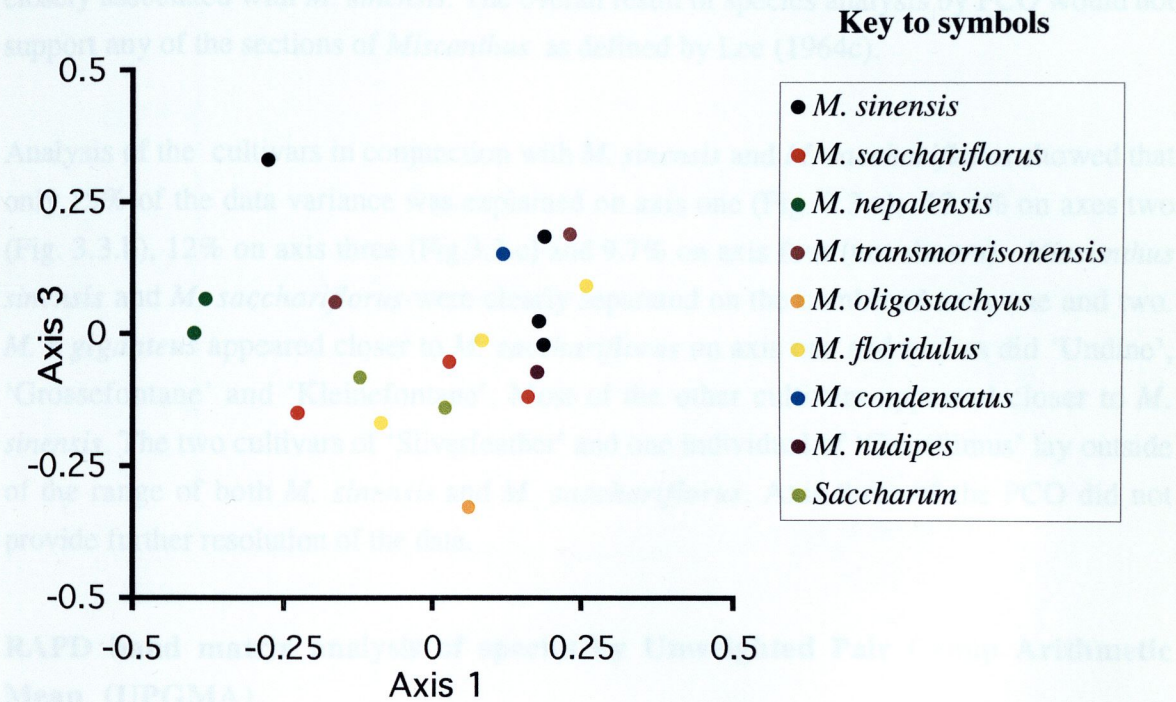


FIG 3.2. Principal Coordinate analysis of RAPD data for all *Miscanthus* species with primers OPA16/17 & 20 (c) axis 1 & 3



appears no be no clear clustering of the species into any recognisable pattern. *Miscanthus nepalensis* is the only species separated on axis one of the PCO. Axis two did not provide any clear separation of *Saccharum* from *Miscanthus* or any separation within *Miscanthus*. Axis three separated *M. sinensis* from *M. sacchariflorus*. *Miscanthus transmorissonensis* always appeared close to *M. sinensis* as did *M. condensatus* on each of the three axes. The three individuals of *M. floridulus* showed a considerable amount of variation and were not closely associated with *M. sinensis*. The overall result of species analysis by PCO would not support any of the sections of *Miscanthus* as defined by Lee (1964c).

Analysis of the cultivars in conjunction with *M. sinensis* and *M. sacchariflorus* showed that only 15% of the data variance was explained on axis one (Fig. 3.3.a), 13.4% on axes two (Fig. 3.3.b), 12% on axis three (Fig.3.3.c) and 9.7% on axis four (not shown). *Miscanthus sinensis* and *M. sacchariflorus* were clearly separated on the combined axes one and two. *M. x giganteus* appeared closer to *M. sacchariflorus* on axis one and two as did 'Undine', 'Grossefontane' and 'Kleinefontane'. Most of the other cultivars appeared closer to *M. sinensis*. The two cultivars of 'Silverfeather' and one individual of 'Gracillimus' lay outside of the range of both *M. sinensis* and *M. sacchariflorus*. Axis three of the PCO did not provide further resolution of the data.

RAPD band matrix analysis of species by Unweighted Pair Group Arithmetic Mean (UPGMA).

Fig. 3.4. illustrates the unrooted phenogram calculated by UPGMA analysis of the RAPD matrix data. As with PCO analysis no clear groupings were obvious from the dendrogram. The only groupings with bootstrap support of over 50% are the group containing *M. nepalensis* and a second group containing one individual of *M. floridulus* from Mangereva and one of *M. sinensis*. The second individual of *M. floridulus* was grouped with *M. condensatus*. No clear separation of *M. sinensis* and *M. sacchariflorus* was evident from UPGMA analysis. *Miscanthus nudipes* had the longest branch length.

RAPD band matrix analysis of species by Neighbor-Joining.

Neighbor-Joining (NJ) analysis produced a similar result to UPGMA analysis (Fig 3.5.). The two individuals of *M. nepalensis* formed a group with 94% bootstrap support and *M. sinensis* and *M. floridulus* from Mangereva a second group with 76% support. The other species were not resolved into any additional clusters.

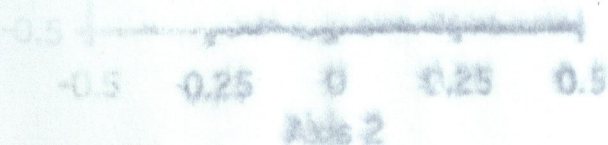


Fig 3.3. Principal Coordinate analysis of RAPD data for all *Miscanthus* cultivars with primers OPA16/17 & 20. (a) axis 1 & 2 (b) axis 2 & 3.

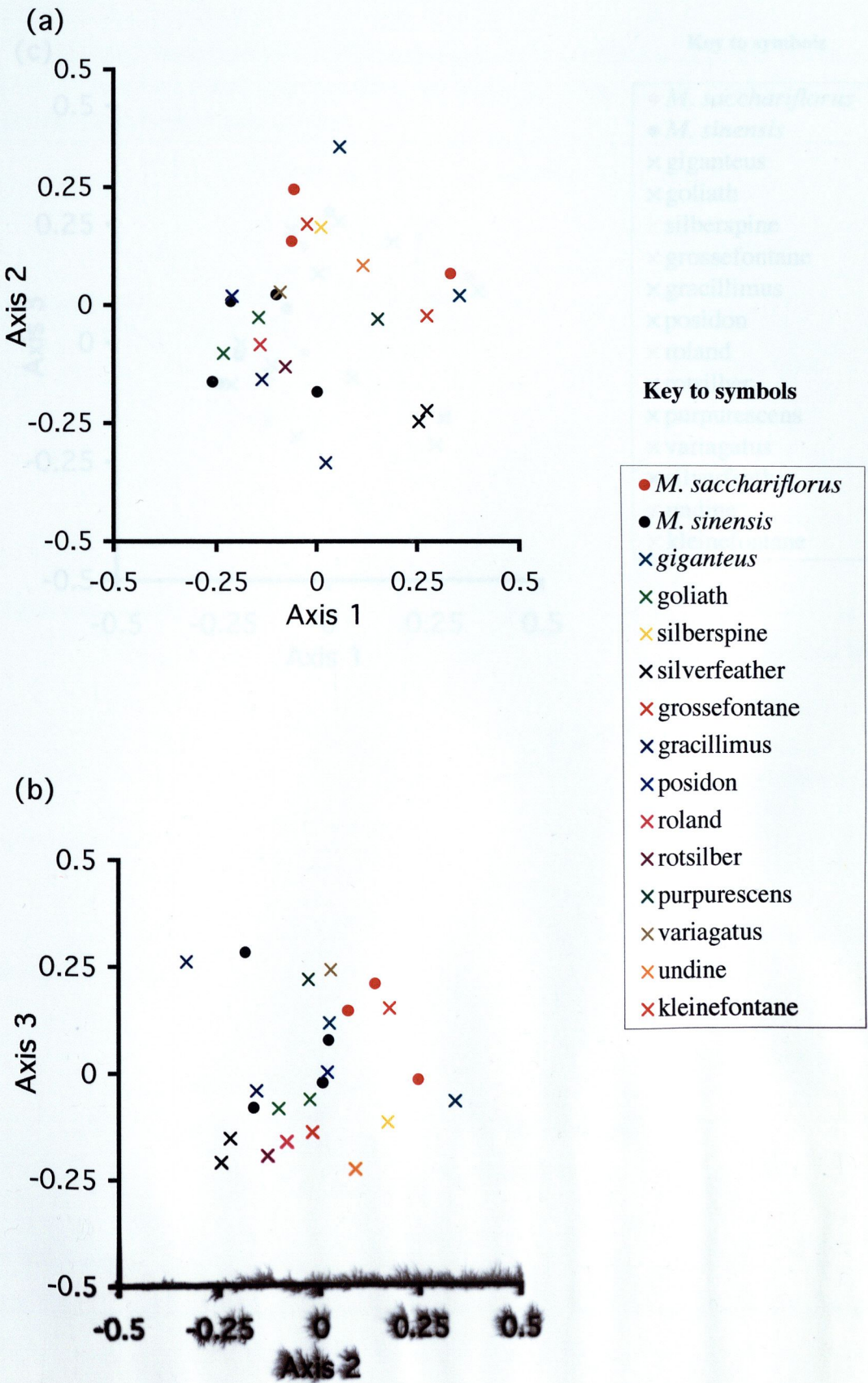


Fig 3.3. Principal Coordinate analysis of RAPD data for all *Miscanthus* cultivars with primers OPA16/17 & 20. (c) axis 1 & 3.

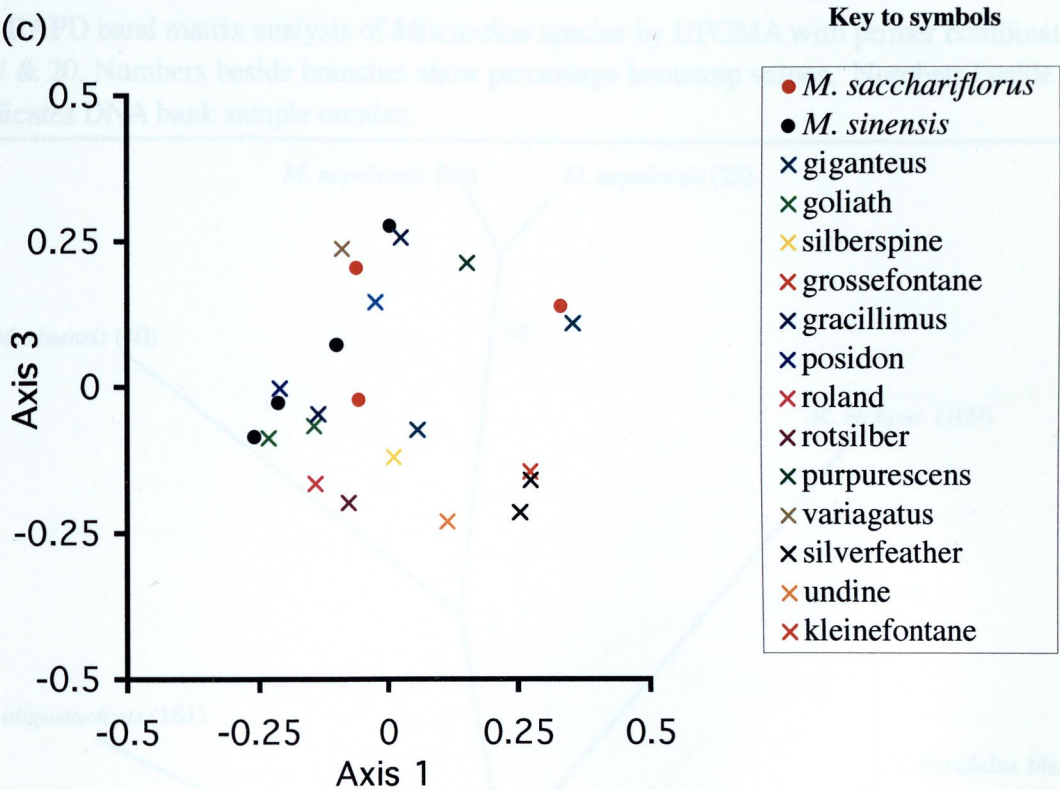


Fig. 3.4. RAPD band matrix analysis of *Miscanthus* species by UPGMA with primer combination OPA16/17 & 20. Numbers beside branches show percentage bootstrap values. Numbers beside species names indicates DNA bank sample number.

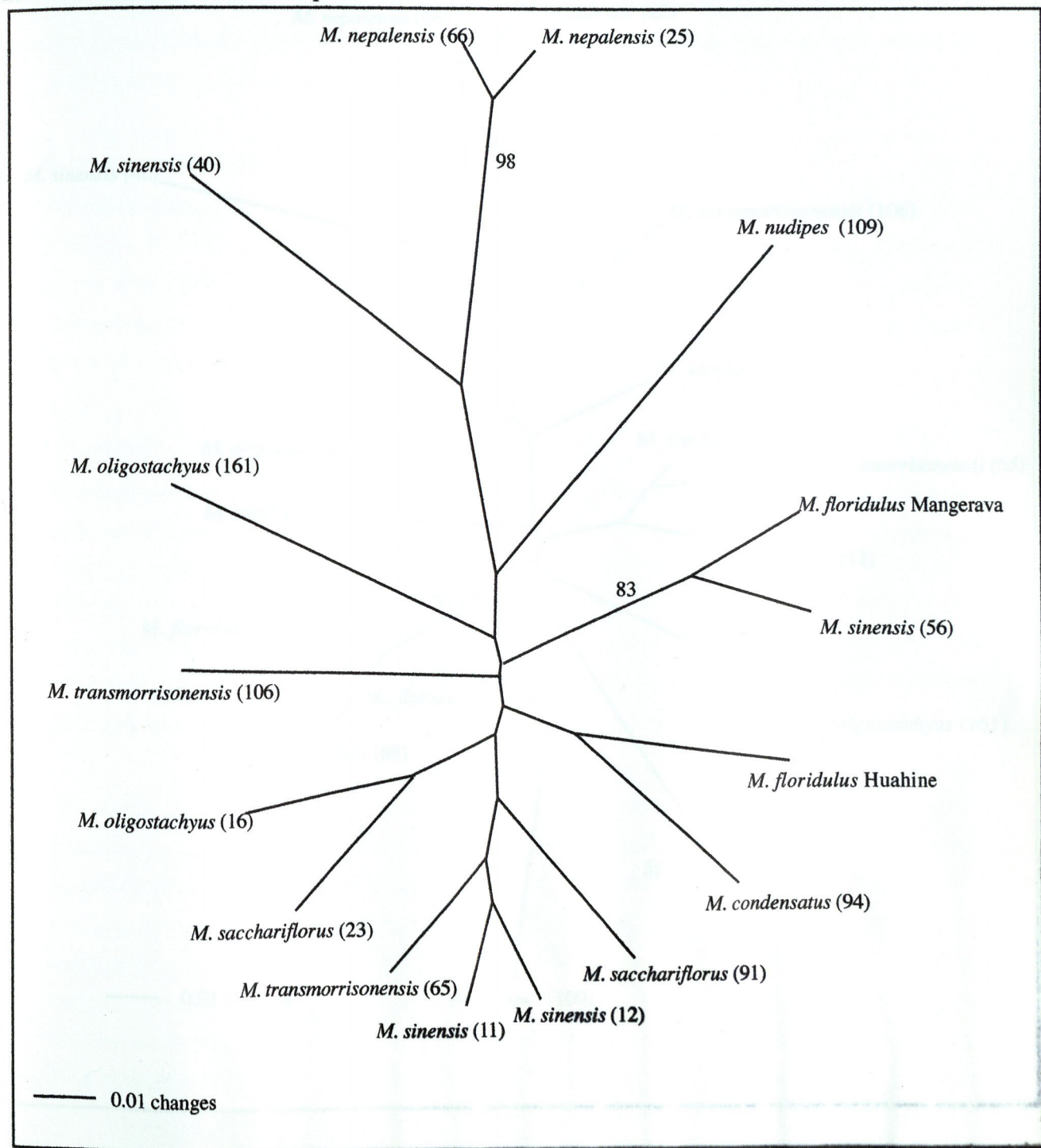
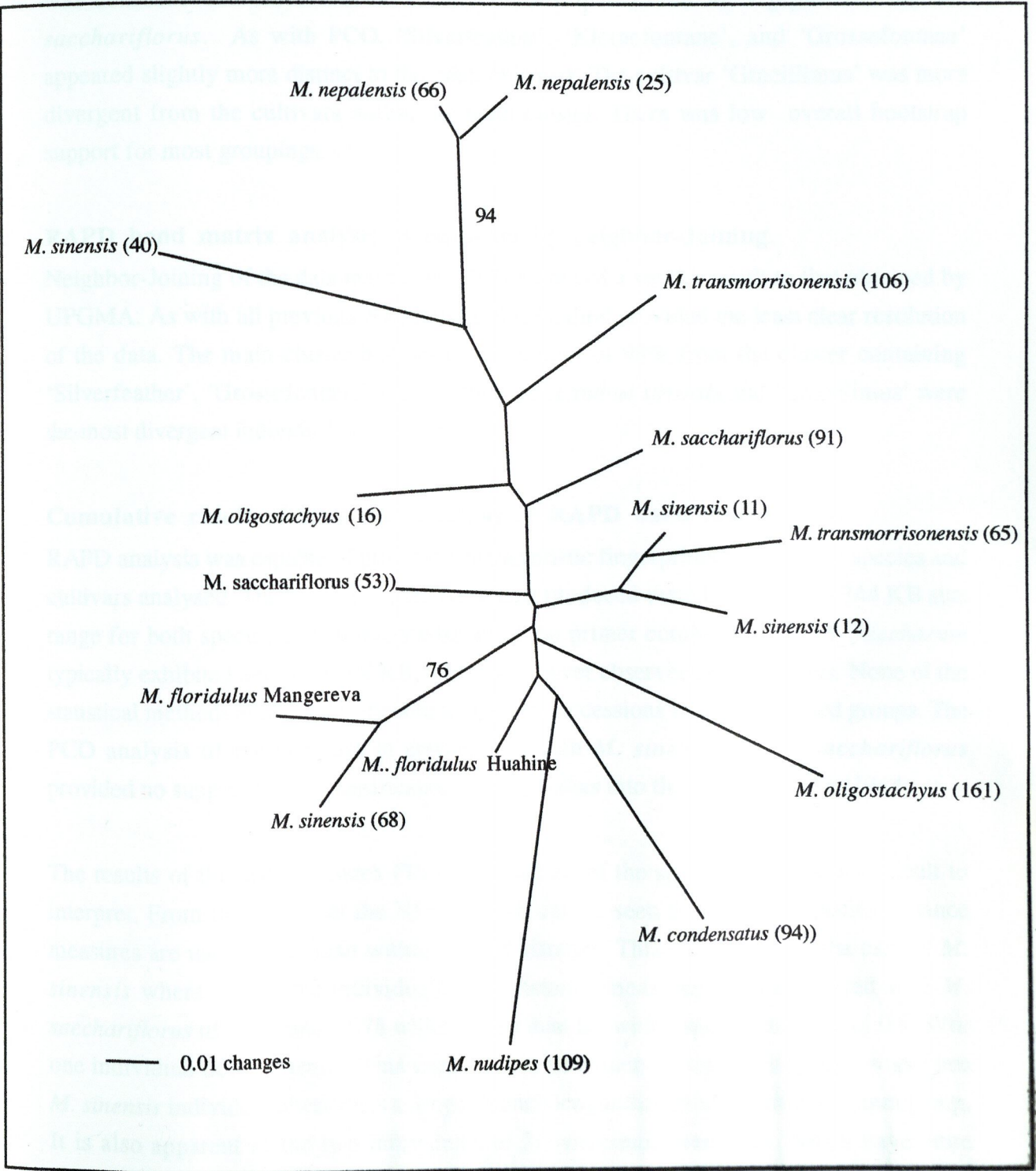


Fig. 3.5. RAPD band matrix analysis of *Miscanthus* species by Neighbor Joining with primer combination OPA 16/17 &20. Numbers beside branches show bootstrap value. Number beside species names indicates DNA bank sample number.



RAPD analysis of cultivars by Unweighted Pair Group Arithmetic Mean (UPGMA).

The results of this analysis (Fig. 3.6) did not present any clearer resolution of the association of the cultivars of *Miscanthus* to each other or to *M. sinensis* or *M. sacchariflorus* than that produced by PCO analysis. Again the distinction between *M. sinensis* and *M. sacchariflorus* was confused and there was no clear relationship between *M. x giganteus* and *M. sacchariflorus*. As with PCO, 'Silverfeather', 'Kleinefontane', and 'Grossefontane' appeared slightly more distinct to the other cultivars. The cultivar 'Gracillimus' was more divergent from the cultivars within the main cluster. There was low overall bootstrap support for most groupings.

RAPD band matrix analysis of cultivars by neighbor-Joining.

Neighbor-Joining of the data matrix (Fig. 3.7) produced a similar result to that obtained by UPGMA. As with all previous NJ analyses, this method provided the least clear resolution of the data. The main cluster had bootstrap support of 98% from the cluster containing 'Silverfeather', 'Grossefontane' and 'Undine'. *Miscanthus sinensis* and 'Gracillimus' were the most divergent individuals in the analysis

Cumulative result of statistical analysis of RAPD band matrix data.

RAPD analysis was capable of providing characteristic fingerprints for all of the species and cultivars analysed. The majority of the fragments produced were in the 1,635 - 344 KB size range for both species and cultivars with all of the primer combinations used. *Saccharum* typically exhibited a band of 298 KB, which was never observed in *Miscanthus*. None of the statistical methods employed were able to place the accessions in well supported groups. The PCO analysis of the cultivars in conjunction with *M. sinensis* and *M. sacchariflorus* provided no support for the classification of *Miscanthus* into the sections of Lee (1964).

The results of the distance index (Table 3.1) for all of the species analysed is difficult to interpret. From this and from the NJ analysis it can be seen that between species distance measures are usually less than within species distance. This can be seen in the case of *M. sinensis* where two of the individuals have distance measures when compared with *M. sacchariflorus* of 0.084 and 0.078 which is less than the within species measure of 0.143 for one individual of *M. sinensis*. This situation is also evident in the dendrograms where one *M. sinensis* individual always has a longer branch length than others within the main group. It is also apparent in the two individuals of *M. transmorrisonensis* which have quite different distances measures one being closer to *M. sinensis* while the other is similar to *M. nepalensis*.

Fig. 3.6 RAPD band matrix analysis of *Miscanthus* cultivars by UPGMA with primer combinations OPA16/17 & 20. Numbers beside branches show percentage bootstrap support. Number beside species names indicates DNA bank sample number.

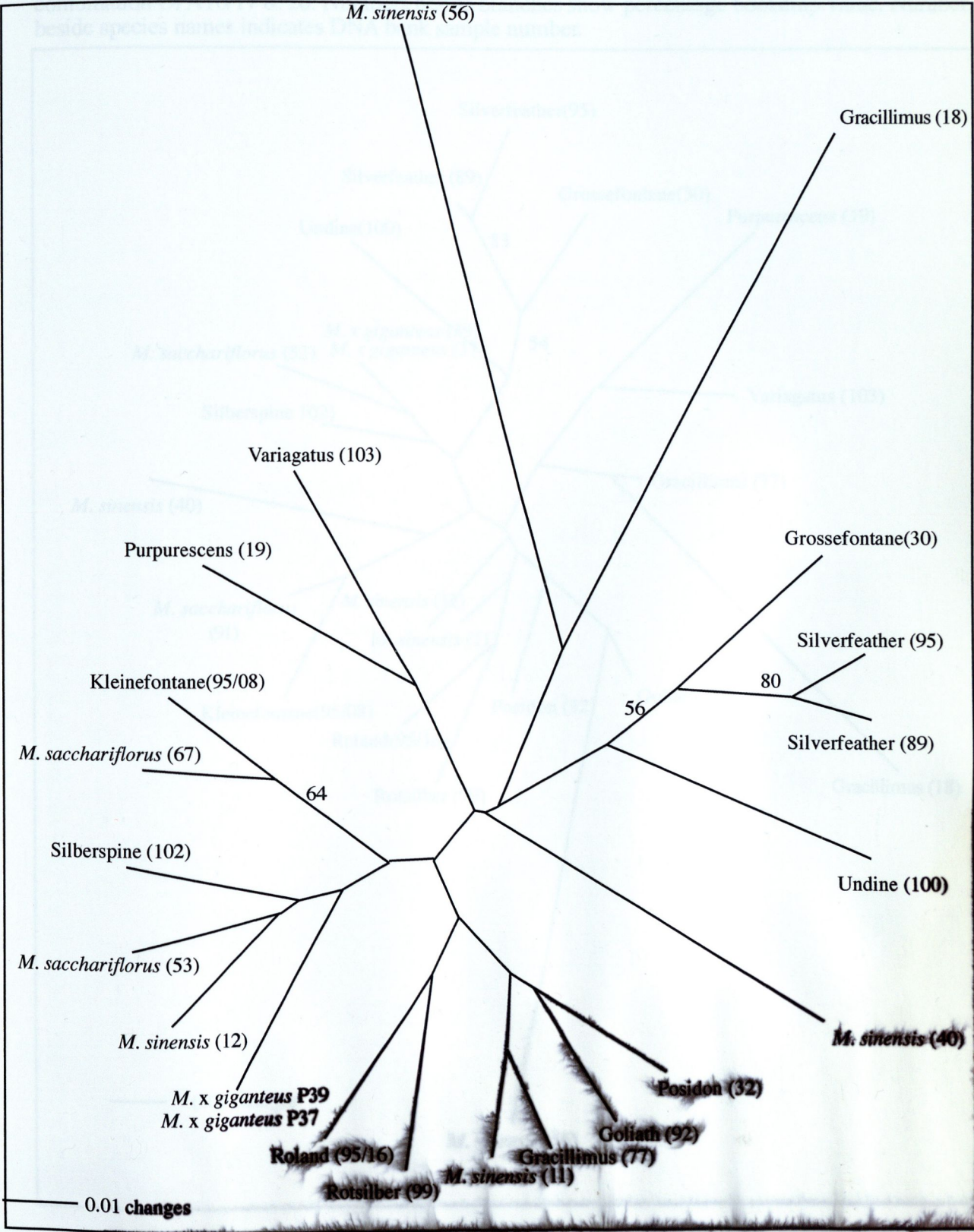


Fig. 3. 7. RAPD band matrix analysis of *Miscanthus* cultivars by Neighbor Joining with primer combination OPA16/17 & 20. Numbers beside branches show percentage bootstrap value. Number beside species names indicates DNA bank sample number.

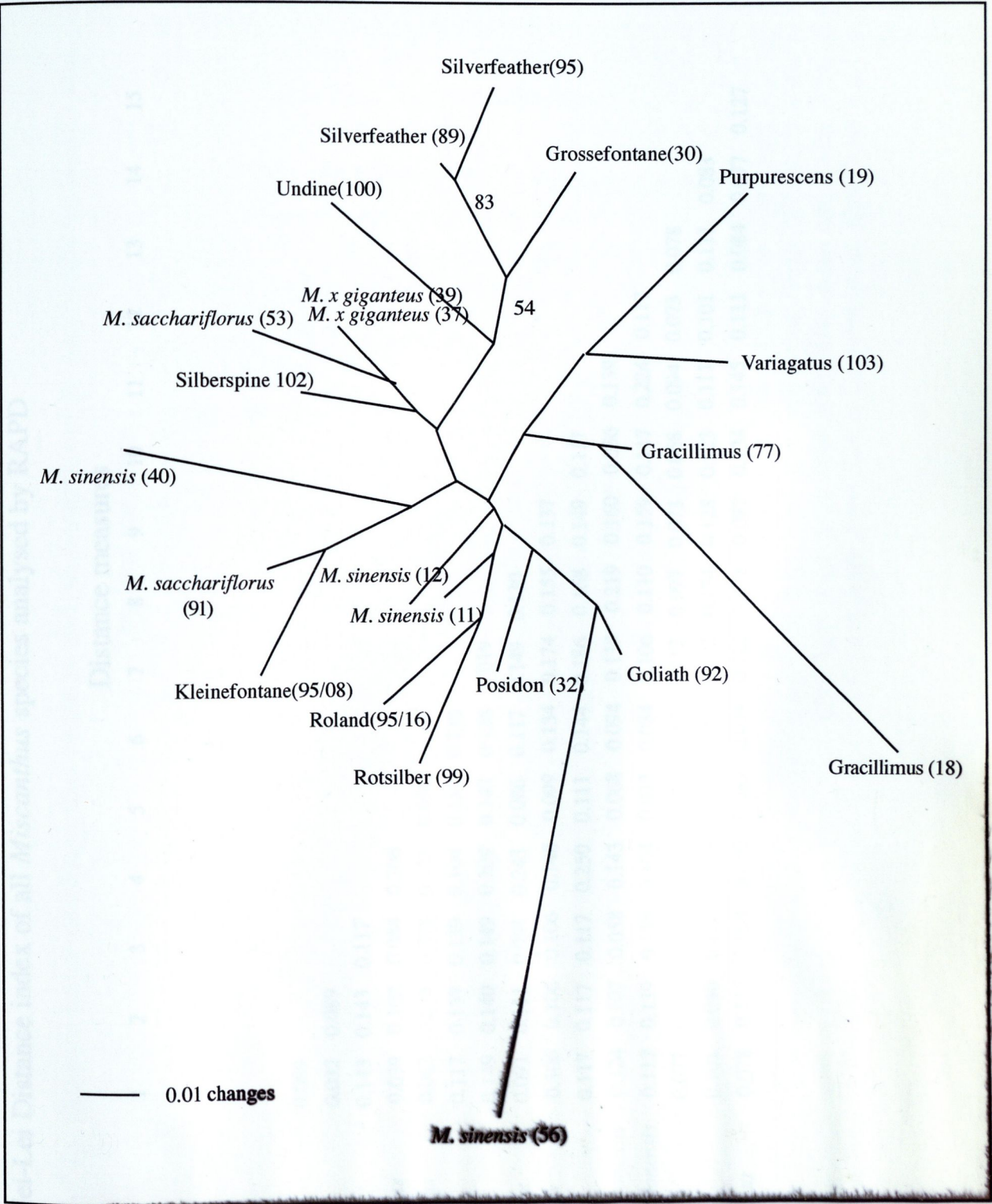


Table 3.1 Nei-Lei Distance index of all *Miscanthus* species analysed by RAPD

Table 3.2 Nei-Li Distance Index of *Miscanthus* cultivars and *M. sinensis* and *M. sacchariflorus* RAPD data

Species	Distance measures														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. <i>M. sinensis</i>															
2. <i>M. sinensis</i>	0.089														
3. <i>M. sinensis</i>	0.032	0.089													
4. <i>M. sinensis</i>	0.143	0.143	0.117												
5. <i>M. sacchariflorus</i>	0.039	0.102	0.084	0.206											
6. <i>M. sacchariflorus</i>	0.063	0.078	0.078	0.123	0.058										
7. <i>M. nepalensis</i>	0.117	0.139	0.139	0.104	0.134	0.122									
8. <i>M. nepalensis</i>	0.149	0.140	0.149	0.109	0.143	0.128	0.019								
9. <i>M. oligostachyus</i>	0.091	0.111	0.091	0.243	0.086	0.117	0.149	0.130							
10. <i>M. condensatus</i>	0.106	0.106	0.106	0.148	0.099	0.134	0.174	0.155	0.137						
11. <i>M. nudipes</i>	0.117	0.117	0.117	0.250	0.111	0.144	0.186	0.168	0.149	0.117					
12. <i>M. transmorrisonensis</i>	0.324	0.107	0.058	0.143	0.068	0.094	0.139	0.219	0.160	0.086	0.139				
13. <i>M. transmorrisonensis</i>	0.117	0.139	0.139	0.104	0.073	0.084	0.106	0.110	0.159	0.117	0.224	0.117			
14. <i>M. floridulus</i>	0.072	0.103	0.059	0.134	0.068	0.092	0.112	0.097	0.073	0.068	0.094	0.073	0.078		
15. <i>M. floridulus</i>	0.101	0.040	0.102	0.206	0.088	0.089	0.192	0.174	0.128	0.123	0.111	0.101	0.111	0.083	
16. <i>M. oligostachyus</i>	0.078	0.155	0.094	0.010	0.089	0.054	0.122	0.128	0.097	0.134	0.145	0.111	0.084	0.077	0.127

Table 3.2 Nei-Li Distance Index of *Miscanthus* cultivars and *M. sinensis* and *M. sacchariflorus* RAPD data.

Cultivar	Distance measures																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. <i>M. x giganteus</i>																				
2. <i>M. x giganteus</i>	0.000																			
3. <i>M. 'Silberspine'</i>	0.050																			
4. <i>M. 'Goliath'</i>	0.081	0.062																		
5. <i>M. 'Silverfeather'</i>	0.137	0.086	0.100																	
6. <i>M. 'Grossefontane'</i>	0.081	0.062	0.094	0.062																
7. <i>M. sinensis</i>	0.080	0.048	0.057	0.097	0.111															
8. <i>M. sinensis</i>	0.100	0.097	0.134	0.139	0.111	0.089														
9. <i>M. sinensis</i>	0.100	0.079	0.043	0.097	0.111	0.032	0.089													
10. <i>M. sinensis</i>	0.179	0.161	0.097	0.161	0.155	0.143	0.143	0.177												
11. <i>M. sacchariflorus</i>	0.040	0.043	0.086	0.134	0.086	0.040	0.102	0.084	0.206											
12. <i>M. sacchariflorus</i>	0.068	0.068	0.079	0.145	0.097	0.063	0.078	0.078	0.123	0.058										
13. <i>M. 'Gracillimus'</i>	0.094	0.091	0.052	0.091	0.106	0.053	0.122	0.027	0.168	0.079	0.089									
14. <i>M. 'Posidon'</i>	0.073	0.073	0.040	0.150	0.122	0.054	0.117	0.042	0.128	0.050	0.087	0.050								
15. <i>M. 'Rotsilber'</i>	0.097	0.078	0.089	0.078	0.089	0.073	0.087	0.047	0.192	0.083	0.108	0.083	0.068							
16. <i>M. 'Roland'</i>	0.088	0.068	0.062	0.086	0.080	0.063	0.097	0.048	0.130	0.073	0.102	0.057	0.073	0.050						
17. <i>M. 'Purpurescens'</i>	0.100	0.117	0.111	0.117	0.111	0.089	0.127	0.127	0.143	0.084	0.112	0.102	0.083	0.121	0.139					
18. <i>M. 'Variagatus'</i>	0.106	0.084	0.117	0.145	0.139	0.063	0.094	0.094	0.180	0.073	0.054	0.073	0.087	0.108	0.102	0.063				
19. <i>M. 'Kleinefontane'</i>	0.081	0.080	0.056	0.149	0.117	0.073	0.091	0.091	0.124	0.068	0.035	0.106	0.102	0.150	0.100	0.134	0.079			
20. <i>M. 'Gracillimus'</i>	0.213	0.192	0.155	0.160	0.155	0.145	0.171	0.102	0.168	0.166	0.107	0.079	0.132	0.117	0.160	0.122	0.107	0.186		
21. <i>M. 'Silverfeather'</i>	0.111	0.089	0.102	0.021	0.040	0.099	0.137	0.099	0.155	0.112	0.121	0.078	0.126	0.081	0.089	0.083	0.121	0.145	0.112	
22. <i>M. 'Undine'</i>	0.062	0.080	0.074	0.080	0.074	0.111	0.134	0.091	0.243	0.086	0.097	0.086	0.102	0.089	0.123	0.134	0.139	0.094	0.186	0.068

The distance index for the analysis of the cultivars (Table 3.2) provided a clearer picture than that for the species. The Nei -Li genetic distance between *M. sinensis* and *M. x giganteus* (0.048-0.179) is greater than the distance between *M. sacchariflorus* and *M. x giganteus* (0.043-0.068), indicating that *M. x giganteus* is more closely related to *M. sacchariflorus*.

3.3.2 Results of Simple Sequence Repeat (SSR) -Anchored PCR.

Trials of SSR-anchored PCR were not successful. The clarity of the banding patterns was much poorer than that achieved by RAPD and the reproducibility of the technique was poor. For this reason the results of the trials were not considered for inclusion in the analysis. Further work to perfect this technique is required, but was beyond the scope of this project.

3.4 Discussion

The two main constraints with regard to RAPD technology are generally considered to be reproducibility and the dominance of RAPD bands. In terms of reproducibility, it has been established that the results of RAPD analysis, even when using the same samples and standardisation of protocols, are difficult to reproduce between laboratories (Jones *et al.*, 1997). The first step in the analysis presented here was therefore to establish if the technique could be standardised to produce reproducible results within the laboratory. This aim was easily achieved with RAPD. While it is accepted that this reproducibility may not be achievable in another laboratory, within laboratory reproducibility allowed characterisation of the accessions at Trinity College Dublin and to use this information to screen accessions used in our research groups field trials and cold tolerance experiments.

With regard to the dominance effect of RAPD markers, this only becomes an issue if one is attempting to use the results of RAPD analysis for detailed population genetics or phylogenetic studies. If the bands are simply scored as presence/absence polymorphisms then scoring RAPD bands is no different to scoring presence/absence characteristics in morphology. However, other researchers, ignoring this fact, have used RAPD data to infer phylogenies. Halward *et al.* (1991) used RAPD data to examine the phylogeny of wild and cultivated strains of peanut. The authors, while acknowledging the fact that the use of arbitrarily primed PCR markers to infer phylogenetic relationships had not been fully determined, used the data to infer such relationships. The data they produced supported RFLP work on the same species, and while this may lend some support for using the data in this manner, it still ignores one of the fundamental principles of analysing dominant molecular markers, their conclusion being that the data supported previous molecular and morphological work and is therefore justified for creating phylogenies. Others who have used RAPD to infer phylogeny have ascertained the homology of bands by excising them from the gel and labelling them before using them as hybridisation probes against the blotted amplified fragments of their respective RAPD reactions (Catalan *et al.*, 1995). By testing the homology of bands in this way the authors were able to establish that RAPD could be used for phylogenetic studies in *Brachypodium* species. This approach was not investigated here, as it would have negated the original intent of the technique, which was to investigate if a simple and rapid technique such as RAPD would allow the genetic characterisation of the genus. All of the RAPD data analysed were treated in the strictest sense, that is, the presence of a band was considered as a dominant marker, and no inferences to phylogeny were made. In this way comparisons could be made with the morphological analysis already carried out by numerical taxonomic methods.

The use of RAPD analysis in cultivar identification has been widely reported. Graham *et al.* (1994) used the technique to successfully identify red raspberry (*Rubus* L.) cultivars and to examine their relatedness to each other using nine random primers. In the analysis of rye (*Secale cereale* L.), Iqbal & Rayburn (1994) demonstrated the utility of RAPD for cultivar identification. Marsolais *et al.* (1993) used RAPD to distinguish between different hybrids of lilac (*Syringa* L.) and to assess the relatedness of these hybrids to their putative parental species. Because of the success other researchers had achieved with RAPD as an aid to cultivar identification, the technique was applied to the cultivars of *Miscanthus*.

RAPD analysis of cultivars in conjunction with *M. sinensis* and *M. sacchariflorus* was designed to assess if any relationship existed between the cultivars and either of the two species. The actual data variance was much lower for this analysis by PCO than that achieved when the species were analysed alone. However the PCO showed a clearer resolution of *M. sinensis* from *M. sacchariflorus*. In addition *M. x giganteus*, which is postulated to be a hybrid involving *M. sacchariflorus* (Greef *et al.*, 1997), did group much closer to *M. sacchariflorus* than *M. sinensis*. As *M. x giganteus* is an allotriploid with genomes from both *M. sinensis* and *M. sacchariflorus*, and is a sterile hybrid, these results would indicate that dominance from *M. sacchariflorus* is exhibited. Three other cultivars, 'Undine', 'Grossefontane' and 'Kleinefontane' also grouped closer to *M. sacchariflorus*. In general there was a wide spread of variation among the cultivars and varieties by PCO analysis, but this spread of variation is also reflected in *M. sinensis* when analysed by RAPD or morphological methods. Cluster analysis by either UPGMA or NJ did not assist in the elucidation of the cultivars or varieties in relation to either *M. sinensis* or *M. sacchariflorus*.

A further aim of this chapter was to examine if RAPD could characterise the different species of *Miscanthus* available and to test if the results obtained from this analysis would confirm the positioning of the species within the relevant sections of *Miscanthus* as proposed by Lee (1964c). In addition it was planned to examine the RAPD data in context of the results already obtained from the morphological investigation and to investigate if these data confirmed or otherwise the results obtained from morphological examination. In this regard the results of RAPD analysis indicated that *M. sinensis* and *M. sacchariflorus* could be distinguished from each other by PCO but not by cluster analysis. However, this separation only held true when these two species were analysed in conjunction with the cultivars. When analysed in conjunction with the other species the separation of *M. sinensis* from *M. sacchariflorus* was much weaker. This contrasts with the morphological analysis which showed a strong separation of *M. sinensis* and *M. sacchariflorus* by PCO. When all of the other species were examined by PCO of the RAPD data set, the only species which was clearly defined was *M. nepalensis*. Although axis one of the data set provided for over 30% of the data variance, the results of the morphological analysis provided a much clearer

resolution. This result may be a reflection of the low number of interpretable bands achieved using RAPD, even though the level of polymorphism among these bands was high. In a total of fortyfour interpretable bands, thirtyfive of these were polymorphic between species. A similar situation was found in the analysis of genetic variation between different populations of *Saxifraga oppositifolia* (Gugerli *et al.*, 1999). The authors found that in a total of 86 interpretable RAPD bands, with only two of them being monomorphic, that no obvious groups were found. Principal Component Analysis, showed that only 12% of the data variance was explained by the first two axes. It therefore appears that RAPD data is too variable, particularly for those primers tested in this analysis, for use at the species level in *Miscanthus* and that their real value is in the characterisation of cultivars.

The analysis of the RAPD data by PCO does not support any of the sections of *Miscanthus* as assigned by Lee (1964c). This contrasts with the morphological analysis of *Miscanthus* which supported all of the sections, with the exception of *Diandra*. Analysis of the data set by either UPGMA or NJ provided no clear resolution of the species, with the exception of *M. nepalensis* which appeared to be distinct from all other species within *Miscanthus*.

The results of the RAPD analysis carried out here support the conclusions of those who have suggested that the use of RAPD markers for quantitative genetic diversity and genetic distance is limited (Bachmann, 1994). The analyses carried out here suggest that the power of RAPD markers lies in their ability to provide qualitative markers. The main purpose of using RAPD analysis, especially in relation to the cultivars was to examine whether they could be uniquely identified for the purpose of screening for potential as biomass crops. In this respect the technique was highly successful as the RAPD analysis produced a unique fingerprint for all of the cultivars available for analysis. A further extension of this work would be to excise the bands designated as markers so that they could be used as hybridisation probes to identify any new cultivars being examined in the field trials. Diagnostic bands could be excised from the gel re-amplified and used as hybridisation probes when screening other genotypes to check their identity. This avoids the problems of inter-laboratory reproducibility, and would allow development of markers for the various species and cultivars of *Miscanthus*. The development of such markers would provide a useful diagnostic tool for the screening of *Miscanthus*, not only in this laboratory but also by other researches conducting trials on the suitability of *Miscanthus* as a biomass crop. Therefore the "marker" as opposed to the profile has essentially been identified by RAPD and could be used as a diagnostic tool. To conclude, therefore the RAPD analysis proved useful for cultivar identification but was less useful at higher taxonomic levels.

CHAPTER 4

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

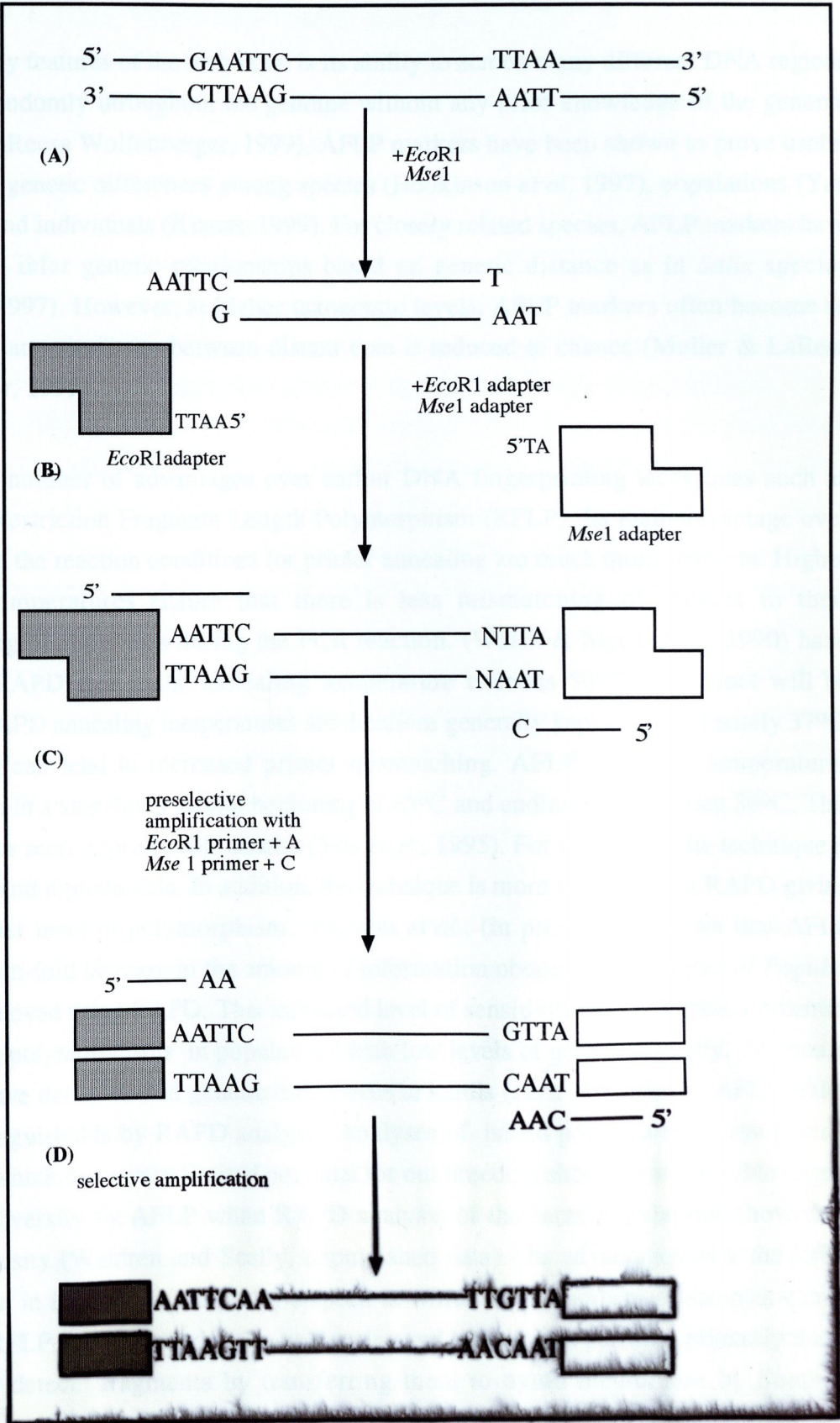
4.1 INTRODUCTION

Amplified Fragment Length Polymorphism (AFLP) is a DNA fingerprinting technique first developed at the Keygene Corporation in the Netherlands (Vos *et al.*, 1995). According to the authors, the technique 'provides a novel and very powerful DNA fingerprinting technique for DNA of any origin or complexity'. Essentially AFLP is a combination of Restriction Fragment Length Polymorphism (RFLP) and the Polymerase Chain Reaction (PCR). The technique draws on the reliability of RFLP and the power of PCR. The strength of AFLP is its ability to screen high numbers of polymorphic loci in a single analysis, while being highly reproducible and requiring no prior knowledge of the sequence being analysed.

The process of AFLP fingerprinting is graphically illustrated in figure 4.1. Total genomic DNA is digested with restriction endonucleases to produce a set of restriction fragments. Typically a frequent cutting enzyme such as *Mse*I and a rare cutter such as *Eco*R1 are used in combination. Frequent cutting enzymes, such as *Mse*I, generally find the recognition sequence at which they cut in many more locations along the genome than do the rare cutting enzymes such as *Eco*R1. The recognition sequences for these enzymes are shown in table 4.1. The frequent cutter will generate fragments of optimal size for PCR amplification that will separate well on denaturing gels. The rare cutter will reduce the number of fragments produced because only rare cutter/frequent cutter fragments will be amplified. Double stranded adapters are then ligated to the ends of the DNA fragments produced from the digestion. The adapters (sequences shown in table 4.1) contain a core sequence and an enzyme specific sequence, this sequence allows them to recognise the complementary sequence on the restriction fragments produced. The sequence of the adapter and the adjacent restriction site then serve as primer binding sites for amplification of the restriction fragments (Vos *et al.*, 1995). Selective nucleotides extending into the restriction fragments are then added to the 3' ends of the primers so that only a subset of the restriction fragments are recognised. Only restriction fragments in which the nucleotides flanking the restriction site matching the selective nucleotides will be amplified. These amplified fragments can then be separated and visualised by gel electrophoresis. The primers used are essentially arbitrary primers, and require no prior knowledge of the sequence of the fragments. Typically 50-100 restriction fragments are produced and detected on denaturing polyacrylamide gels. AFLP technology is currently covered by patents or patent applications owned by Keygene n.v. The technology can however be used if the reagents required for AFLP analysis are purchased from licensed suppliers such as Life Technologies when used in conjunction with

Figure. 4.1 Illustration of the steps of the AFLP technique

(A) Restriction digest with *EcoRI* & *MseI* (B) Ligation of adapters
(C) Preselective amplification (D) Selective amplification.



an authorised thermal cycler such as Perkin - Elmer. It is worth mentioning here that different primer combinations may give conflicting results for the analysis of the same DNA sample. Breyne *et al.*, (1999) have found conflicting results based on the analysis of the same DNA by different primer combinations and different restriction enzyme combinations.

One of the key features of the technique is its ability to screen many different DNA regions distributed randomly throughout the genome without any prior knowledge of the genome (Muller & LaReesa Wolfenberger, 1999). AFLP markers have been shown to prove useful for assessing genetic differences among species (Hodkinson *et al*, 1997), populations (Yan *et al*, 1999) and individuals (Krauss, 1999). For closely related species, AFLP markers have been used to infer genetic relationships based on genetic distance as in *Salix* species (Beismann, 1997). However, at higher taxonomic levels, AFLP markers often become so variable that any similarity between distant taxa is reduced to chance (Muller & LaReesa Wolfenberger, 1999).

AFLP has a number of advantages over earlier DNA fingerprinting techniques such as RAPD and Restriction Fragment Length Polymorphism (RFLP). Its main advantage over RAPD is that the reaction conditions for primer annealing are much more stringent. Higher annealing temperatures ensure that there is less mismatching of primers to their corresponding binding sites during the PCR reaction. (Walsh & McClelland, 1990) have shown for RAPD that if the annealing temperature exceeds 50°C no product will be produced. RAPD annealing temperatures are therefore generally kept at approximately 37°C. This in turn can lead to increased primer mismatching. AFLP annealing temperatures generally run in a step down format beginning at 65°C and ending no lower than 56°C. This in turn creates more reproducible results (Vos *et al.*, 1995). For this reason the technique is more robust and reproducible. In addition, the technique is more sensitive than RAPD giving a much higher level of polymorphism. Cervera *et al.*, (in press) have shown that AFLP produced a ten-fold increase in the amount of information obtainable in analysis of *Populus* than was achieved using RAPD. This increased level of sensitivity provides greater potential for detecting polymorphisms in populations with low levels of genetic diversity. Sharma *et al.* (1996) have demonstrated genetic differences in lentils (*Lens culinaris*) by AFLP which were indistinguishable by RAPD analysis. Analyses of island populations of rye (*Secale cereale* L.) which had a very limited potential for out breeding showed a measurable degree of genetic diversity by AFLP when RAPD analysis of the same populations showed no genetic diversity (Waldren and Scally, unpublished data). Its advantages over the RFLP technique lie in its ease of use and the speed at which large numbers of samples can be processed. RFLP are particularly time consuming and laborious to perform, primarily due to the need to detect fragments by transferring them to nylon membranes by Southern

hybridisation (Southern, 1975). The membrane must then be hybridised, usually with a radioactively labelled single stranded DNA probe.

Dominance of AFLP markers still presents a problem and the original authors of the technique (Vos *et al.*, 1995) have clearly stated that AFLP are presence /absence polymorphisms. However, it has been shown that co-dominant AFLP markers have been found at frequencies of 4-15% among all polymorphic AFLP markers (Waugh, 1997). In addition, since AFLP are capable of distinguishing one base pair size differences, it is highly likely that bands comigrating to the same position on a gel are in fact homologous. Since the publication of the original technique, developments in software for the image analysis of fluorescent PCR products has improved and it may be possible to utilise such analysis with AFLP which would allow co-dominant markers to be scored (Ridout & Donini, 1999). Despite the fact that AFLP are dominant markers many researchers have chosen to ignore this fact and continue to create phylogenies based on AFLP data (Sharma *et al.*, 1996; (Heun, 1997); Hodkinson *et al.*, 2000). Restriction Fragment Length Polymorphism (RFLP) and microsatellites (Jarne & Lagoda, 1996) remain the fingerprinting techniques of choice currently available for the determination of codominant markers and to distinguish homozygote from heterozygote loci. A comparison of AFLP and RFLP for the analysis of population genetics of the yellow fever mosquito in Trinidad showed that RFLP provided better estimates of population genetic variability and offered more statistical power for detecting population genetic structure than AFLP (Yan *et al.*, 1999). However RFLP are laborious and time consuming thereby limiting the number of samples that can be analysed. Microsatellites, being PCR based, are easier to perform but require prior knowledge of the sequence on either side of the repeat sequence in order to design a suitable primer (Streiff *et al.*, 1998).

One of the disadvantages of the technique, as described here, is the requirement for radioactively labelled isotopes. This obviously requires great care when handling the isotopes and a suitable laboratory facility in which to use them. While this facility was available for use on this project, it may not be available to every laboratory utilising AFLP. For this reason a silver staining protocol was investigated as an alternative technique. Although Keygene do not recommend this technique as it has not been tried and tested by them, silver staining has been used with success for the staining of polyacrylamide gels. Bassam *et al.* (1991) successfully used a modification of the silver staining technique of Merril *et al.* (1981) to detect as low as 1 pg/mm² band cross-section of double stranded DNA. They were also successful in applying their technique to DNA fragments produced from DNA Amplification Fingerprinting (DAF), although they do not state if their method was as sensitive as using radioisotopic methods for the same technique.

Fluorescent techniques for the visualisation of AFLP bands are now available and the method can be automated for use on a DNA sequencer for reading the gels, a facility that was not an option in this project. Automated techniques may however be superior in other respects as an automated sequencer actually reads the bands, thereby increasing the efficiency of the technique while reducing scoring error. Fluorescent labelling and automated analysis of AFLP bands was used by Hodkinson *et al.*, (1997) in their analysis of *Miscanthus*. This system allowed them to analyse over 100 bands per primer and has the advantage of allowing bands of higher molecular weight to be scored. When using manual analysis the high molecular bands sometimes have to be disregarded as the spacing between them is so small they become unreadable. Automated sequencing of AFLP bands should provide a very useful tool in the area of systematics as it will enable researchers to vastly increase data sets, or scorable characters, with greater accuracy and efficiency than is currently available by radioactive labelling and manual scoring. In addition, Lynch & Milligan (1994) have suggested that two to ten times more individuals should be sampled per RAPD locus than per RFLP isozyme loci due to the dominance effect, the same criterion should therefore be applied to AFLP. With the advent of fluorescent labelling and automated analysis this option should be realised more easily, thereby increasing the accuracy of data sets.

Attempts to assess the genetic diversity of *Miscanthus* have been made by others. Greef *et al.* (1997) examined eleven accessions of *M. sinensis*, two of *M. sacchariflorus* and 31 of *M. x giganteus* by AFLP analysis. They found that *M. sinensis* and *M. sacchariflorus* were easily distinguished by their AFLP profiles and that most of the *M. x giganteus* accessions were closer to *M. sacchariflorus*. Their results also showed that there were no molecular differences between rhizome and micro-propagated strains of *M. x giganteus*. However, they did not examine any of the other species within the genus or a large sample of the varieties or cultivars of *Miscanthus*. Hodkinson *et al.* (1997) also used AFLP analysis to investigate the systematics of the genus and included many of the other species within their analysis. They found the technique to be successful in aiding in the identification of *Miscanthus* accessions and intend to use the data to build an AFLP database for *Miscanthus*.

The aim of the analyses undertaken in this chapter was to examine if AFLP could distinguish between the different species of *Miscanthus* and to test the different sections of the genus as assigned by Lee (1964c) based on their AFLP profiles. The potential of the AFLP technique for distinguishing between varieties and cultivars of *Miscanthus* and their relationship to *M. sinensis* and *M. sacchariflorus* was also investigated. In particular, the putative hybrid *M. x giganteus* was examined by AFLP to investigate its relationship to *M. sinensis* and *M. sacchariflorus*, the two species believed to be involved in its hybridisation (Greef & Deuter, 1993).

4.2.1 AFLP technique

AFLP technology is licensed by Karygene Corporation, Life Technologies, Inc. and is used to supply the reagents required for AFLP analysis in kit form. For this reason all kit

Table 4.1 Structure of AFLP adapters and primers (Adapted from Vos *et al*, 1995).

Adapter sequences

AFLP adapters consist of a core sequence and an enzyme specific sequence.

*Eco*RI-adapter:

5-CTCGTAGACTGCGTACC

CATCTGACGCATGGTTAA-5

*Mse*I-adapter:

5-GACGATGAGTCCTGAG

TACTCAGGACTCAT-5

Primer sequences

AFLP primers consist of a core sequence, an enzyme specific sequence and a selective extension. The selective extensions are normally either two or three bases long depending on the species being analysed, They are shown here as NNN.

	Core sequence	Enzyme sequence	Extension
<i>Eco</i> RI	5-GACTGCGTACC	AATTC	NNN-3
<i>Mse</i> I	5-GATGAGTCCTGAG	TAA	NNN-3

Note the recognition sequence of *Eco*RI is G/AATTC and that of *Mse*I is T/TAA

4.2 Materials and Methods

4.2.1 AFLP technique

AFLP technology is licensed by Keygene Corporation. Life Technologies, Inc. are licensed to supply the reagents required for AFLP analysis in kit form. For this reason all kits required for AFLP analysis in this project were purchased through Life Technologies, Inc. The exact components of the solutions marked with an asterisk are given at the end of the section.

The DNA analysed by the AFLP technique was extracted by the protocol outlined in chapter three of this thesis. Additional DNA, extracted by Dr. T. Hodkinson at the Royal Botanic Garden, Kew was also included in the analysis.

The technique involved the following steps:

Restriction Digestion of DNA

A master mix of the following components was prepared. The following are the volumes required per reaction.

5X reaction buffer*	5 μ l
<i>Eco</i> R1/ <i>Mse</i> I (1.25 units/ μ l)	2 μ l
Distilled water	15.5 μ l

2.5 μ l of sample DNA (100 ng μ l⁻¹) was added to a 1.5 ml micro centrifuge tube and 22.5 μ l of the master mix was added, giving a total volume of 25 μ l. The contents were gently mixed and the reaction collected by brief centrifugation. The reaction was then incubated at 37°C for 2 hours on a hot block. Immediately after the incubation period the block temperature was ramped up to 70°C and held for 15 min to inactivate the restriction enzymes. The tubes were then placed on ice and the reaction collected by brief centrifugation (approximately 10 seconds at 13, 000rpm).

Ligation of Adapters

24 μ l of ligation solution* and 1 μ l of T4 DNA ligase were added to each tube. The tubes were mixed gently at room temperature and centrifuged briefly to collect the contents. The reaction was then incubated at 20°C $\pm$ 2°C for two hours (this was normally achieved by simply placing the tubes in a laboratory cupboard for the time period). Following the incubation period a 1:10 dilution of the ligated solution with TE buffer was made. Any undiluted ligated solution was stored at -20°C in case it was required for future use.

Pre-amplification Reactions

The following components were added to a 0.5 ml PCR tube:

Diluted template from the ligation reaction	5 µl
Pre-amplification primer mix (patented product, exact components unknown)	40 µl
10x PCR buffer*	5 µl
Taq DNA polymerase (6 units µl ⁻¹)	0.4 µl

The tubes were mixed gently and centrifuged briefly to collect the reaction. The reaction was overlaid with 2 drops of mineral oil to prevent evaporation. 20 cycles of the PCR reaction were performed in a Perkin - Elmer 480 thermal cycler using the following parameters:

- 94°C for 30 sec
- 56°C for 60 sec
- 72°C for 60 sec

Following PCR a 1:50 dilution of the PCR products was made with TE buffer. The remainder of the product was frozen at -20°C for future use if required. Both diluted and undiluted portions can be stored at -20°C at this stage.

Primer Labelling

Primer labelling can be achieved using either Y-³³P or Y-³²P. It is recommended however that Y-³³P is used as it gives better resolution of the PCR products on the gels. Also, the reaction products are less prone to autoradiolysis.

The following components were added to a 1.5 ml micro centrifuge tube: The following are the volumes required for one reaction, in most cases a master mix was made with enough reagent for all reactions being carried out.

EcoR1 primer	18 µl
5X kinase buffer*	10 µl
[Y- ³³ P]ATP (2,000 Ci/mmol)	10 µl
T4 kinase*	2 µl
Total volume	50 µl

An endless list of possible primer pair combinations can be used for AFLP analysis. Trials conducted on various plant species by Keygene have greatly assisted in narrowing this list.

Keygene have produced recommendations on potentially useful combinations from their trials and this proved to be a very useful starting point for choosing a suitable primer combination. Initial trials involved the use of a number of primer pair combinations (table 4.2). To increase the level of polymorphism detected it is recommended that a primer combination with a two base paired anchor *Mse* primer is used. However, when this combination was used with *Miscanthus* the level of polymorphism was so high that the autoradiographs produced were extremely difficult to read. In order to reduce the number of bands per gel three base pair primers were then chosen. These produced autoradiographs with fewer bands but a sufficiently high level of polymorphism. Following this initial investigation the primer combinations *EcoRI*ACA/*Mse*IACC and *EcoRI*ACC/*Mse*ICAT were found to be most suitable and were therefore used in all subsequent analysis.

Table 4.2: Sequence of primers investigated

- EcoRI* Primer TG combined with *Mse* ICTT
- EcoRI* Primer AAG combined with *Mse* ICAC
- EcoRI* Primer ACA combined with *Mse* ICAA
- EcoRI* Primer ACA combined with *Mse* IACC
- EcoRI* Primer ACC combined with *Mse* ICAT

Selective amplification

To prepare the selective amplifications two initial master mixes were prepared.

Mix 1: 5 µl of labelled *EcoRI* primer from the above step was combined with 45 µl of *MseI* primer (which also contains the dNTPs).

Mix 2: Distilled water 79 µl, 10X PCR buffer 290 µl, *Taq* DNA Polymerase (6 units µl⁻¹) 1 µl.

Each amplification reaction was prepared by combining the following in a 0.5 ml PCR tube: Diluted template from pre amplification reaction 5 µl

Mix 1 (primers/dNTPs) 5 µl

Mix 2 (*Taq* DNA polymerase/buffer) 10 µl

The reaction was mixed gently and overlaid with 2 drops of silicone oil. Each reaction was then subjected to the following PCR conditions. All reactions were performed on a Perkin Elmer 480 thermal cycler:

94°C for 60 sec, 65°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 64°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 63°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 62°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 61°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 60°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 59°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 58°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 57°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 60 sec, 56°C for 60 sec, 72°C for 90 sec for 1 cycle

94°C for 30 sec, 56°C for 30 sec, 72°C for 60 sec for 23 cycles

Soak temperature 4°C

Following PCR, 20 µl of formamide dye (98% formamide, 10mM EDTA, bromophenol blue, xylene cyanol) was added to each reaction. The formamide prevents reassociation of the two DNA strands following denaturation and the two dye system facilitates loading of the samples onto the gel and tracking of the run as it progresses. Xylene cyanol runs through the gel at a slower speed than bromophenol blue allowing lower molecular weight fragments to be tracked. The sample tubes were then heated to 90°C for 3 min to denature the DNA and then immediately placed on ice.

4.2.2. Quality Control

A sample of tomato DNA was provided with the AFLP kit and this served as an external positive control which was analysed and run alongside each set of samples. In addition an internal positive control of *M. sinensis* was run with each batch of samples analysed. Reagent blanks were also included with each run. In initial trials all samples were run in duplicate to test for reproducibility of banding patterns, but samples were reduced to one per analysis in the final assays so that all samples for a particular primer combination could be run on the same gel, once reproducibility had been assured

4.2.3 Polyacrylamide gel electrophoreses.

Prior to pouring the gel the glass plates were washed thoroughly with soap and water and then rinsed in distilled water. When dry both plates were wiped with ethanol, the contact

side of the smaller plate was then wiped with silane to prevent the gel adhering to this surface.

A 6% polyacrylamide gel was prepared by mixing 60 ml of premixed Seguagel® (National Diagnostics) with 480 µl of 10% ammonium persulphate and 48 µl of TEMED*. 0.4 mm spacers and sharktooth combs were used on a model S2 gel apparatus. The running buffer used was 1 x TBE*. The gel was pre-run at 70 volts for 30 min, to ensure the gel temperature was constant throughout the run. A 5 µl aliquot of each sample were loaded onto the gel and then run at 70 volts for about 2 hours, until the slower xylene cyanol dye was about two-thirds down the length of the gel. Following electrophoresis the plates were separated and the signal from the gel was measured with a gieger counter. The strength of the signal allowed the length of subsequent exposure to X - ray film to be determined. The gel was then removed from the plate by laying a sheet of Whatmann 3mm® filter paper over the gel and then carefully removing the paper with the gel adhering to its surface. A piece of Clingfilm® was then placed over the gel for protection.

The gel was then dried on an automatic, vacuum-aided gel drier. When dry, the gel was exposed on Agfa Curix RP1 Plus medical grade X-ray film at room temperature overnight, or in some cases for 48 hours if the signal strength was weak.

4.2.4 Scoring of autoradiographs

All samples for a given primer combination were loaded on the same gel. This allowed comparisons to be made directly across the gel between samples. If the number of samples run precluded them all from being loaded on a single gel scoring of the bands would have been much more problematic. Approximately 60 bands per lane were produced. Those closer to the top of the gel were unreadable due to lack of adequate separation and would have required the gel to have been run for a longer period in order for them to be readable. However due to the high number of bands and the adequate levels of polymorphism observed this was not necessary. Instead a section of the gel with good resolution was taken and the bands in this section were scored as present or absent.

4.2.5. Silver staining

Although this technique was not used for the final analysis of AFLPs it was investigated as a possible means of eliminating the need to use radioactively labelled primers in this project. Two basic approaches to the silver staining technique were used. The first involved the use of the Bio-Rad® silver staining kit. This is a pre-prepared kit in which all of the reagents required are supplied, although information on the chemical composition of all of the

reagents is not supplied. The second method required all of the reagents to be made up from first principles.

Components of solutions used in AFLP analysis.

5X PCR reaction buffer: 50 mM Tris-HCl (pH 7.5), 50 mM Magnesium-acetate, 250 mM potassium-acetate.

Adapter/ligation solution: *EcoRI*/*MseI* adapters, 0.4 mM ATP, 10 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 50% (V/V) glycerol.

T4 DNA ligase: 1 unit/ μ l in 10 mM Tris-HCl (pH) 7.5, 1 mM DTT, 50 mM KCl, 50% glycerol (v/v).

TE buffer: 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA

1x TBE buffer: 10.8g Tris, 5.5g boric acid, 0.92g EDTA-Na in 1000ml

T4 kinase: 10 units/ μ l in 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 1 mM 2-mercaptoethanol, 0.1 μ M ATP, 50% (v/v) glycerol.

5X Kinase buffer: 350 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 500 mM KCl, 5 mM 2-mercaptoethanol

Bio-RAD® protocol for silver staining:

1. After electrophoresis the plates were separated and the gel was fixed in Acetic acid: ethanol 2:50 for 15 min. The fixative was then poured off and the wash repeated for a further 15 min.
2. The fixative was poured off and an oxidiser solution was applied to the gel for 5 min.
3. The gel was then washed in distilled water for 10 min.
4. Silver reagent was then applied to the gel for 20 min.
5. Following silver staining the gel was washed in distilled water very briefly.
6. Developer was then applied to the gel (recommended time 1-10 min.)

Protocol for Silver staining (pers comm Dr Ronan Loftus, TCD)

1. Prior to pouring the gel the larger plate was wiped with bind-silane (Sigma). The smaller plate (the one to be removed from the gel) was then treated by washing with normal Silane.
2. After electrophoresis the plates were separated. The plate with the gel attached was then washed in two washes of a 400 ml solution of fixative (ethanol, 40 ml; acetic acid, 2 ml; water 358 ml) for 3 min. per wash.
3. The fixative solution was then poured off and the gel washed in 200 ml of silver nitrate (1g/l) for 10 min.

4. The gel was then washed twice in two separate washes of distilled water (10 sec per wash).

5. Finally, the gel was washed in 200 ml of a solution of 3g sodium hydroxide, 800 µl formaldehyde and 0.2 g borohydrate.

All washes were conducted at ambient temperature with gentle shaking. This protocol proved much more successful than the Bio-Rad protocol. The bind - silane ensured the gel remained in place throughout the washing stages and very little background staining occurred.

4.2.6. Statistical Analysis of AFLP Autoradiographs

A band presence/absence matrix was compiled from all of the samples analysed. Three methods of statistical analysis were employed to analyse the band matrix. Principal Coordinate Analysis (PCO), as outlined in chapter two, was used to produce an ordination of the bands. Unweighted Pair-Group method using Arithmetic Mean (UPGMA) and neighbor joining (NJ) software packages, also described in chapter three, were used to provide clustering methods of analysis using the PAUP computer program version 4.0b2 for Macintosh (Swofford, 1999). When analysing the data by UPGMA and NJ each primer combination was analysed separately and then both primer combinations were analysed together. This allowed a comparison to be made between different primer pairs, to ascertain if the data produced from each primer was homologous, while the combined analysis provided a larger number of scored characters for the analysis. Species were analysed separately to cultivars. When the cultivars were being analysed *M. sinensis* and *M. sacchariflorus* were also included in the analysis. This allowed the position of the cultivars to be examined in relation to the two species they were most likely to have been derived from. If a sample failed to produce a successful AFLP profile with one primer it would have to have been excluded from the analysis for both primer pairs. By analysing the primer combinations separately all data was allowed to be included in at least one analysis. In addition, parsimony analysis using a heuristic search (fully described in chapter 5) was also applied to the data for comparison with subsequent sequence analysis.

4.3 Results

The AFLP technique was highly successful in establishing genetic differences between species and between cultivars in all of the samples analysed. The level of polymorphism detected was considerably higher than that achieved by the RAPD technique. Approximately 60 bands per sample were produced, compared to approximately 10 by RAPD analysis. Analysis of duplicate samples and quality control checks demonstrated the reproducibility of the technique. In no case did sample blanks show any contamination. The tomato DNA sample run as an external standard and the *M. sinensis* sample run as an internal standard were consistent in each run. The technique, while considerably more time consuming and complicated than RAPD was extremely reproducible.

4.3.1 AFLP band analysis by Principal Co-Ordinate Analysis (PCO) of all species.

4.3.1a Primer combination *EcoRIACA/MseICAC*

Principal Co-ordinate Analysis (PCO) of all species analysed by AFLP with the primer combination *EcoRIACA/MseICAC* showed that 20.2% of the data variance was explained on axis one (fig 4.2.a), 18% on axis two (fig 4.2.b), 14.5% on axis three (fig. 4.2.c) and 11.7% on axis four (not presented as the data was not easily interpretable). Axis one of the PCO separated one individual of *M. condensatus* and *M. oligostachyus* from all other species, but not from each other. The second individual of *M. condensatus* was closer to the *M. sinensis* cluster. This axis showed only a marginal separation of *M. sinensis* and *M. sacchariflorus*. Axis two produced a much clearer separation of *M. sinensis*, *M. sacchariflorus* and *M. floridulus*. Axis three of the PCO was more difficult to interpret, but produced a slightly better resolution of one individual of *M. condensatus* and *M. oligostachyus*. Axis four did not provide any interpretable resolution of the data. The combined axes one and two supported section *Triarrhena* as defined by Lee (1964c). Support for the other sections remained unresolved.

4.3.1b Primer combination *EcoRIACC/MseICAT*

Analysis of the data with the primer combination *EcoRIACC/MseICAT* showed a much clearer resolution of the species to that obtained with the combination *EcoRIACA/MseICAC*. 27% of the data variance was explained on axis one (Fig. 4.3.a), 19% on axis two (Fig. 4.3.b), 14.7% on axis three (Fig 4.3.c) and 9.5% on axis four (not presented). Axis one separated *M. sinensis*, *M. sacchariflorus*, *M. floridulus* and *M. transmorrisonensis*. However one individual of *M. sacchariflorus* appeared closer to *M. floridulus*, and *M. nudipes* was close to *M. sacchariflorus* on this axis. Axis two separated *M. oligostachyus*

Fig. 4.2 Ordination of *Miscanthus* species by PCO of AFLP data with primer combination *Eco* RIACA/*Mse* ICAC.(a) axis one & two (b) axis two & three (c) axis one & three

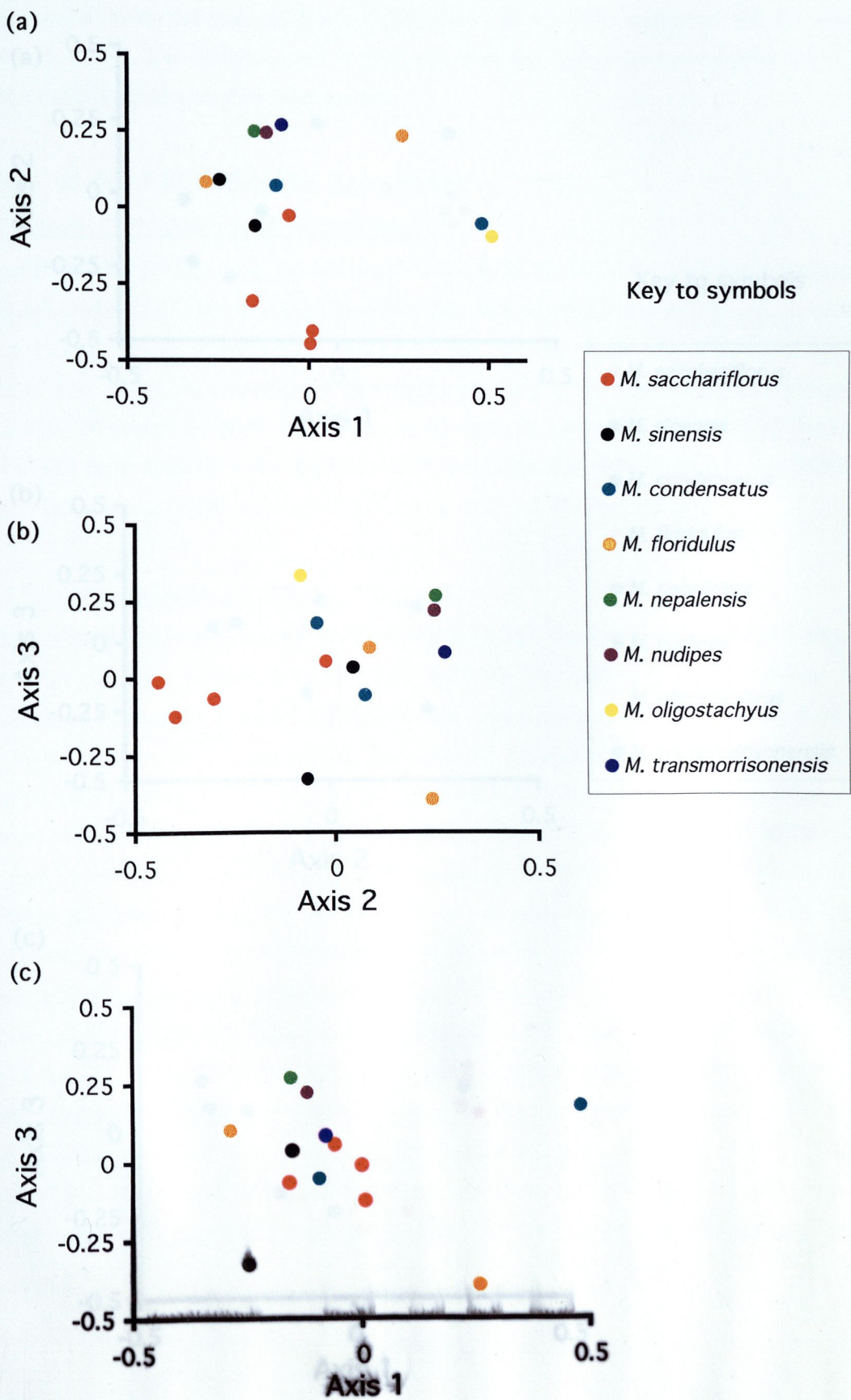
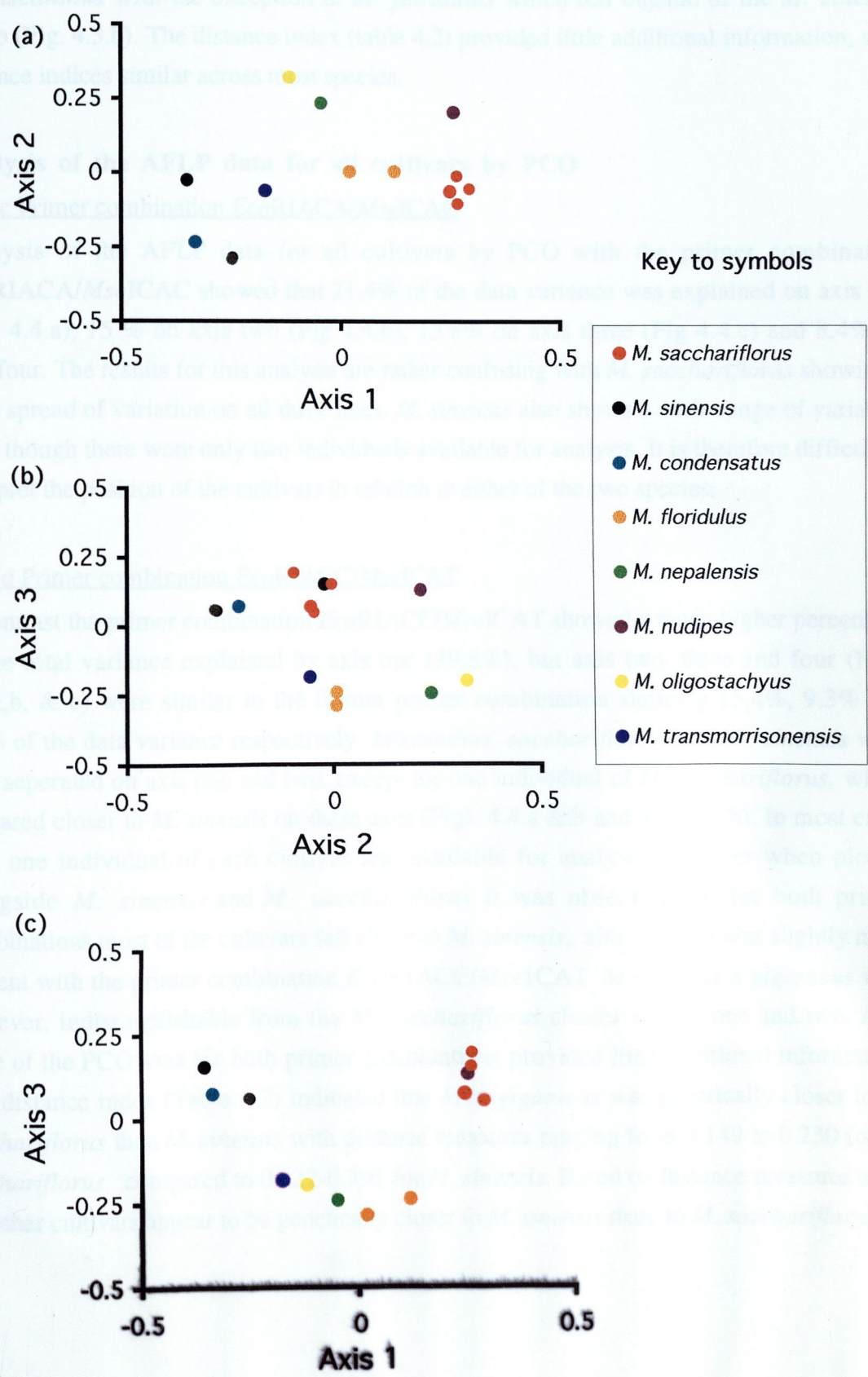


Fig. 4.3 Ordination of *Miscanthus* species by PCO of AFLP data with primer combination *Eco* RIACC/*Mse* ICAT. (a) axis one & two (b) axis two & three (c) axis one & three.



and *M. nepalensis* from each other but not from *M. nudipes*. Axis three showed *M. floridulus*, *M. nepalensis*, *M. oligostachyus* and *M. transmorrisonensis* to be distinct from the other species but not from each other. As with the previous primer combination axis four presented no clear resolution of the data. Axis one and two broadly supported the sections of *Miscanthus* with the exception of *M. floridulus* which fell outside of the *M. sinensis* group (Fig. 4.3.b). The distance index (table 4.2) provided little additional information, with distance indices similar across most species.

Analysis of the AFLP data for all cultivars by PCO

4.3.1c Primer combination *EcoRIACA/MseICAC*

Analysis of the AFLP data for all cultivars by PCO with the primer combination *EcoRIACA/MseICAC* showed that 21.4% of the data variance was explained on axis one (Fig. 4.4.a), 15 % on axis two (Fig 4.4.b), 13.8% on axis three (Fig 4.4.c) and 8.4% on axis four. The results for this analysis are rather confusing with *M. sacchariflorus* showing a wide spread of variation on all three axes. *M. sinensis* also shows a wide range of variation even though there were only two individuals available for analysis. It is therefore difficult to interpret the position of the cultivars in relation to either of the two species.

4.3.1d Primer combination *EcoRIACC/MseICAT*

In contrast the primer combination *EcoRIACC/MseICAT* showed a much higher percentage of the total variance explained by axis one (39.8%), but axis two, three and four (Figs. 4.5.a,b, & c) were similar to the former primer combination showing 15.4%, 9.3% and 7.6% of the data variance respectively. *Miscanthus. sacchariflorus* and *M. sinensis* were well separated on axis one and two, except for one individual of *M. sacchariflorus*, which appeared closer to *M. sinensis* on these axes (Figs. 4.4.a &.b and 4.5. a & b). In most cases only one individual of each cultivar was available for analysis, however when plotted alongside *M. sinensis* and *M. sacchariflorus* it was observed that for both primer combinations most of the cultivars fell closer to *M. sinensis*, although this was slightly more evident with the primer combination *EcoRIACC/MseICAT*. *Miscanthus x giganteus* was, however, indistinguishable from the *M. sacchariflorus* cluster on axis one and two. Axis three of the PCO axes for both primer combinations provided little additional information. The distance index (Table 4.3) indicated that *M. x giganteus* was genetically closer to *M. sacchariflorus* than *M. sinensis* with distance measures ranging from 0.149 to 0.230 for *M. sacchariflorus*. compared to 0.322-0.391 for *M. sinensis*. Based on distance measures all of the other cultivars appear to be genetically closer to *M. sinensis* than to *M. sacchariflorus*

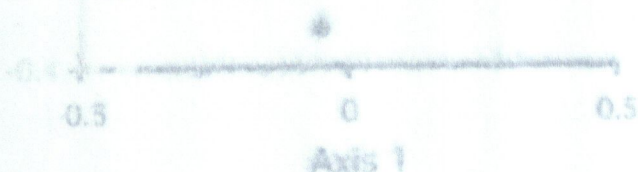


Fig. 4.4 Ordination of *Miscanthus* cultivars by PCO of AFLP data with primer combination *Eco* R1ACA/*Mse* 1CAC (a) axis one & two (b) axis two & three (c) axis two & three.

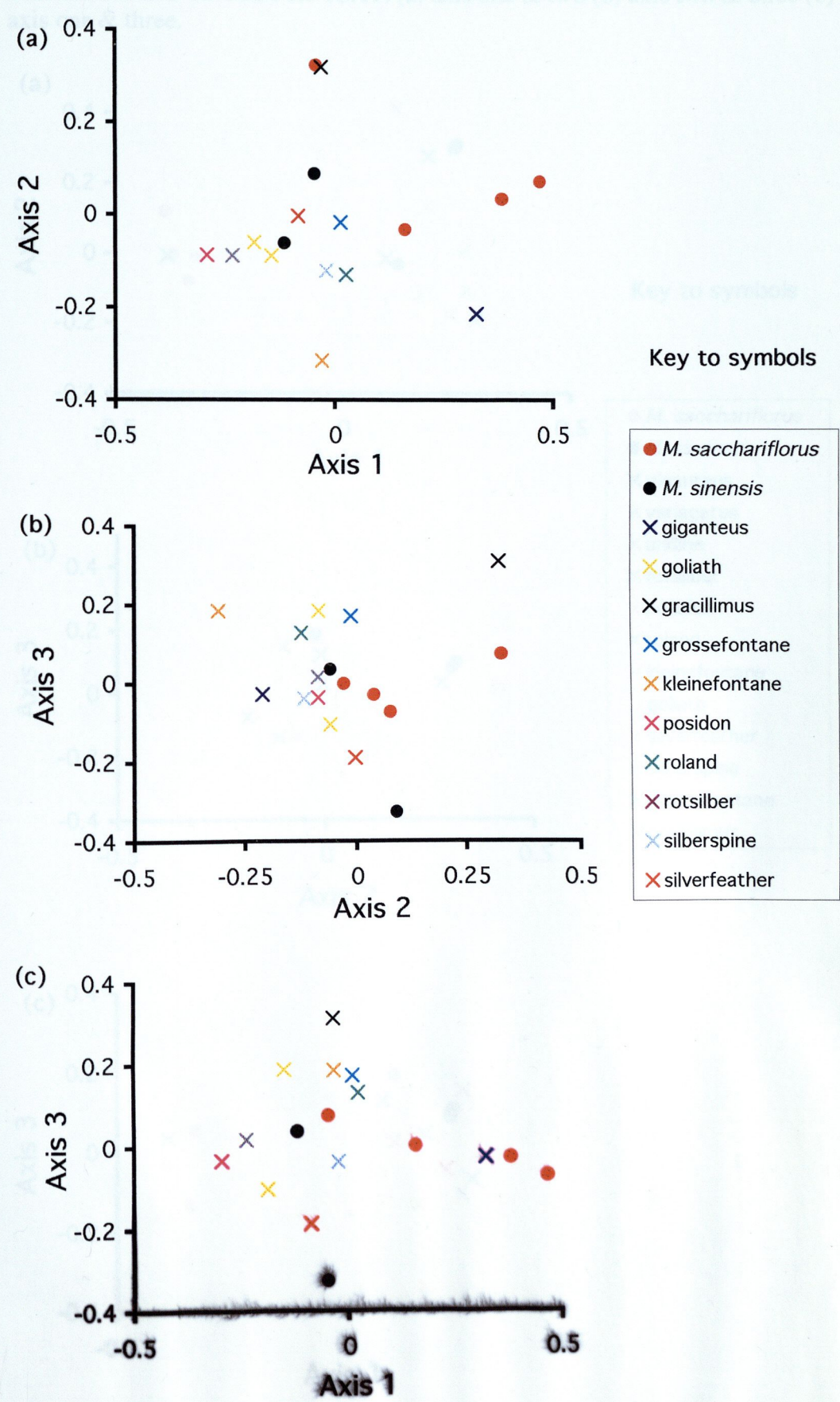


Fig. 4.5 Ordination of *Miscanthus* cultivars by PCO of AFLP data with primer combination *Eco* RIACC/*Mse* ICAT. (a) axis one & two (b) axis two & three (c) axis one & three.

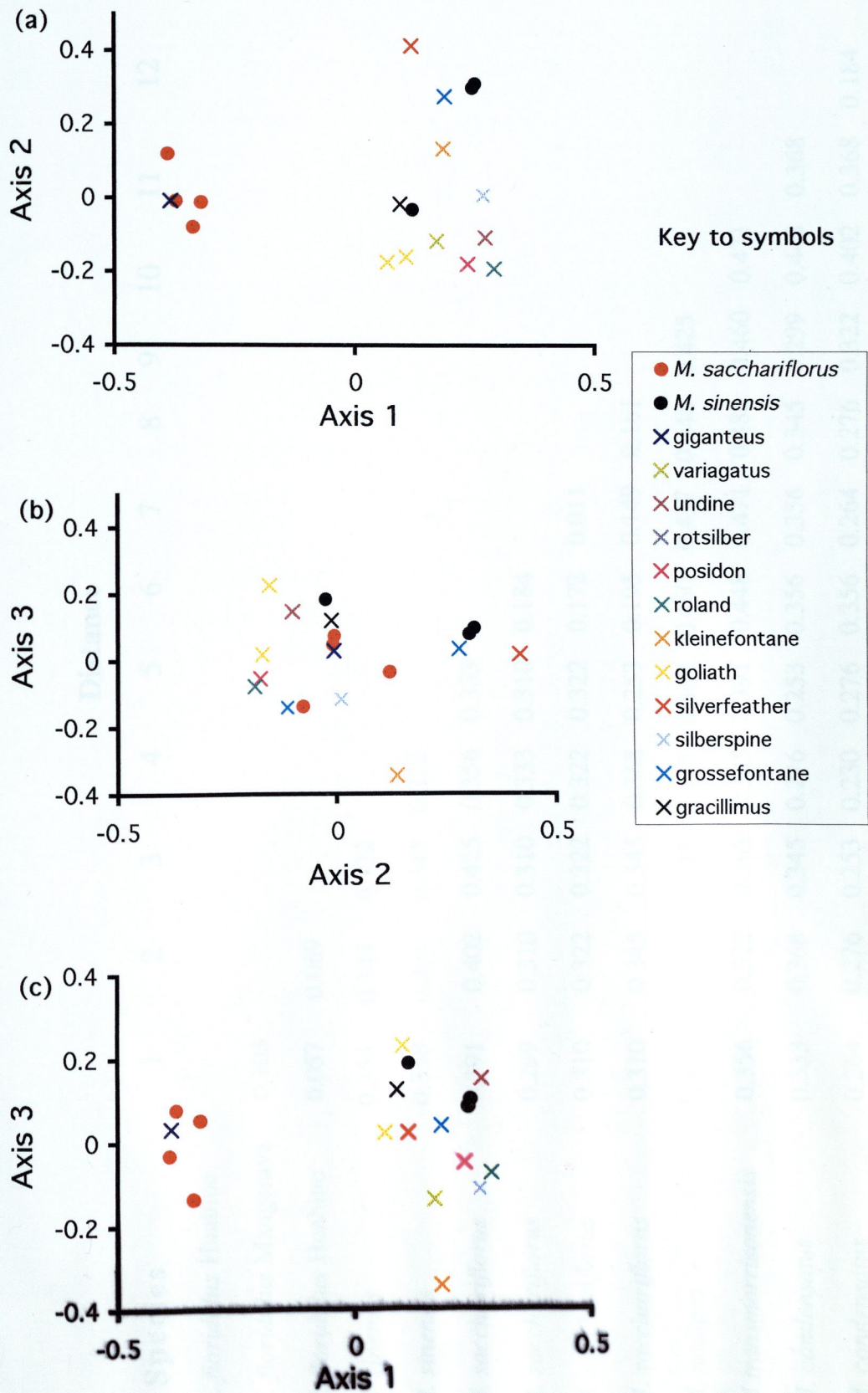


Table 4.2 Nei-Lei distance index for *Miscanthus* species of AFLP data

Species	Distance												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1. <i>M. floridulus</i> Huahine													
2. <i>M. floridulus</i> Mangerava	0.103												
3. <i>M. floridulus</i> Huahine	0.057	0.069											
4. <i>M. sinensis</i>	0.356	0.345	0.322										
5. <i>M. sinensis</i>	0.356	0.391	0.345	0.322									
6. <i>M. sacchariflorus</i>	0.391	0.402	0.425	0.356	0.333								
7. <i>M. sacchariflorus</i>	0.299	0.310	0.310	0.333	0.310	0.184							
8. <i>M. sacchariflorus</i>	0.310	0.322	0.322	0.322	0.322	0.172	0.011						
9. <i>M. sacchariflorus</i>	0.310	0.345	0.345	0.368	0.253	0.195	0.149	0.161					
10. <i>M. nudipes</i>	0.345	0.356	0.333	0.425	0.402	0.460	0.437	0.448	0.425				
11. <i>M. transmorrisonensis</i>	0.356	0.322	0.368	0.345	0.391	0.448	0.471	0.483	0.460	0.471			
12. <i>M. condensatus</i>	0.333	0.368	0.345	0.276	0.253	0.356	0.356	0.345	0.299	0.448	0.368		
13. <i>M. condensatus</i>	0.264	0.276	0.253	0.230	0.276	0.356	0.264	0.276	0.322	0.402	0.368	0.184	
14. <i>M. nepalensis</i>	0.345	0.333	0.310	0.333	0.356	0.483	0.414	0.425	0.425	0.299	0.356	0.356	0.310

Table 4.3 Nei-Lei Distance index for *Miscanthus* cultivars, *M. sinensis* and *M. sacchariflorus* for AFLP data

Cultivar	Distance														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. <i>M. sacchariflorus</i>															
2. <i>M. sacchariflorus</i>	0.149														
3. <i>M. sacchariflorus</i>	0.195	0.184													
4. <i>M. sacchariflorus</i>	0.136	0.180	0.205												
5. <i>M. sinensis</i>	0.318	0.368	0.333	0.356											
6. <i>M. sinensis</i>	0.300	0.253	0.310	0.333	0.322										
7. <i>M. 'Goliath'</i>	0.293	0.322	0.287	0.310	0.345	0.276									
8. <i>M. 'Silverfeather'</i>	0.361	0.391	0.402	0.425	0.391	0.368	0.299								
9. <i>M. 'Silberspine'</i>	0.257	0.391	0.379	0.402	0.322	0.276	0.276	0.368							
10. <i>M. 'Grossefontane'</i>	0.389	0.345	0.379	0.379	0.322	0.299	0.322	0.414	0.230						
11. <i>M. 'Gracillimus'</i>	0.458	0.414	0.402	0.425	0.299	0.414	0.368	0.483	0.414	0.253					
12. <i>M. x giganteus</i>	0.246	0.230	0.218	0.149	0.322	0.391	0.345	0.391	0.414	0.391	0.414				
13. <i>M. 'Rotsilber'</i>	0.309	0.322	0.379	0.287	0.345	0.345	0.276	0.437	0.276	0.276	0.322	0.368			
14. <i>M. 'Posidon'</i>	0.295	0.345	0.356	0.379	0.276	0.299	0.138	0.368	0.276	0.299	0.299	0.414	0.230		
15. <i>M. 'Roland'</i>	0.392	0.333	0.322	0.368	0.301	0.287	0.159	0.287	0.287	0.287	0.287	0.333	0.333	0.287	
16. <i>M. 'Kleinefontane'</i>	0.300	0.287	0.299	0.299	0.333	0.310	0.264	0.379	0.310	0.287	0.333	0.264	0.264	0.241	0.230

4.3.1.e *Miscanthus* sections examined by PCO with primer combination *EcoRIACC/MseICAT*

When the sections of *Miscanthus* are plotted against axis one and two of the PCO (Fig 4.6), all sections are well defined with the exception of section *Triarrhena*, where one individual overlaps the range of section *Miscanthus*. Section *Miscanthus* has a wider spread of variation than any of the other section.

4.3.2 AFLP band analysis of *Miscanthus* species UPGMA.

4.3.2a primer combination *EcoRIACC/MseICAT*

The phenogram constructed on the basis of shared fragments using the primer combination *EcoRIACC/MseICAT* divided the species into a number of groups, two of which have high bootstrap support: a *M. sacchariflorus* group and a *M. floridulus* group (Fig. 4.7). The remaining species were not clearly resolvable, although branch lengths suggested that *M. nepalensis*, *M. oligostachyus*, *M. transmorrisonensis* and *M. nudipes* were the most genetically distinct of these species. It must be noted, however that the DNA from the *M. nudipes* individual was extracted from herbarium material (courtesy of Dr. T.R. Hodgkinson), and although the AFLP profile for this individual was clear and strong, it was the only individual in the analysis represented by herbarium extracted DNA. The bootstrap value for the main *M. sacchariflorus* group was 83%. This group was subdivided into two separate groups for which the bootstrap support remained high. It is interesting to note that the *M. sacchariflorus* individual which appeared closer to *M. floridulus* by axis one of the PCO does not appear to be distinct from the other *M. sacchariflorus* individuals by UPGMA. The *M. floridulus* group, supported by 84% was composed of two separate sub-divisions, one of these contained a *M. floridulus* individual from the island of Huahine in the south Pacific, while the other contained a second individual from Huahine and one from the island of Mangerava also in the southern Pacific ocean.

4.3.2b primer combination *EcoRIACA/MseICAC*

Analysis of the same group of species with the primer combination *EcoRIACA/MseICAC* (Fig.4.8) showed a similar result to that obtained with the primer combination *EcoRIACC/MseICAT*. *Miscanthus sacchariflorus* formed a cluster with a bootstrap support of 75%. The *M. floridulus* cluster had support of 98%, however in this analysis the two *M. floridulus* individuals from the island of Huahine were grouped together with support of 83%. *Miscanthus nepalensis* and *M. nudipes* were grouped together in this analysis with support of 57%, unfortunately the DNA from *M. oligostachyus* failed AFLP analysis by this primer combination and so is not included in the result. The final group contained *M. sinensis* and all other individuals. There was no further support for this division and little difference in branch length between the representatives, indicating little genetic distance between these species.

Fig. 4.6 Ordination of *Miscanthus* sections by PCO of AFLP data with primer combination (a) *Eco* RIACC/*Mse* ICAT and (b) *Eco* RIACA/*Mse* ICAC. Axis one & two shown.

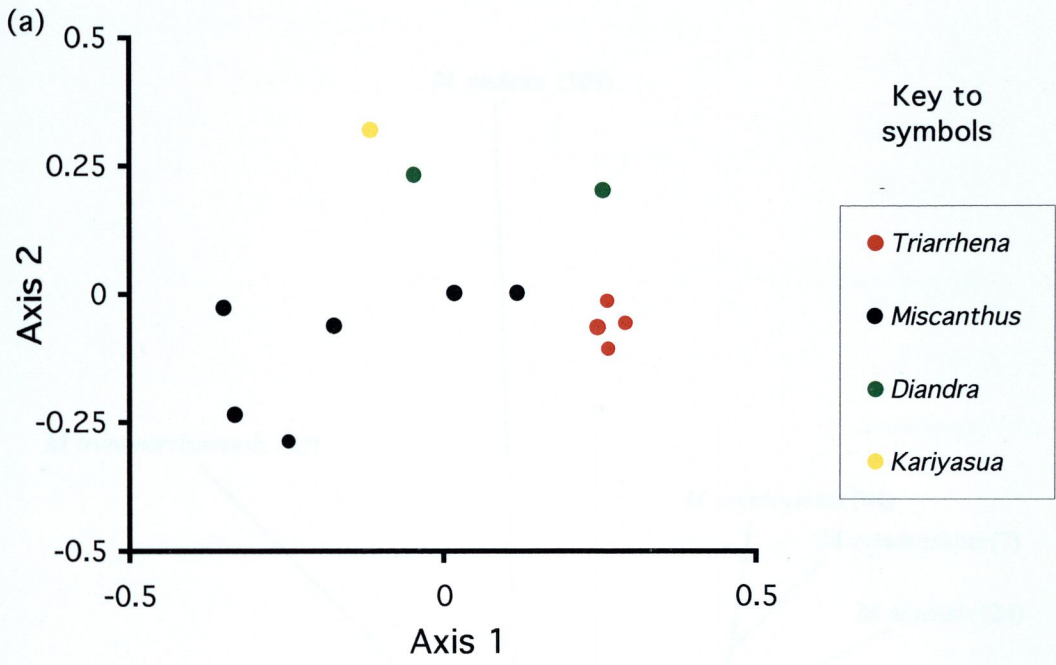


Fig. 4.7. AFLP band matrix analysis of *Miscanthus* species by UPGMA with primer combination *EcoRI*ACC/*MseI*CAT. Numbers beside branches show percentage bootstrap value. Numbers after species name indicate DNA bank sample number.

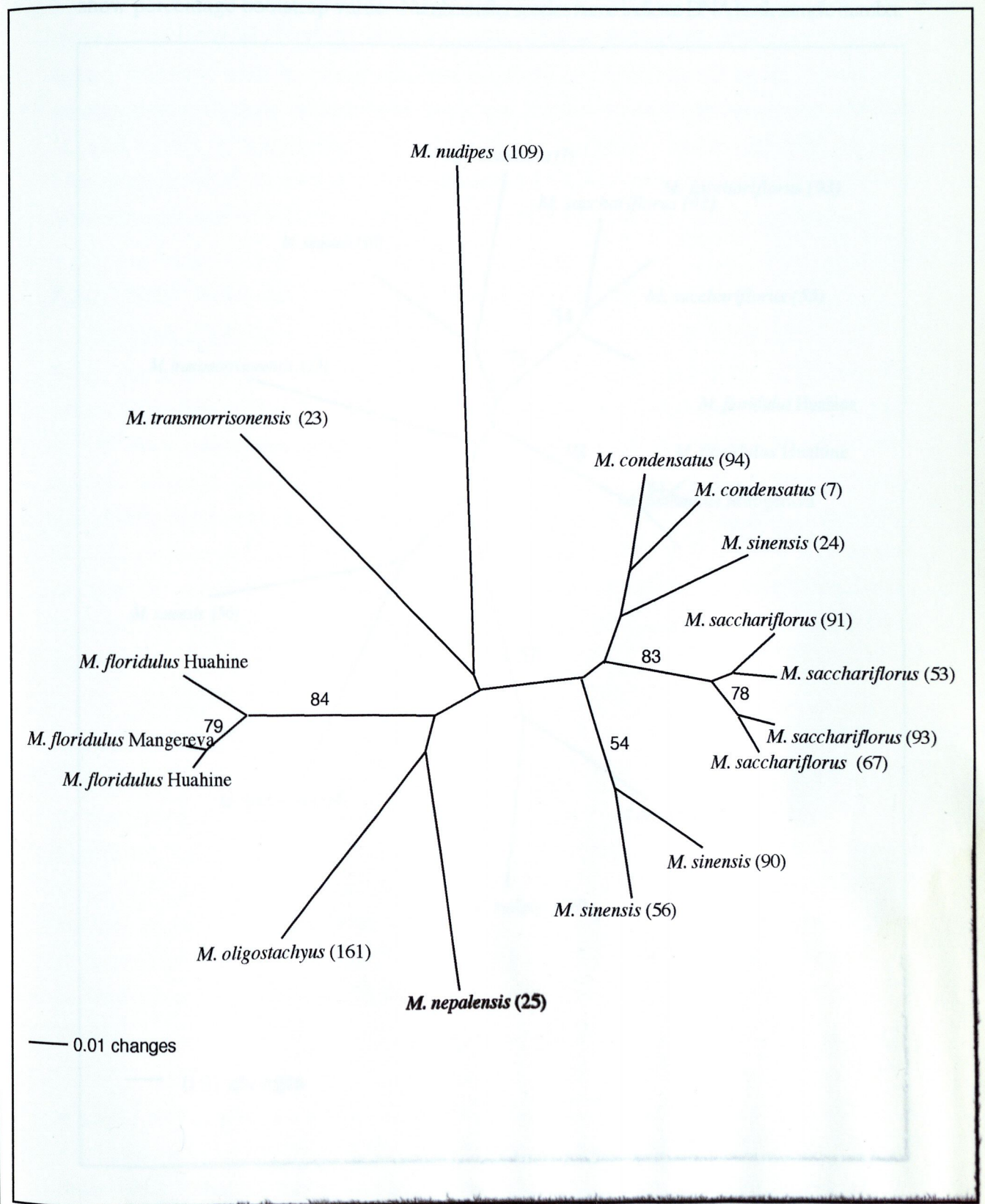
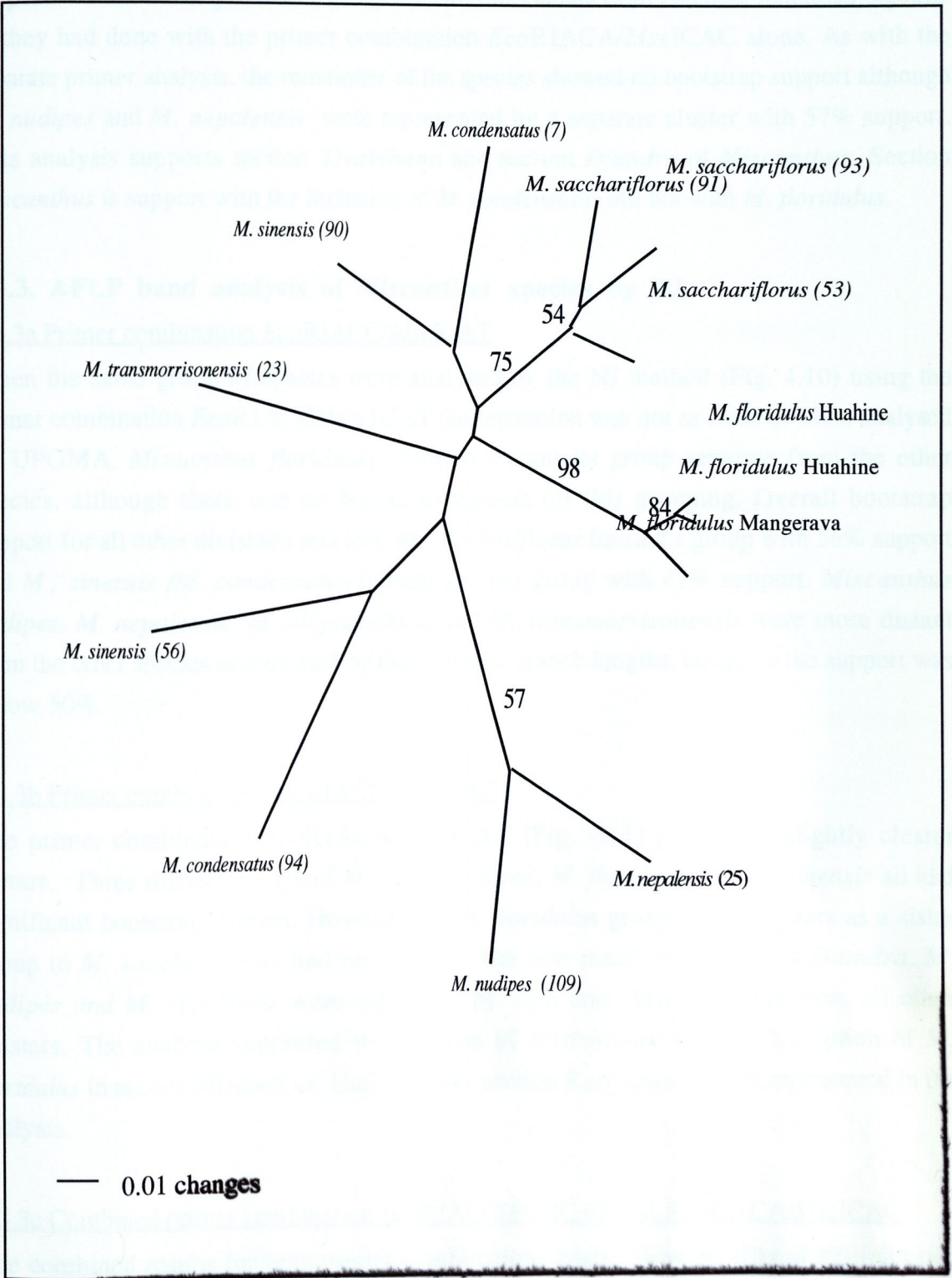


Fig. 4.8 AFLP band matrix analysis of *Miscanthus* species by UPGMA using primer combination *EcoRI*ACA/*MseI*CAC and *EcoRI*ACC/*MseI*CAT. Numbers beside branches show percentage bootstrap value. Numbers after species name indicate DNA bank sample number.



4.3.2c Combined primer combinations *Eco*RIACA/*Mse*ICAC and *Eco*RIACC/*Mse*ICAT

When the two primer combinations were analysed together (Fig.4.9) there was little difference in the overall result. *Miscanthus sacchariflorus* remained as a distinct group with bootstrap support of 75%. *Miscanthus floridulus* also remained as a distinct group with 98% support. Within the *M. floridulus* group the two individuals from Huahine remained together as they had done with the primer combination *Eco*RIACA/*Mse*ICAC alone. As with the separate primer analysis, the remainder of the species showed no bootstrap support although *M. nudipes* and *M. nepalensis* were represented by a separate cluster with 57% support. This analysis supports section *Triarrhena* and section *Diandra* of *Miscanthus*. Section *Miscanthus* is support with the inclusion of *M. condensatus* but not with *M. floridulus*.

4.3.3. AFLP band analysis of *Miscanthus* species by NJ

4.3.3a Primer combination *Eco*RIACC/*Mse*ICAT

When the same group of species were analysed by the NJ method (Fig. 4.10) using the primer combination *Eco*RIACC/*Mse*ICAT the separation was not as clear as when analysed by UPGMA. *Miscanthus floridulus* formed a compact group separate from the other species, although there was no bootstrap support for this grouping. Overall bootstrap support for all other divisions was low. *M. sacchariflorus* formed a group with 56% support and *M. sinensis* /*M. condensatus* formed another group with 61% support. *Miscanthus nudipes*, *M. nepalensis*, *M. oligostachyus* and *M. transmorrisonensis* were more distant from the other species as indicated by their relative branch lengths, but again the support was below 50%.

4.3.3b Primer combination *Eco*RIACA/*Mse*ICAC

The primer combination *Eco*RIACA/*Mse*ICAC (Fig. 4.11) provided a slightly clearer picture. Three distinct groups of *M. sacchariflorus*, *M. floridulus* and *M. sinensis* all had significant bootstrap support. However the *M. floridulus* group which appears as a sister group to *M. sacchariflorus* had no support. The two members of section *Diandra*, *M. nudipes* and *M. nepalensis* were supported by 72% and clearly distinct from all other clusters. The analysis supported the sections of *Miscanthus* with the exception of *M. floridulus* in section *Miscanthus*. Unfortunately section *Kariyasua* was not represented in the analysis.

4.3.3c Combined primer combination *Eco*RIACC/*Mse*ICAT and *Eco*RIACA/*Mse*ICAC

The combined results for both primer combinations analysed by NJ (Fig. 4.12) indicated three main divisions all with high bootstrap support. One group composed of *M. sacchariflorus* had 93% support, the group containing *M. nepalensis* and *M. nudipes*, 77% and the group containing *M. condensatus*, 62%. This analyses shows a *M. condensatus* group

Fig. 4.9 AFLP band matrix analysis of *Miscanthus* species by UPGMA with primer combination *EcoRI*ACA/*MseI*CAC. Numbers beside branches show bootstrap value. Numbers after species name indicate DNA bank sample number.

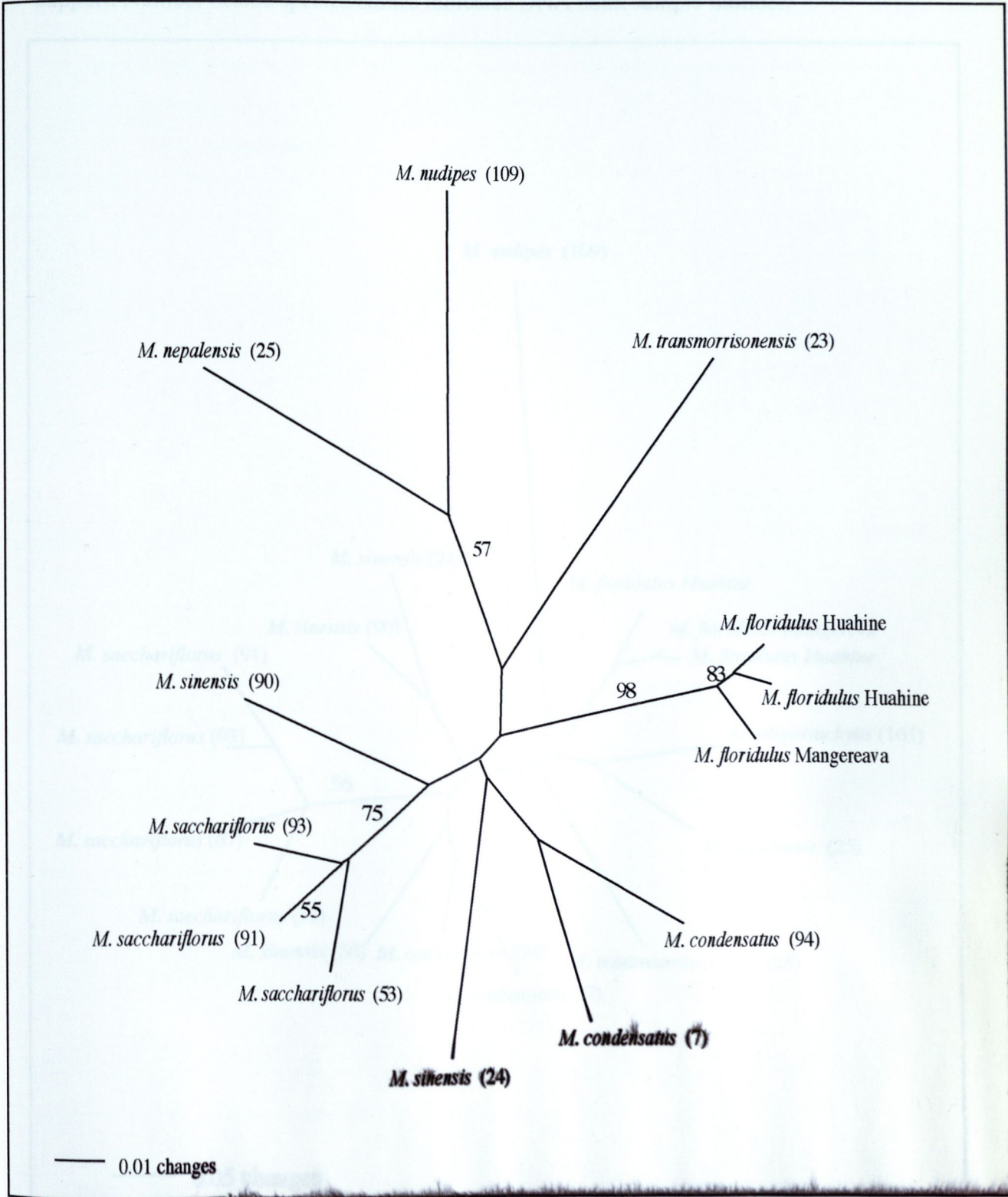


Fig. 4.10 AFLP band matrix analysis of *Miscanthus* species by Neighbor Joining with primer combination *EcoRI*ACC/*MseI*CAT. Numbers beside branches show percentage bootstrap support. Number beside species names indicates DNA bank sample number.

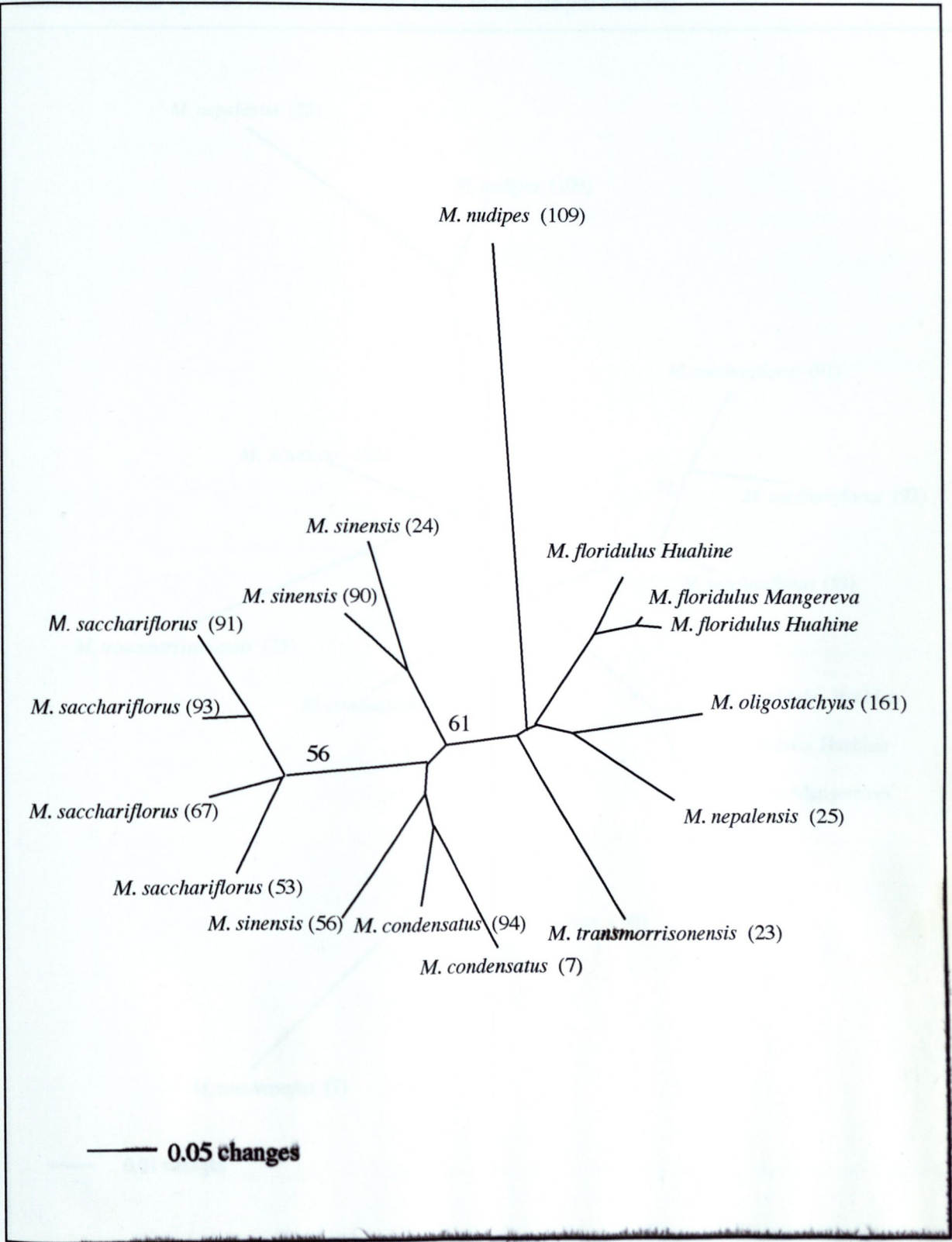


Fig. 4.11 AFLP band matrix analysis of *Miscanthus* species by Neighbor Joining with primer combination *EcoRI*ACA/*MseI*CAC. Numbers beside branches show percentage bootstrap value. Numbers beside species names indicates DNA bank sample number.

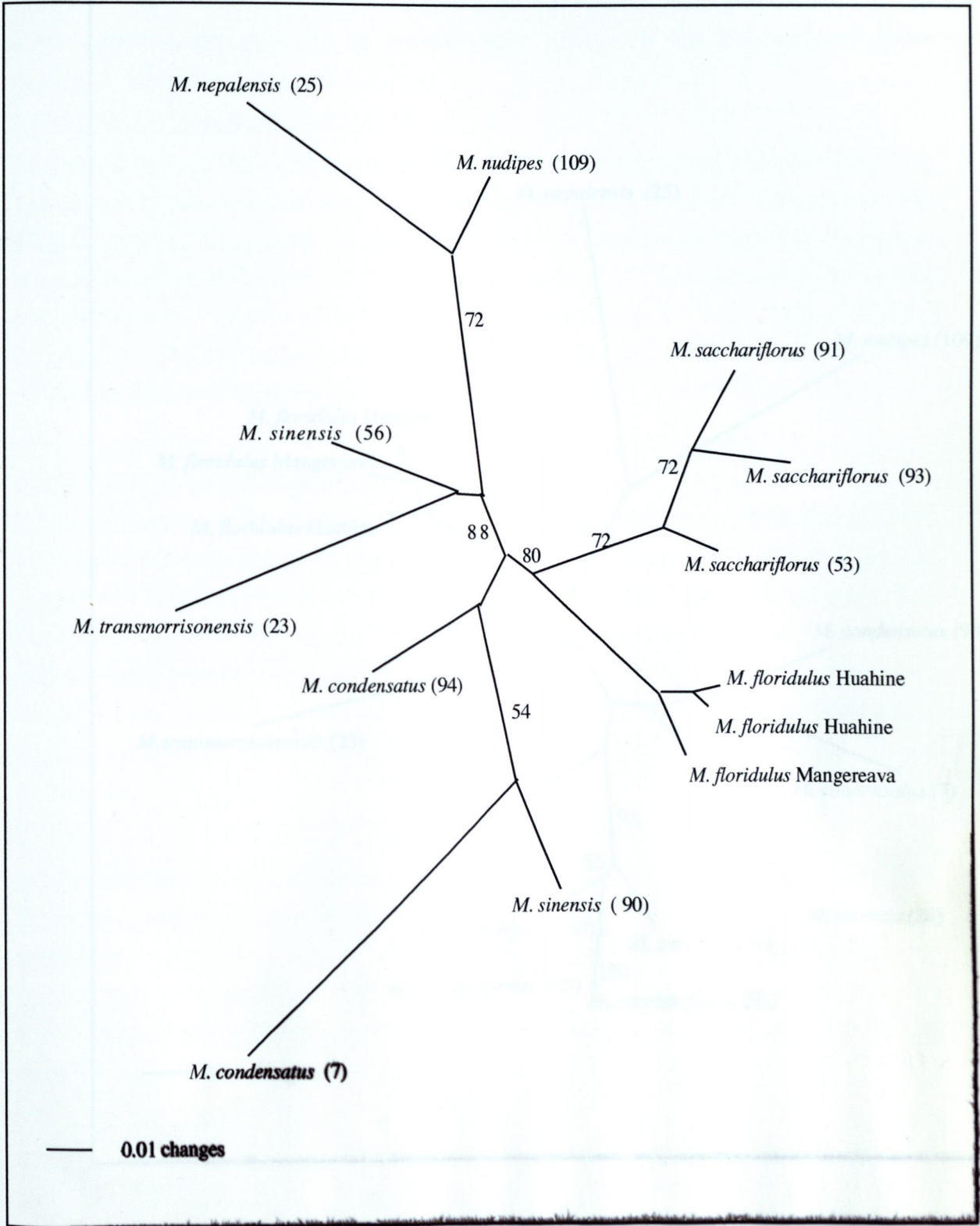
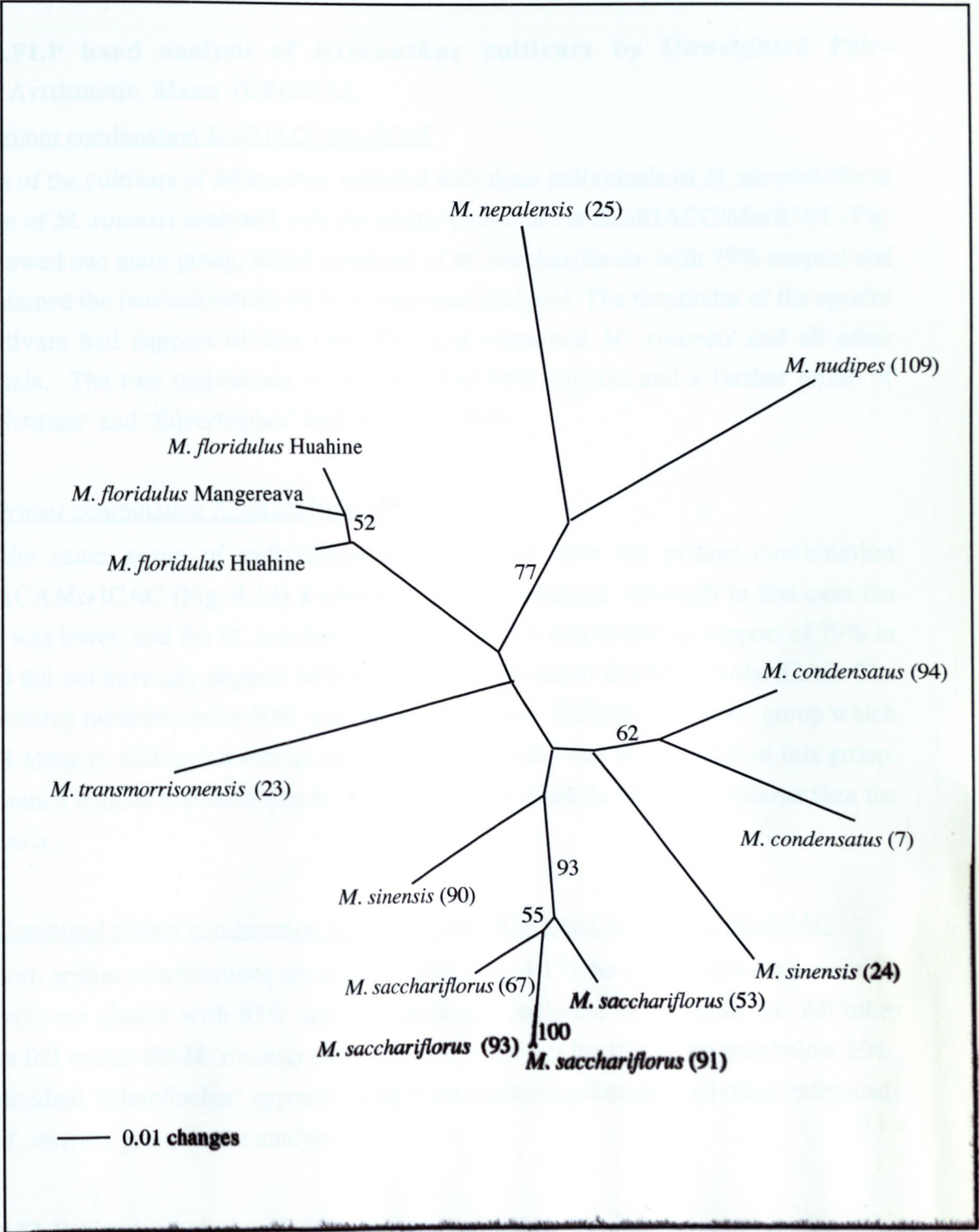


Fig. 4.12 AFLP band matrix analysis of *Miscanthus* species by Neighbor Joining with primer combination *EcoRI*ACC/*MseI*CAT and *EcoRI*ACA/*MseI*CAC. Numbers beside branches show percentage bootstrap value. Numbers beside species names indicates DNA bank sample number.



with 62% support, closely related to but distinct from *M. sinensis*. *Miscanthus transmorrissonensis* appears more distant to all of the other species. The support for all other species was 98% (not shown on graph, for practical reasons). This analyses supports the sections *Triarrhena*, *Diandra* and *Miscanthus*, with the exception of *M. floridulus*, of *Miscanthus*. Unfortunately section *Kariyasua* was not represented in the analysis

4.3.4 AFLP band analysis of *Miscanthus* cultivars by Unweighted Pair-Group Arithmetic Mean (UPGMA).

4.3.4a Primer combination *EcoRIACC/MseICAT*

Analysis of the cultivars of *Miscanthus* included with three individuals of *M. sacchariflorus* and three of *M. sinensis* analysed with the primer combination *EcoRIACC/MseICAT* (Fig. 4.13) showed one main group, which consisted of *M. sacchariflorus* with 79% support and also contained the two individuals of *M. x giganteus* analysed. The remainder of the species and cultivars had support of less than 50% and contained *M. sinensis* and all other individuals. The two individuals of 'Goliath' had 66% support and a further group of 'Grossefontane' and 'Silverfeather' had support of 59%.

4.3.4b Primer combination *EcoRIACAMseICAC*

When the same group of individuals were analysed with the primer combination *EcoRIACAMseICAC* (Fig. 4.14) a similar result was obtained, although in this case the support was lower, and the *M. sacchariflorus* group which had bootstrap support of 79% in Fig 4.13 did not have any support with the primer combination *EcoRIACAMseICAC*. The only bootstrap measure above 50% was that shown for the 'Goliath'/'Posidon' group which had 61% support, although a second individual of 'Goliath' was well outside of this group. Again branch lengths indicated that *M. x giganteus* was closer to *M. sacchariflorus* than the *M. sinensis*.

4.3.4c Combined primer combination *EcoRIACC/MseICAT* and *EcoRIACAMseICAC*

When both primer combinations are used together (Fig. 4.15) the results indicate a main *M. sacchariflorus* cluster with 85% support which also included *M. x giganteus*. All other cultivars fell within the *M. sinensis* cluster although support for this group was below 50%. One individual 'Silverfeather' appeared to be more genetically distant to all other individuals in the *M. sinensis* group in the analysis.

4.3.5 AFLP band analysis of *Miscanthus* cultivars by NJ

4.3.5a Primer combination *EcoRIACC/MseICAT*

NJ analysis of the cultivars analysed with the primer combination *EcoRIACC/MseICAT* (Fig. 4.16) showed a similar result to that obtained by UPGMA analysis. Again *M.*

Fig. 4. 13 AFLP band matrix analysis of *Miscanthus* cultivars by UPGMA with primer combination *EcoRI*ACC/*MseI*CAT. Numbers beside branches show bootstrap values. Numbers beside species names indicates DNA bank sample numbers.

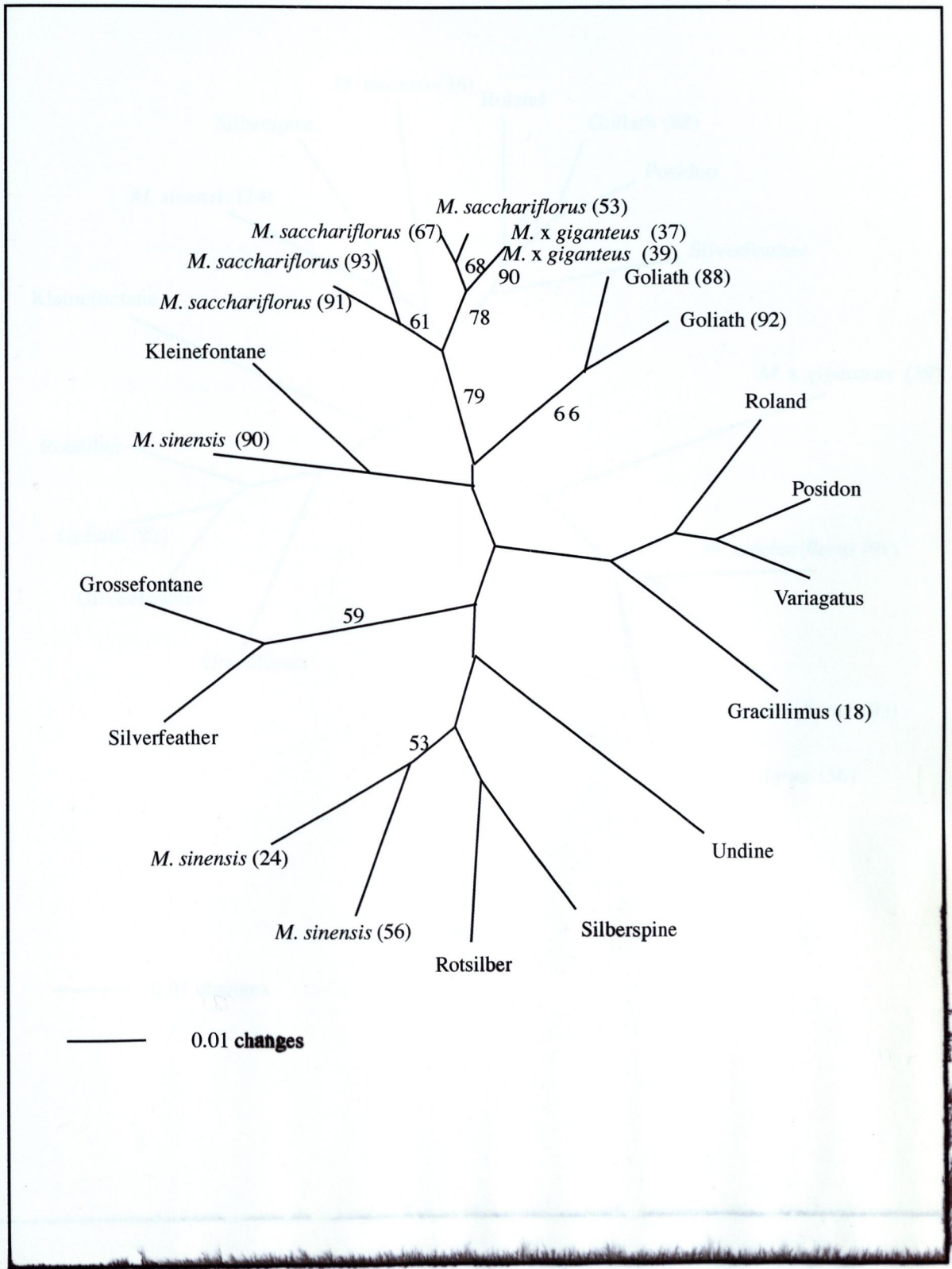


Fig. 4.14 AFLP band matrix analysis of *Miscanthus* cultivars by UPGMA with primer combination *Eco*RIACA/*Mse*IACAC. Numbers beside branches show percentage bootstrap value. Numbers beside species names indicates DNA bank sample number.

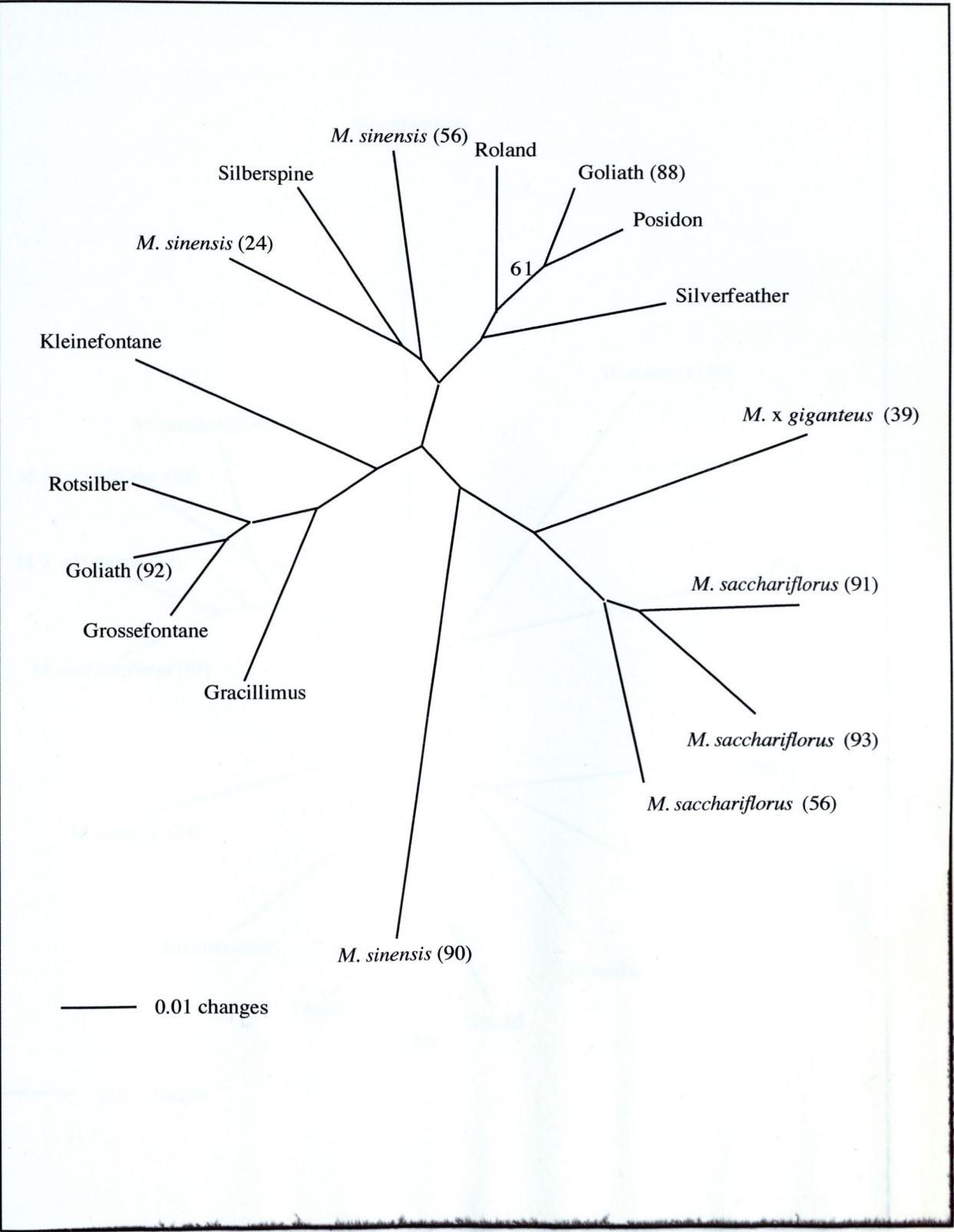


Fig. 4.15. AFLP band matrix analysis of *Miscanthus* cultivars by UPGMA with primer combination *Eco*RIACC/*Mse*ICAT and *Eco*RIACA/*Mse*ICAC. Numbers beside branches show percentage bootstrap value. Numbers beside species names indicates DNA bank sample number.

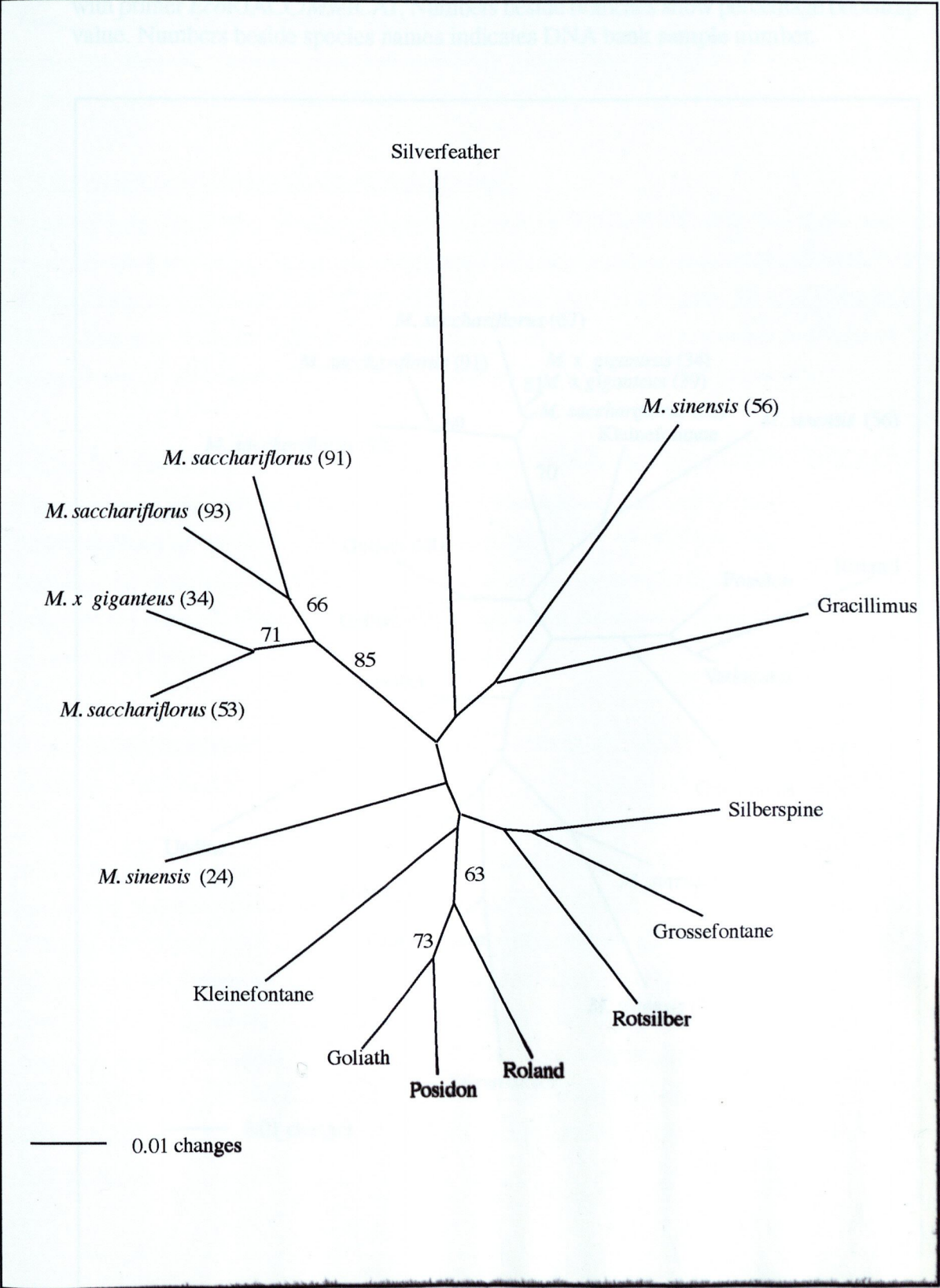
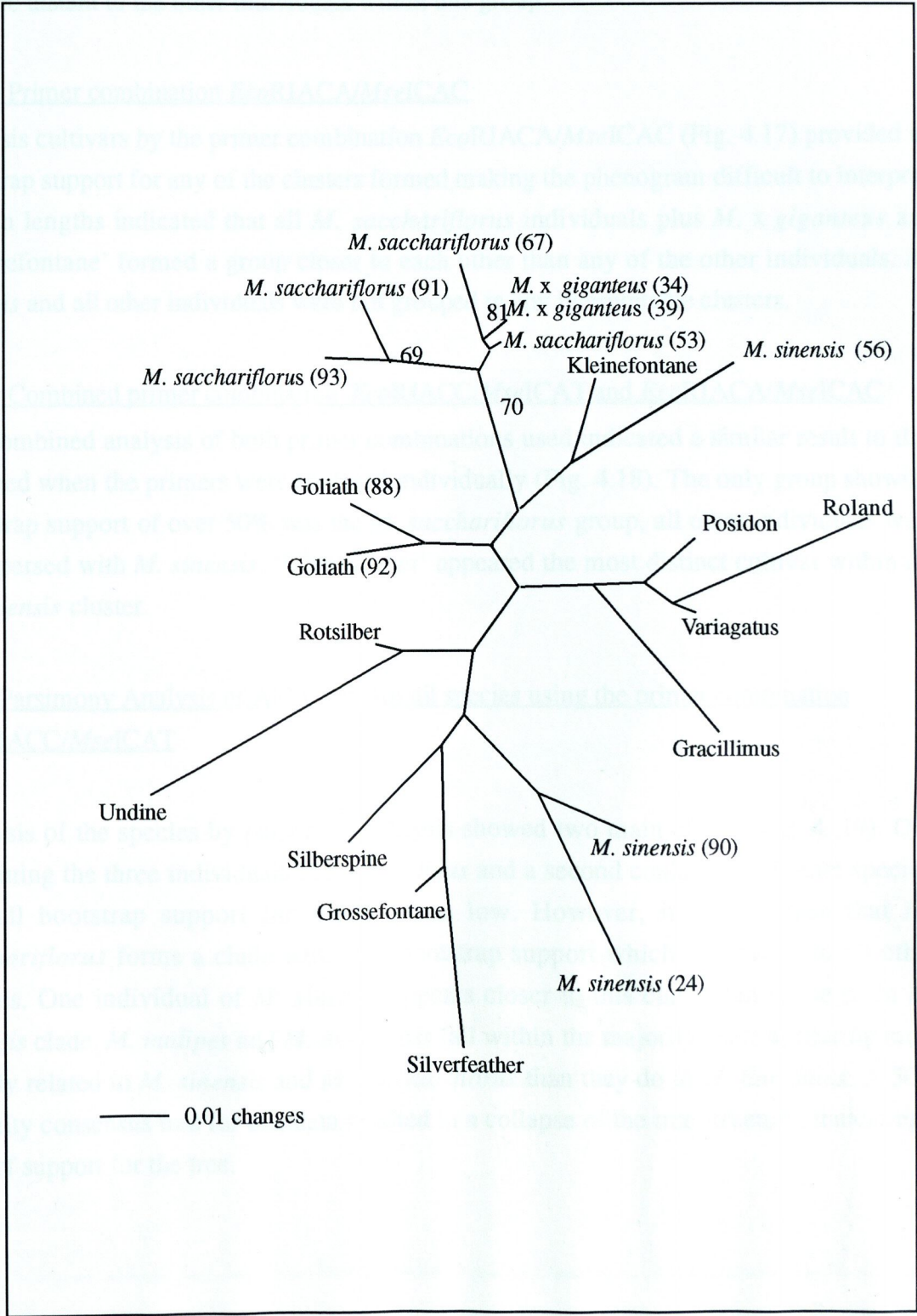


Fig. 4.16. AFLP band matrix analysis of *Miscanthus* cultivars by Neighbor Joining with primer *EcoRI*ACC/*MseI*CAT. Numbers beside branches show percentage bootstrap value. Numbers beside species names indicates DNA bank sample number.



sacchariflorus formed a discreet cluster with 70% bootstrap support and *M. x giganteus* was grouped within this cluster. All of the cultivars were outside the *M. sacchariflorus* group. Bootstrap support for this grouping was however less than 50% in most cases. 'Silverfeather' formed a separate cluster with 70% support and the two individuals of 'Goliath' were grouped together with 50% support. 'Undine' and 'Silverfeather' while within the main *M. sinensis* cluster had relatively longer branch lengths indicating they may be more distant to the other individuals within this group.

4.3.5b Primer combination *EcoRIACA/MseICAC*

Analysis cultivars by the primer combination *EcoRIACA/MseICAC* (Fig. 4.17) provided no bootstrap support for any of the clusters formed making the phenogram difficult to interpret. Branch lengths indicated that all *M. sacchariflorus* individuals plus *M. x giganteus* and 'Kleinefontane' formed a group closer to each other than any of the other individuals. *M. sinensis* and all other individuals were not grouped in any interpretable clusters.

4.3.5c Combined primer combination *EcoRIACC/MseICAT* and *EcoRIACA/MseICAC*

The combined analysis of both primer combinations used indicated a similar result to that obtained when the primers were analysed individually (Fig. 4.18). The only group showing bootstrap support of over 50% was the *M. sacchariflorus* group, all other individuals were interspersed with *M. sinensis*. 'Silverfeather' appeared the most distinct cultivar within the *M. sinensis* cluster.

4.3.6 Parsimony Analysis of AFLP data for all species using the primer combination *EcoRIACC/MseICAT*

Analysis of the species by parsimony analysis showed two main clades (Fig. 4. 19). One containing the three individuals of *M. floridulus* and a second containing all other species. Overall bootstrap support for the tree was low. However, it can be seen that *M. sacchariflorus* forms a clade with 64% bootstrap support which is separate to all other species. One individual of *M. sinensis* appears closer to this clade than to the main *M. sinensis* clade. *M. nudipes* and *M. nepalensis* fall within the majority clade appearing more closely related to *M. sinensis* and *M. sacchariflorus* than they do to *M. floridulus*. A 50% majority consensus tree for this data resulted in a collapse of the tree structure, indicating a lack of support for the tree.

Fig. 4.17. AFLP band matrix analysis of *Miscanthus* cultivars by Neighbor Joining with primer combination *EcoRI*ACA/*MseI*CAC. Numbers beside branches show percentage bootstrap value. Numbers beside species names indicates DNA bank sample number.

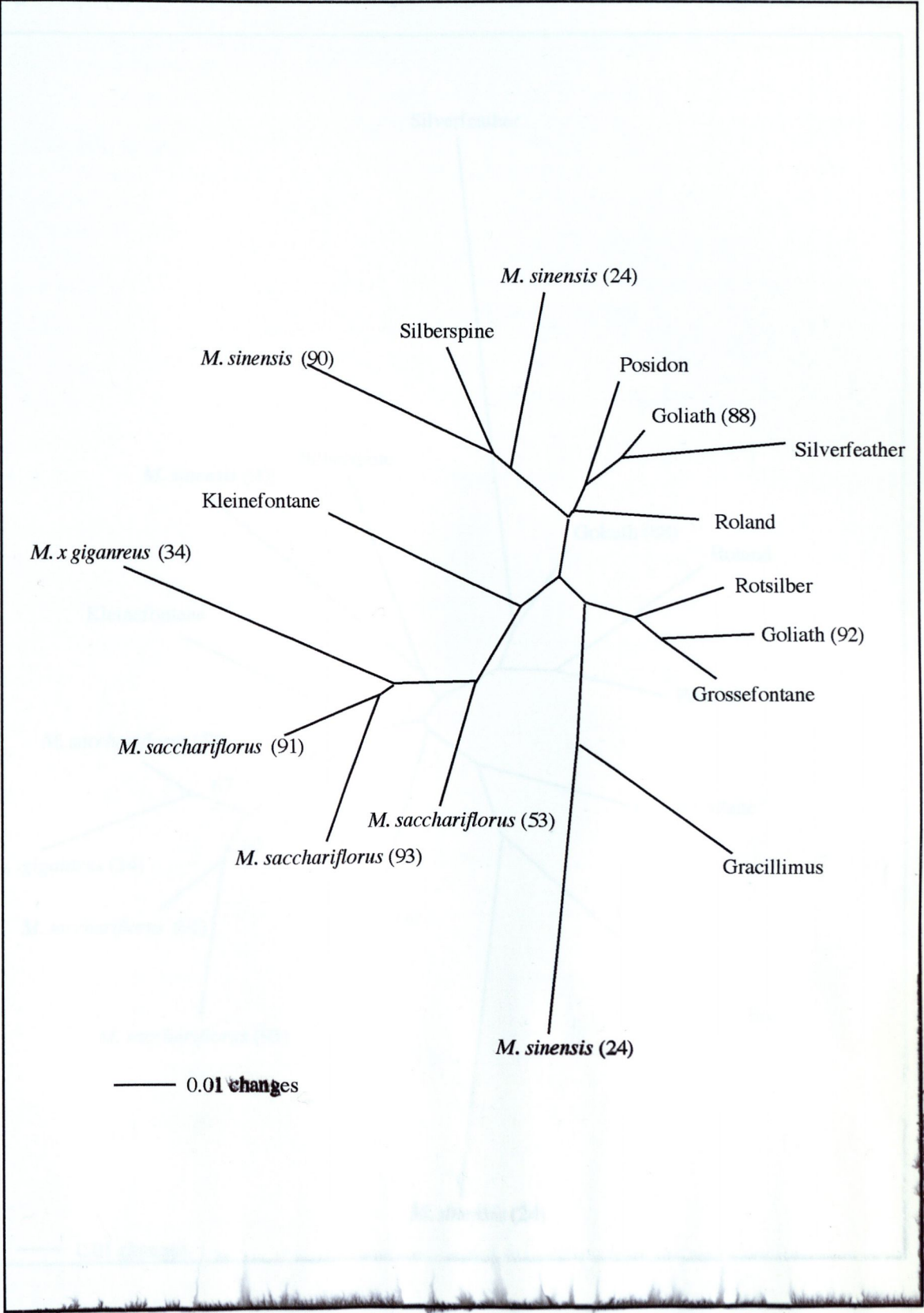


FIG. 4.18 AFLP band matrix analysis of *Miscanthus* cultivars by Neighbor Joining with primer combination *EcoRIACC/MseICAT* and *EcoRIACA/MseICAC*. Numbers beside branches show percentage bootstrap value. Numbers beside species names indicates DNA bank sample number.

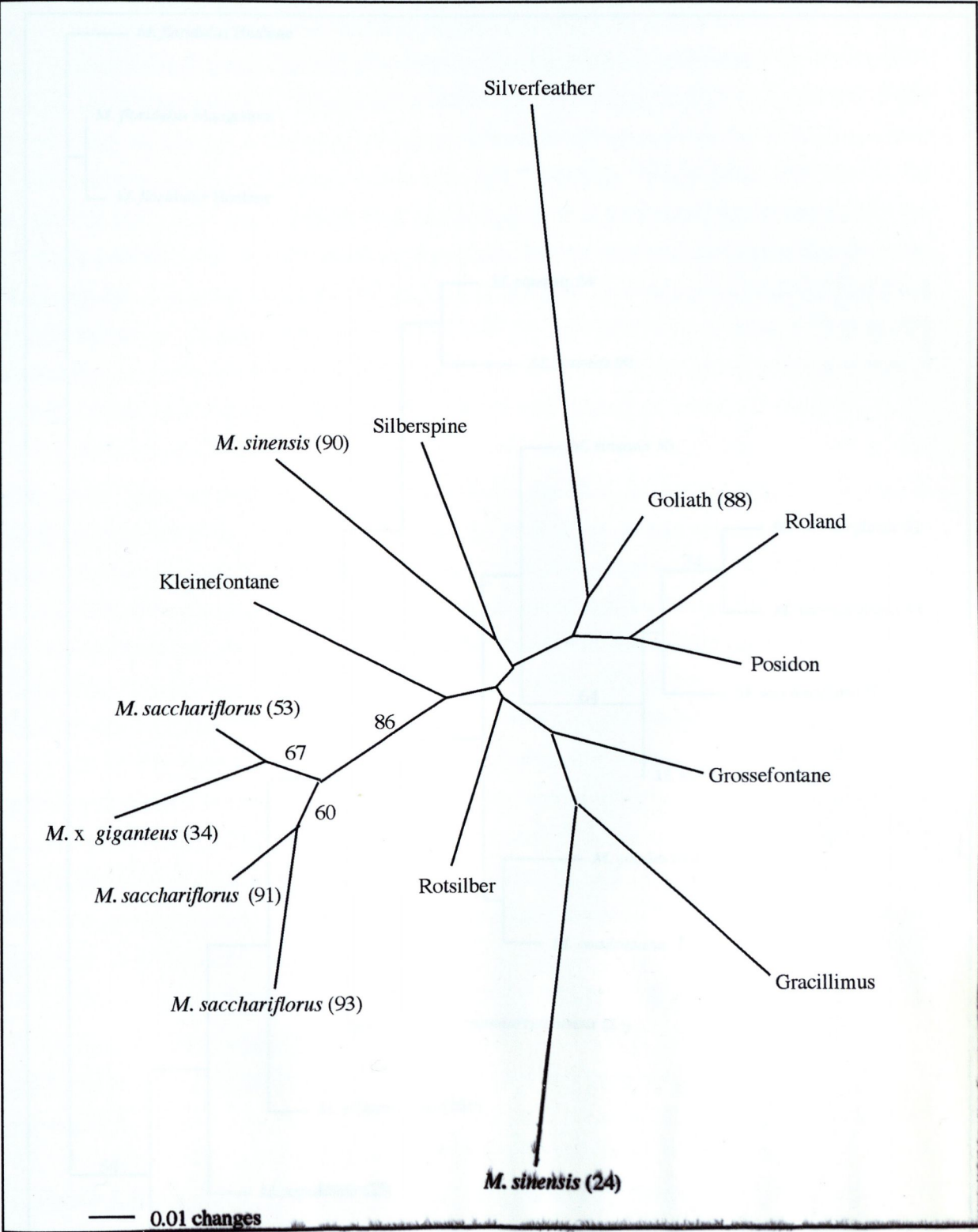
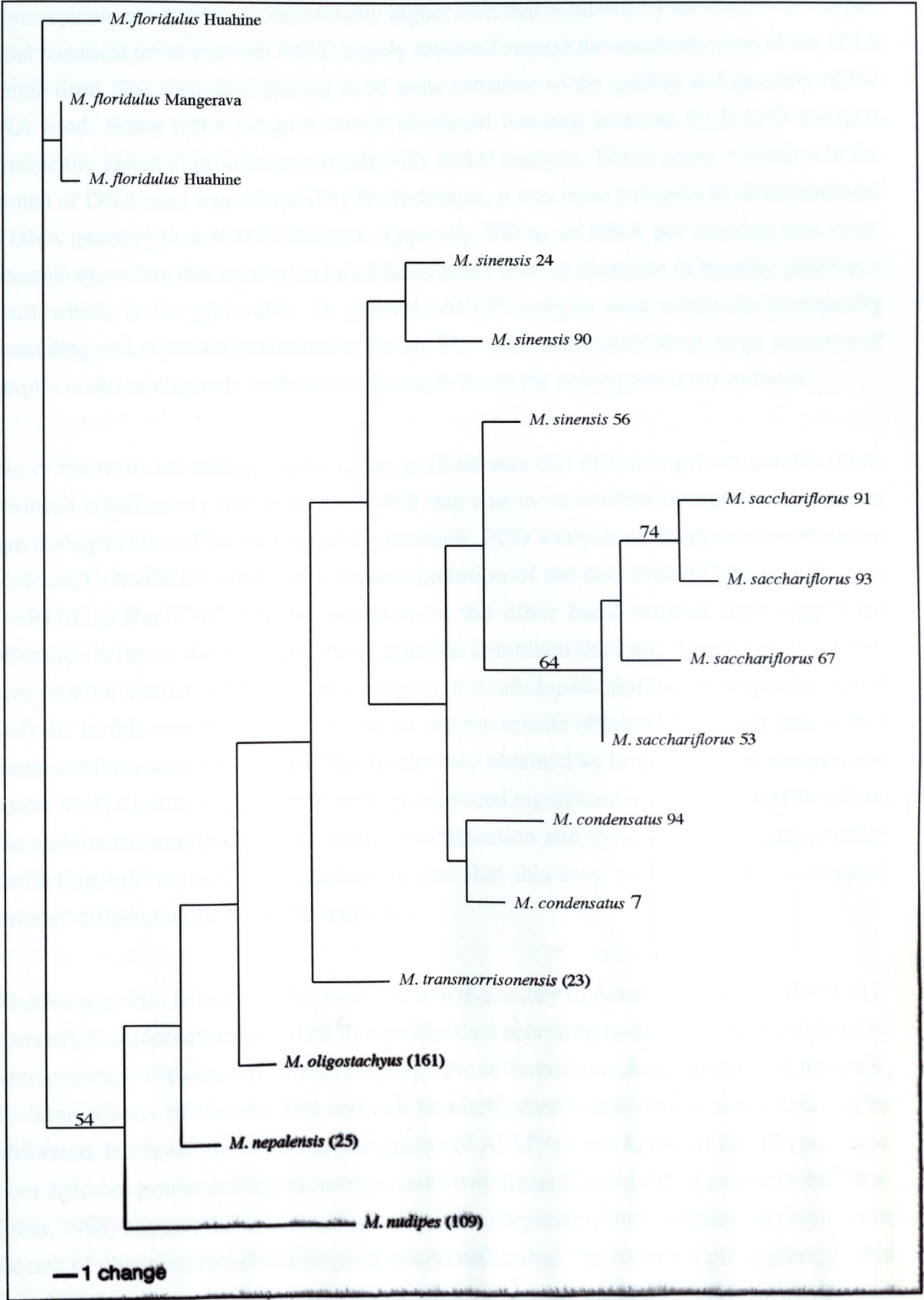


Fig. 4.19. One of 89 maximally parsimonious trees, using tree bisection and reconnection of AFLP data for the primer combination *EcoRI*ACC/*MseI*CAT. Figures above branches indicate percentage bootstrap support. Numbers beside species names indicates DNA bank sample number.



4.4 Discussion

The AFLP technique was highly successful in detecting genetic differences between species and between cultivars and varieties in all of the samples analysed. The level of polymorphism achieved was considerably higher than that achieved by the RAPD technique. Initial technical problems with AFLP largely revolved around the standardisation of the DNA sample used. The technique proved to be quite sensitive to the quality and quantity of the DNA used. Some DNA samples which produced banding patterns by RAPD analysis consistently failed to produce any result with AFLP analysis. While some variation in the amount of DNA used was tolerated by the technique, it was more stringent in its requirement of DNA quantity than RAPD analysis. Typically 250 ng of DNA per reaction was used. Amounts exceeding this resulted in failed lanes rather than an alteration in banding patterns, a result which is far preferable. In general, AFLP analysis was relatively technically demanding and expensive technique to set up. However, once established, large numbers of samples could be routinely analysed by the method and the subsequent costs reduced.

One of the most interesting results of the analysis was that different primer combinations produced significantly different results and this was more evident through PCO analysis than through either of the two clustering methods. PCO analysis of the primer combination *EcoRIACC/MseICAT* produced a clearer resolution of the data than did the combination *EcoRIACA/MseICAC*. Cluster analysis, on the other hand showed little significant difference between the two data sets or with the combined data set. Breyne *et al.*, (1999) have found a similar situation in the analysis of *Arabidopsis thaliana* ecotypes by AFLP analysis. In this case the authors choose to use the results obtained from only one of two primer combinations investigated. The results they obtained by both ordination analysis and cluster analysis with two different primers produced significantly different classifications. The authors showed that both the enzyme combination and the primers used can generate conflicting information. They further suggest that this may be a result of non-random genomic distribution of the AFLP fragments.

Whether it is wise to combine the two data sets is a matter of debate (Bull *et al.*, 1993). It is generally considered inappropriate to combine data sets from two distinct genes within the same genotype because different genes may have different branching histories. If however, the homogeneity of the two data sets can be established, combination of the data can be performed. However, due to the random nature of AFLP it is not known if the data produced from different primer combinations represents two distinct parts of the genome. (Johnson & Soltis, 1998) suggest that it is better to analyse data separately and compare the results with the risk of obtaining two different phenograms rather than one incorrect phenogram. In this way differing phenograms can be compared to other sources of data on the same taxa to

assess congruence. For this reason the data produced from AFLP analysis in this chapter was analysed both separately and in combination. In this way it could be examined whether the increased number of data points in the combined data set balanced out any ambiguities between the two separate data sets. The combined (larger) data set should also lead to a less biased representation of genetic variation. AFLP markers are dominant *i.e* if one of the “alleles” at an AFLP site is unamplifiable, then homozygotes cannot be distinguished from heterozygotes. Lynch & Milligan (1994) have calculated that when analysing dominant markers (as compared with co-dominant RFLP or isozyme markers) an order of 2 to 10 times more individuals should be sampled per locus.

When compared to the results of the RAPD analysis (chapter 3), AFLP produced a much clearer picture of the relationships between species and between cultivars and species. Firstly the level of polymorphism achieved with AFLP was far greater than RAPD. Although this may be as a result of the larger number of bands produced with AFLP (eighty seven bands were scored for AFLP analysis with two different primer combinations, of these 63 were polymorphic between species and cultivars, while forty four were scored for RAPD with three different primers, thirty nine of these were polymorphic between species and cultivars). RAPD analysis did not provide any clear resolution of any of the species and was consequently incapable of resolving the sections. AFLP analysis provided a much clearer resolution of the species. Analysis of the cultivars in conjunction with the species by RAPD analysis by PCO provided slightly more information than achieved for the species, but cluster analysis of the same data was not capable of indicating any relatedness of the cultivars to *M. sinensis* or *M. sacchariflorus*. AFLP analysis of the same data provided clearer evidence of the position of the cultivars and varieties in relation to these two species.

While some success was achieved using silver staining the results were not as successful as those achieved using radioactively labelled probes. Firstly, the gel did not remain in position on the plate during the procedure. This made it impossible to carry out the technique as recommended. Although Bio-Rad claim the technique can be used on thin denaturing gels, this is obviously not the case where the gel size exceeds dimensions of approximately 10cm x 10cm. Larger gels simply fold over on themselves making staining and washing impossible. Success in ensuring the gel remained in position was however obtained after the application of a solution of Bind-Silane (Sigma) to the plates prior to pouring the gel. The second problem encountered was that of high background staining when using the Bio-Rad kit. To overcome this problem Bio-Rad recommend washing the plates in acid before use. Even after the plates had been acid soaked in 10% nitric acid overnight, the problem of high background still remained. Due to these problems it was decided to attempt a silver staining protocol which had been in use at the Genetics Department, Trinity College Dublin. This technique was much more successful, the level of background staining was much lower than

that achieved with the Bio-Rad kit and the bands obtained were much clearer and sharper. The level of sensitivity was still lower than that achieved using radioactivity and for this reason silver staining was not used to visualise the AFLP bands.

The use of Principal Co-ordinate Analysis and various methods of cluster analysis to establish genetic relatedness with AFLP has been well established (Travis *et al.*, 1996; Muluvi *et al.*, 1999; O'Hanlon *et al.* 1999). However, the work of Sharma *et al.* (1996) appeared to confuse the techniques of Principal Co-ordinate analysis (PCO) and Principal Component Analysis (PCA) and it is not clear from their work which technique they actually used. PCA is not suitable for use with bivariate data such as that obtained from AFLP analysis. PCO can be applied to bivariate data, providing a suitable distance or similarity matrix is used. The AFLP data discussed in this chapter were analysed by cluster analysis and PCO in order to assess the genetic relatedness of species and varieties. Although phylogeny analysis is not generally considered appropriate for AFLP data. Sharma *et al.*, 1996 and Hodkinson *et al.*, (2000) have used parsimony techniques to infer phylogenies. It is generally considered inappropriate to analyse AFLP data by parsimony analysis, due to the possibility of dominance in co-migrating bands. However Hodkinson *et al.*, (2000), used parsimony analysis in a phylogenetic study of *Phyllostachys* (Bambusoideae, Poaceae) and found the results of this analysis to be largely congruent with those obtained from ITS nuclear rDNA sequence data, while also providing clearer resolution and better bootstrap support for the major groupings. The parsimony analysis of the AFLP data generated in this study did not show a particularly clear resolution of the data and bootstrap support was quite low. However, the analysis did compliment the distance analysis of the same data set showing *M. floridulus* to be separate from *M. sinensis* and most all other species analysed. This aspect is further discussed in relation to the sequence data presented in chapter five.

Cluster analysis using UPGMA is commonly used for the analysis of molecular bivariate data such as AFLP (Breyne *et al.*, 1999; Lerceteau & Szmidt, 1999). However, this technique relies on the data being ultrametric, *i.e.* that the pairwise distances fit a constant molecular clock and it cannot be ascertained that this was the case with the AFLP data. Therefore the less frequently used technique of NJ was also applied to the data. The results indicated little difference between the two techniques which gives a measure of the robustness of the data.

It was found that the combined primer combination provided a clearer resolution of the data than *EcoRIACA/MseICAC* or *EcoRIACC/MseICAT* and therefore the following consensus of the genus is based on the combined primer combination. The results of AFLP analysis of *Miscanthus* species indicated one distinct grouping which included *M. sacchariflorus* and *M. x giganteus*. The remainder of the species, including *M. sinensis* were loosely associated with each other but did not form a distinct grouping. The *M. sacchariflorus* group was

consistently separate from all other species within the genus. AFLP analysis would therefore support section *Triarrhena* as assigned by Lee (1964c). This supports the work of Greef *et al.* (1997), who obtained a similar result using AFLP analysis to examine genetic variation within and between different accessions of *M. sinensis*, *M. sacchariflorus* and *M. x giganteus*. Greef *et al.* (1997) also found a broader range of genetic variation within the accessions of *M. sinensis* than within *M. sacchariflorus*. This they attributed to the fact that many more clones of *M. sinensis* have been spread across Europe by horticultural breeders than have *M. sacchariflorus* clones, thereby providing the potential for greater genetic variation within *M. sinensis* European clones. The analysis carried out in this chapter also showed a very narrow range of variation within the five accessions of *M. sacchariflorus* examined and a wider range within *M. sinensis*. It must be noted, however, that only three individuals of *M. sinensis* were available for AFLP analysis while Greef *et al.* (1997) analysed eleven *M. sinensis* clones. Conversely five individuals of *M. sacchariflorus* were analysed compared with two by Greef *et al.* (1997). In addition the accessions of *M. sinensis* and *M. sacchariflorus* examined here were wild collected in China and Japan and only one *M. sacchariflorus* accession was of European cultivated origin. This would indicate that the variation within *M. sinensis* may not simply be a result of horticultural breeding but the fact that *M. sinensis* has a broader genetic base originating from its wide geographical spread across Asia and Polynesia.

When the other species were examined by AFLP analysis in the context of the sections of *Miscanthus* the consensus is not quite as clear. Section *Miscanthus* is classified as comprising of *M. sinensis*, *M. floridulus* and *M. condensatus*. AFLP analysis using either primer combination showed *M. floridulus* to be distinct from *M. sinensis* and not any closer to it than *M. sacchariflorus*, which belongs to a different section. *Miscanthus condensatus* did appear to be closely related to *M. sinensis* by cluster analysis but PCO analysis showed one individual to be outside the range of *M. sinensis*. Only one species representing the section *Kariyasua*, *M. oligostachyus*, was available for analysis. This species lay outside of the range of sections *Miscanthus* and *Triarrhena* (Fig. 4.10), however it generally appeared more closely related to *M. nepalensis* which belongs to the section *Diandra*. *M. nepalensis* and *M. nudipes* which belong to *Diandra* generally grouped together by both PCO and cluster analysis and would appear to constitute a separate grouping. *Miscanthus transmorrisonensis*, a species which is now believed to be *M. sinensis* (Clayton & Renvoize, 1986), did appear to be distinct from *M. sinensis* (Fig 4.10 & 4.11). However, as only one individual was available for analysis no conclusive support for keeping it as a separate species can be drawn from AFLP analysis.

The species *M. nepalensis* should according to (Hodkinson *et al.*, 1997) be separated from *Miscanthus* based on AFLP and sequence data. These data indicate that *M. nepalensis* is

clearly separate from sections *Miscanthus* and *Triarrhena* but not from *M. nudipes* within section *Diandra* or *M. oligostachyus* within section *Kariyasua*. Strong bootstrap support of 77% with the combined primer combination showed *M. nepalensis* and *M. nudipes* formed a distinct group separate to all other species within the genus, although as only one individual was available for analysis it would be inappropriate to draw a strong conclusion from this.

One of the aims of AFLP analysis was to enable characterisation of the species within the genus. This proved to be successful, with all species producing unique fingerprinting profiles. This data will be an aid to researchers currently assessing the potential of different species of *Miscanthus* as suitable for European biomass production. In addition the analysis allowed an assessment of the genetic variation within certain sections of the genus to be examined.

Apart from the AFLP analysis of *M. x giganteus* by Greef *et al.* (1997), there is no other published account of the genetic profiles of the various varieties and cultivars of *Miscanthus* by AFLP analysis. This analysis provided unique AFLP profiles for a number of such accessions. It also allowed an assessment of the relatedness of these accessions to *M. sinensis* and *M. sacchariflorus* to be made. In general the analysis showed all of the cultivars and varieties to be closer to *M. sinensis* than *M. sacchariflorus*. The only exception to this was *M. x giganteus* which was consistently closer to *M. sacchariflorus*. Greef *et al.* (1997) found a very low level of genetic diversity among clones of *M. x giganteus*. They also found this hybrid to be genetically closer to *M. sacchariflorus* than *M. sinensis*. The accessions of *M. x giganteus* examined here would support these data, both accessions always clustered closer to *M. sacchariflorus* than *M. sinensis* when analysed together. Although only two accessions of *M. x giganteus* were examined, these individuals showed very little genetic variation between each other. They were indistinguishable by cluster analysis and only marginally different by PCO analysis. It is not surprising to find such a low level of genetic diversity among *M. x giganteus* accessions as this hybrid is sterile and can only be propagated by rhizome cuttings or by micro propagation. It is interesting to note that no molecular differences were found between the micro and rhizome propagated clones of *M. x giganteus* when analysed by AFLP by Greef *et al.* (1997). However, rhizome propagated *M. x giganteus* survived outdoor field trials where micro-propagated individuals failed within the first year of planting under Irish climatic conditions (Clifton-Brown, 1997). Unique AFLP profiles were identified for all other varieties and cultivars examined. It is envisaged that these data will be of use to researchers investigating the potential of different *Miscanthus* cultivars as potential biomass crops. It will also assist in determining the identity of clones already being tested in field trials.

The main problems with the above consensus of the classification of the genus and the cultivars was the lack of representatives of some of the sections of *Miscanthus*, especially the

section *Kariyasua*. Collecting trips to obtain some of these species was prohibitively expensive and contacts with Botanic gardens in the areas where these species are found failed to assist in obtaining them. Their representation in the various herbaria contacted was also low, excluding the possibility of obtaining DNA from herbarium material. However, even if this had been a possibility DNA extracted from herbarium material is generally not suitable for AFLP analysis as it is generally of lower molecular weight than that collected from living material.

AFLP analysis of *Miscanthus* did prove to be useful in assessing the relatedness of the cultivars to *M. sinensis* and *M. sacchariflorus*. It also excluded *M. floridulus* from section *Miscanthus*, although there was no bootstrap support for this exclusion. Future work including more representation of the other species constituting sections *Diandra* and *Kariyasua* to examine the robustness of these sections and the genetic variability of the species within them is required to thoroughly assess these sections. It would also be interesting to examine genetic diversity within the various cultivars and varieties and to compare them with the AFLP profiles of artificially produced hybrids to ascertain their true origin.

CHAPTER 5

DNA SEQUENCING: a molecular phylogeny of *Miscanthus* with particular reference to *M. floridulus*, *M. sinensis* and *M. sacchariflorus*.

5.1 INTRODUCTION

All of the molecular and morphological approaches investigated in the thesis have allowed the range of variation within the species and sections of *Miscanthus* to be examined. Most of the data collected precluded a phylogenetic hypothesis from being constructed, the data being more suited to a phenetic approach. It was therefore decided to examine DNA sequencing as a potential method of obtaining information which would be suited to phylogenetic reconstruction. However, it must be noted that DNA sequencing is not the only method of molecular phylogenetic construction available. DNA restriction site data and DNA-DNA hybridisation studies have provided much phylogenetic evidence in the past and restriction site data in particular is still widely used in phylogenetic studies. Most studies have examined variation in chloroplast DNA or nuclear ribosomal DNA for phylogenetic studies (Smith & Doyle, 1995; Smith *et al.*, 1993), and for studies on genetic diversity at the population level (Mason-Gamer *et al.*, 1995; Neal *et al.*, 1986). However, with the advent of the PCR and automated sequencing instrumentation, DNA sequencing is rapidly superseding restriction site analysis for the construction of phylogenies.

DNA sequencing is a technique which has advanced the work of molecular systematics significantly. This is mainly because of the enormous data sets it is capable of providing in a single analysis. Some nuclear genomes have 10^{11} nucleotide pairs per haploid genome (Judd *et al.*, 1999). One of the main attractions of sequence data is that it allows the examination of the basic units of inherited information encoded in organisms. Although sequence data does reflect direct gene level change it is also subject to some of the constraints of morphological data, such as convergence and parallelism (Judd *et al.*, 1999). It does, however produce larger data sets and often more easily interpretable results, and has been of assistance in confirming competing hypotheses based on morphological data. (Hoot *et al.*, 1999) found that sequence evidence based on three separate molecular data sets strongly confirmed the classification and relationships of Eudicots previously established from morphological evidence. On the other hand, Spangler & Olmstead, (1999) found conflicting evidence between the chloroplast encoded gene, *rbcL* and *ndhF* sequence and morphological data in the elucidation of the phylogeny of the Bignoniaceae.

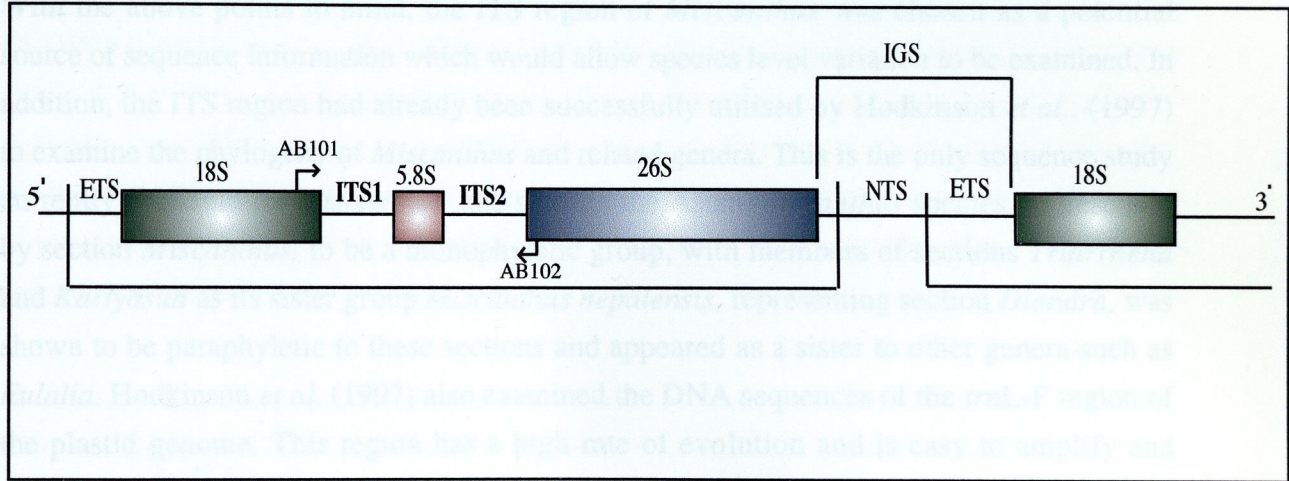
One of the first considerations when initiating DNA sequencing is choosing an appropriate genome and an area of that genome to sequence. The plant cell contains three different genomes, the nuclear, mitochondrial and plastid genomes. Of these the largest is the nuclear genome which is inherited from both parents. The mitochondrial genome, usually inherited from the maternal parent, is considerably smaller in size (200-250 Kbp). The plastid genome, which is the smallest of the three (135-160 Kbp) is usually only inherited from the maternal parent in angiosperms, although there is growing evidence to suggest that paternal inheritance is more widespread than previously thought (Sewell *et al.*, 1993). Sequence information is generally collected from either the plastid or the nuclear genome. Mitochondrial genomes tend to rearrange themselves both within the cell and between species very frequently, making mitochondrial DNA data less practically suitable in assessing genetic relatedness (Judd *et al.*, 1999). Nuclear genomes, are much more stable both within the cell and between species. The plastid genome is the most stable and highly conserved of all, not just across species but between genera. For this reason the plastid genome is often the region of choice for studies of evolutionary history at higher taxonomic levels (Chase *et al.*, 1993; (Morgan & Soltis, 1993; (Price & Palmer, 1993; (Spangler & Olmstead, 1999). The conservative evolution of the plastid genome has limited its use at the population level and among very closely related species. However, different parts of the genome evolve at different rates and some researchers have been successful in finding portions of the plastid genome which have been suitable for use at the species level (Sewell *et al.*, 1996; Soltis *et al.*, 1998).

Within the nuclear genome, most sequence information has been obtained from the genes encoding ribosomal RNA, because these genes occur in such high copy number within the genome that they can be easily isolated. These genes are arranged in tandem repeats, one after the other, along the genome. An illustration of the structure of these genes is shown in Fig. 5.1. Essentially, the ribosomal gene is constructed of three individual parts, the 18s subunit, a larger gene the 26s subunit and the smallest subunit, the 5.8s which separates the two larger genes. The entire set of genes is transcribed as a single unit. Regions known as ITS (Internal Transcribed Spacers) separate each of the three genes. The entire set of genes is separated from the next set by a nontranscribed spacer (NTS) and an external transcribed spacer (ETS), these two sections are referred to cumulatively as the Intergenic spacer (IGS).

The 18s and 26s are highly conserved regions and therefore, as with the chloroplast genome, are of more use at family or tribal levels, as illustrated in the classic work of Soltis *et al.*, (1997). This study demonstrated the suitability of 18s rDNA in elucidating the phylogeny of 223 species representing all major groups of angiosperms and showed that this phylogeny largely confirmed previous angiosperm phylogenies based on chloroplast *rbcL* sequence data. The *rbcL* gene is located in the large single copy region of the plastid genome. This

gene has been shown to be highly useful at the family level and above (Clegg, 1993). The 26S rDNA sequences have been less commonly investigated, largely due to their large size in comparison with the 18S rDNA, although regions within the gene have been sequenced in tandem with the 18S region (Hamby & Zimmer, 1992). The 5.8S rDNA is generally not used for phylogenetic studies as it is highly conserved, and very small. The ITS region has been found to evolve more rapidly than the other sections whilst the nuclear ribosomal DNA and is increasingly becoming the focus of attention at lower taxonomic levels (Baldwin *et al.*, 1995). The ITS region has been found to be polymorphic at species level and has been useful for resolving phylogenetic relationships within genera and among closely related plant species (Baldwin, 1993; Saag *et al.*, 1994).

FIG. 5.1 Structure of the nuclear ribosomal genes and associated ITS region



Key to abbreviations:

- NTS: non-transcribed spacer
- ETS: External transcribed spacer
- ITS: Internal transcribed spacer
- IGS: Intergenic spacer
- 18S: 18S subunit of ribosomal RNA
- 5.8S: 5.8S subunit of ribosomal RNA
- 26S: 26S subunit of ribosomal RNA

gene has been shown to be highly useful at the family level and above (Clegg, 1993). The 26s rDNA sequences have been less commonly investigated, largely due to their large size in comparison with the 18s rDNA, although regions within the gene have been sequenced in tandem with the 18s region (Hamby & Zimmer, 1992). The 5.8s rDNA is generally not used for phylogenetic studies as it is highly conserved, and very small. The ITS region has been found to evolve more rapidly than the other sections within the nuclear ribosomal DNA and is increasingly becoming the focus of attention at lower taxonomic levels (Baldwin *et al.*, 1995). The ITS region has been found to be polymorphic at species level and has been useful for resolving phylogenetic relationships within genera and among closely related plant species (Baldwin, 1992; Sang *et al.*, 1994).

With the above points in mind, the ITS region of *Miscanthus* was chosen as a potential source of sequence information which would allow species level variation to be examined. In addition, the ITS region had already been successfully utilised by Hodkinson *et al.*, (1997) to examine the phylogeny of *Miscanthus* and related genera. This is the only sequence study currently available for *Miscanthus* and showed the Asian *Miscanthus* species, represented by section *Miscanthus*, to be a monophyletic group, with members of sections *Triarrhena* and *Kariyasua* as its sister group *Miscanthus nepalensis*, representing section *Diandra*, was shown to be paraphyletic to these sections and appeared as a sister to other genera such as *Eulalia*. Hodkinson *et al.* (1997) also examined the DNA sequences of the *trnL-F* region of the plastid genome. This region has a high rate of evolution and is easy to amplify and sequence, and found the phylogeny resulting from the analyses was largely congruent with that produced from the ITS sequences. However, the work of Hodkinson *et al.* (1997) lacked sequence data from *M. floridulus* and *M. sacchariflorus* and these species among others were added to this data set in an attempt to further resolve the classification of *Miscanthus*.

DNA sequencing, like most other molecular techniques, initially relies on the PCR to amplify the region of interest; in this case the ITS region. Firstly, ITS sequence primers are annealed to single stranded DNA. The PCR primers widely used were initially developed by (White *et al.*, 1990). Once the target sequence has been amplified it is generally purified using commercially available purification columns before being directly sequenced. Sequencing of the amplification product is usually carried out in the forward direction first. For automated sequencing this is accomplished by carrying out a cycle sequencing reaction in which the forward sequence primer and a sequencing dye mix are incorporated. The dye mix is designed to incorporate coloured florescent dye terminators corresponding to each of the four nucleotides of the sequence. These dyes can later be read by a laser beam on an automated DNA sequencer to provide a chromatogram of the bases. It is worth mentioning here that DNA sequences were generally produced manually until the advent of automated DNA

sequencers in recent years and are still commonly carried out in this way. According to Soltis & Soltis (1998) the results from automated sequencers should be treated with caution and always edited base by base by eye. However, with this in mind, automated sequencing is generally superior to manual sequencing as it allows many more samples to be analysed simultaneously and gives a longer read of sequence bases. This in turn allows replicate samples to be run, which can assist in answering any ambiguities in the sequence. Providing a clear sequence is obtained from the forward strand of the DNA, the reverse strand can then be sequenced in the same way using reverse primers. The sequence of the reverse strand can then be aligned with the forward strand, further confirming the sequence of the bases.

Once a range of sequences have been produced for the taxa in question the data must firstly be aligned and then analysed. This can be done by eye or by using a program such as CLUSTAL (Higgins *et al*, 1992). Once the sequence has been correctly edited Swofford and Olsen (1990) state that any ambiguous or highly diverse regions be eliminated from the analysis. Typically the beginning and end of a sequence is unreliable and it is therefore safer to eliminate it from the analysis. Their justification for eliminating data is that in the choice of the region to be sequenced in the first place most of the information encoded in the rest of the genome has already been eliminated.

In this project parsimony analysis was used to analyse the aligned sequences. In this case the software package PAUP 4.0 (Swofford, 1999) was used. A heuristic search with 1000 replicates of random addition sequence, tree bisection and reconnection (TBR) or with nearest-neighbour interchange (NNI) was performed. Parsimony analysis is a method of producing a tree from the given data in the simplest (shortest number of steps) manner possible. Parsimony analysis follows Ockham's razor rule which states 'what is the simplest explanation of the observations'. Effectively, this means that no hypothesis any more complex than the data demands, should be generated. In this manner, parsimony analysis attempts to find the shortest tree from the data provided. The shortest tree is however the one that maximises character congruence i.e. agreement between characters and it is very powerful.

The word heuristic is defined as "a method of serving or leading to find out, depending on assumptions based on previous experience". Its principles are based on making decisions when all possibilities can not be explained based on guided trial and error. This definition of the term heuristic serves well to explain how the technique actually works in computational terms. Firstly, an initial tree is used to start the process. That tree is then improved upon by rearranging it in a way that reduces its branch lengths. When all possibilities for reducing the branch lengths have been exhausted the process stops. This process is repeated many times to increase the chance of finding the optimal tree (1000 replicates per individual in this study)

However, it is not known whether the final tree is in fact the most optimal one, in this way the search is heuristic i.e. it is based on guided trial and error. Exact methods are obviously the ideal method to search for the maximally parsimonious tree. However exact methods can only be employed if the data set is small, generally containing less than twenty taxa and a limited number of characters (Kitching, 1992), otherwise the computational difficulties result in prohibitively long analysis time.

The actual manner by which the tree is arranged and rearranged can follow a number of different procedures. The method used here was based on random additional sequence. This method chooses three taxa for the original tree, and from this group of three, the taxa that produces the shortest tree is chosen *i.e.* the tree that requires the smallest increase in tree length is chosen. At each successive step all of the remaining taxa in the data matrix are examined for connection to every branch of that tree, and the taxon-branch combination that requires the shortest increase in tree length is chosen (Swofford & Olsen, 1990). In order to improve the original tree, which is in effect a 'best guess,' different forms of branch swapping such as tree bisection and reconnection and nearest-neighbour interchange can be applied to the initial tree. Tree bisection and reconnection splits the original tree in two along a branch producing two separate trees. The two trees are then reconnected by joining any one pair of branches from each tree. This process continues until all possible split "bisections" and pairwise reconnections have been made. The technique of nearest-neighbour interchange works by recognising that each branch of the tree defines a local region containing four subtrees which are all connected to this branch. It then interchanges a subtree from one side of this branch with one from the other *i.e.* its nearest neighbour. According to Swofford & Olsen (1990) either one of these branch swapping techniques amounts to a "stab in the dark" but the hope is that if a better tree exists one of these rearrangements will find it. Finally a consensus tree can be produced as a summarisation of all of the trees produced by a heuristic search. Consensus trees are a summarisation of the trees, rather than the actual data. They are especially useful for comparing trees from different data sets (Siebert, 1992)

Having agreed that the tree produced from the above methods may not be exactly the best, or most correct tree, it is necessary to have some estimate of confidence in the final tree. The groupings can be assessed using bootstrapping (Felsenstein, 1985), described in Chapter three of this thesis. The confidence of the overall tree itself can be measured by consistency indices (CI) and retention indices (RI). Consistency indices and retention indices provide estimates of how well the data fits the tree. The Consistency index for a tree is calculated by the summation of the minimum amount of change possible for a character divided by the actual tree length (the number of genetic changes on the tree). If no homoplasy (non-homology) exists in the data set a CI of one will result, and this value will decrease as homoplasy increases. The retention index is a measure of the amount of synapomorphy

(shared derived character states) in the data set. It is achieved by expressing the amount of homoplasy as a fraction of the maximum possible homoplasy. High RI values indicate changes mainly occurring on internal nodes of the tree and low values indicate changes occurring on branches leading to terminal taxa. The most parsimonious tree will be the tree of shortest length. If the data matrix contains a large number of uninformative characters then a number of trees of similar branch length will result. Tree length therefore, is a measure of the number of steps required to account the number of transformations required to explain the data on the tree. However tree length will be affected by the number of characters in the data matrix, the more characters in the data the larger the tree length will be (Siebert, 1992).

The aim of this analysis was to examine the ITS region of a number of *Miscanthus* species where sequence information had been lacking or was ambiguous in the data collected by Dr. T.R. Hodkinson at the Royal Botanic Garden, Kew. It was hoped that by adding to data already collected a clearer picture of some aspects of *Miscanthus* phylogeny could be obtained and that this analysis would compliment the morphological and fingerprinting data already collected in this project. In particular the position of *M. floridulus*, *M. sinensis* and *M. sacchariflorus* was examined as *M. floridulus* was poorly sampled in the analysis of Hodkinson *et al.*, (1997) and the position of *M. sacchariflorus* is still undetermined.

Chapter 5

5.2 Materials and Methods

All of the DNA used in the sequencing reactions was extracted following the protocol outlined in chapter three. In addition, DNA extracted and sequenced by Dr. T.R. Hodgkinson at the Royal Botanic Garden, Kew was included in the final analysis.

5.2.1 Analytical methods

Amplification of ITS region of nuclear ribosomal DNA for sequencing.

PCR amplification reactions were carried out on a Perkin - Elmer 480 thermal cycler, without a heated lid as follows: 100 μ l reactions were carried out in thin walled 0.5 ml PCR tubes. Each reaction was overlaid with two drops of silicon oil to prevent evaporation of the sample. The PCR cycling conditions were as follows:

Denaturing step: 97°C for 1 minute, 30 cycles

Annealing step: 50°C for 1 minute, 30 cycles

Extension step: 72°C for 3 minutes, 30 cycles

Final extension: 72°C for 7 minutes, 1 cycle

Soak temperature 4°C

The following reaction concentrations were used per sample, although generally a mastermix was prepared containing enough reagent for the number of samples being processed:

	<u>Volume per reaction</u>	<u>Amount in 100 μl</u>
DNA	2 μ l (= 50 ng)	50 ng
Distilled water	77.5 μ l	-
Promega 10x PCR Buffer (10mM)	10 μ l	1 mM
dNTP (10mM)	2 μ l	0.2 mM
Primer AB101 (100ng ⁻¹)	1.0 μ l	100 ng
Primer AB102 (100ng ⁻¹)	1.0 μ l	100 ng
MgCl ₂ (25mM)	6.0 μ l	1.5 mM
Taq polymerase (6 units μ l ⁻¹)	0.5 μ l	3 units

Following the PCR the product was examined to ascertain that only one sharp band corresponding to the amplified region existed. This was achieved by running 6 µl of the PCR reaction plus 1µl of loading dye on a 1.5% mini agarose gel (containing ethidium bromide) at 100 volts for 30 minutes and viewing the gel under ultra violet light.

Purification of PCR product

Successful PCR reactions were then purified using QIAquick PCR purification kits (Qiagen UK. Ltd) as follows:

1. The oil was removed from the PCR reaction and 250 µl of the supplied buffer was added to 50 µl of the PCR product and the solution was mixed gently.
2. A QIAquick spin column was placed in a 2 ml collection tube and the solution from 1. above was applied to the column.
3. The column was spun in a microcentrifuge at 14, 000 rpm for 45 seconds.
4. The flow-through collected in the 2 ml tube was discarded.
5. 0.75 ml of supplied buffer was added to the column and spun at 14, 000 rpm for 60 seconds.
6. The flow-through was discarded and the column spun again for an additional 1 minute at 14, 000 rpm.
7. The column was placed in a fresh 1.5ml tube and the DNA eluted by adding 50 µl of 10mM Tris-HCl, pH 8.5 to the centre of the column and spinning the column for 1 minute at 14, 000 rpm.

Note: The exact formulation of some of the reagents supplied with this kit are not given by the manufacturer.

Following the purification procedure the DNA was checked again for purity and quantity by running a sub-sample of it on a mini agarose gel as described above. A known size standard was run alongside to allow an estimate of the amount of product required in the sequencing reaction to be made.

Cycle sequencing reactions of amplified ITS region.

5 µl sequencing reactions were carried out on an MJ research PTC-200 Peltier thermal cycler with a heated lid. All reactions were carried out in 200 µl thin walled PCR tubes. Initially only the forward strand was sequenced. If this reaction was successfully sequenced the reverse strand was sequenced in exactly the same manner except the reverse sequence primer was used.

The concentrations for the sequencing reactions were as follows:

Purified PCR product from purification procedure 2 µl

ITS primer (AB101 forward sequencing primer) 0.7 µl (at 5 ng/µl)

Sequencing buffer 3.5 µl

*Sequencing dye mix 1 µl

Distilled water 2.8 µl

* The sequencing dye mix used was the *Taq* Dye-Deoxy Terminator Cycle Sequencing Kit of Applied Biosystems International (ABI)

Each of the above components were added as a 'dot' to the side of the wall of the PCR tube which was held on ice throughout the procedure. When all components had been added the tube was given a brief spin to mix the reagents. Tubes were then placed in the thermal cycler and the machine was run according to the following cycling conditions:

24 cycles of:

Denaturing step: 96°C for 10 seconds

Annealing step: 50°C for 5 seconds

Extension step: 60°C for 4 minutes

Soak temperature 4°C

Following the sequencing reaction the product was cleaned using the following procedure:

1. 50 µl of 100% ethanol and 2 µl of 3M sodium acetate were placed in a 0.5 ml tube
2. All of the sequenced product (5 µl) was added to the tube.
3. The tube was left to stand at room temperature for 10 minutes and then placed on ice for a further 10 minutes.

4. The tube was then spun at 13, 000 rpm in a microcentrifuge for 15 minutes and the supernatant was subsequently poured off.
5. The 'pellet' was then washed in 300 µl of 70% ethanol and then spun at 13, 000rpm for 15 minutes and the supernatant drained off.
6. The 'pellet' was washed again in 300 µl of 70% ethanol and spun at 13, 000rpm for 15 minutes, the supernatant was drained off.
7. The 'pellet' was dried by placing it in an oven at 70°C for a maximum of 15 minutes.

Sequencing of amplified product

As an automated sequencer was not available at Trinity College, Dublin, all sequenced products were sent to the Jodrell Laboratory at the Royal Botanic Garden, Kew who processed the sequenced product on an ABI 373A or 377 automated DNA sequencer. The sequence of the product was then returned to us via email whereupon the data was processed.

5.2.2 Parsimony analysis of DNA sequences

The forward and reverse strands of the sequence were assembled using Sequencher version 3.0 (Genecodes Corporation Inc.), followed by manual editing of ambiguous nucleotides. The sequence of the forward strand produced a strong signal from the 5' end of the region which weakened as the sequence progressed. In the same manner the sequence of the reverse strand produced a strong signal from the 3' end. In this way when the two strands are aligned any ambiguities can be resolved by examining the stronger and more accurate signal of the opposing strand. A data matrix of the consensus sequence was assembled in PAUP 4.0 (Swofford, 1999) and is given in Appendix 7. Gaps are placed in the data matrix to account for insertions or deletions which have taken place to allow positions with inferred homology to remain in line. Swofford and Olsen (1990) recommend that areas of the sequence that are very divergent, making alignment by eye a form of guesswork, should be deleted from the sequence matrix. With this in mind, sections of the sequence were eliminated from the analysis. The areas of the sequence which were eliminated only corresponded to the beginning and end of the sequence, any other ambiguities, which amounted to one or two bases, within the sequence were treated as missing data.

Phylogenetic analysis of the aligned sequences were then carried out by parsimony analysis. Two separate analyses were carried out. One of these included the entire ITS sequence for the species analysed matched for alignment with the data matrix of the same region produced

by Dr. T.R. Hodkinson at the Royal Botanic Garden, Kew. As there were some ambiguities between the two data sets, at the beginning and end of the sequences, due to different stop/start positions of the sequence a second analysis was performed on a reduced set of characters where all of the ambiguous bases were removed. These bases included the first seven and final twenty two characters in the matrix.

All analyses carried out on the sequence data were based on rooted trees. For this purpose *Andropogon gerardii* was used as an outgroup. This species was used because it is related to *Miscanthus*, being included in the tribe Andropogoneae but it is not a member of the ingroup (Clayton and Renvoize, 1986). Rooting of trees should however be treated with caution, as incorrect rooting can lead to erroneous phylogenies. If a taxon, thought to be an outgroup is actually part of the ingroup, an incorrect tree will be produced (Swofford & Olsen, 1990). Swofford & Olsen (1990) also suggest that if assignment of an outgroup is to be made, two outgroups are included to test the monophyly of the ingroup. In this analysis *Saccharum officinarum* was also tested as an outgroup. All of the species and genera analysed are shown in Table 5.1.

5.2.3 Distance analysis of DNA ITS sequences

Distance analyses of ITS DNA sequences were carried out using PAUP by neighbor-joining and UPGMA as described in Chapter three. The only difference in the statistical methodology was that mean character differences instead of Nei-Li distances were calculated.

Table 5.1. *Miscanthus* accessions analysed for ITS sequence region

Species	DNA sample code	Analyst
<i>M. floridulus</i>	TCD 69	L. Scally
<i>M. floridulus</i>	TCD 70	L. Scally
<i>M. floridulus</i>	TCD 73	L. Scally
<i>M. sacchariflorus</i>	TCD 53	L. Scally
<i>M. oligostachyus</i>	TCD 35	L. Scally
<i>M. oligostachyus</i>	62	T. Hodkinson
<i>M. floridulus</i>	72	T. Hodkinson
<i>M. sinensis</i> ssp. <i>condensatus</i>	7	T. Hodkinson
<i>M. sinensis</i> 'Roland'	29	T. Hodkinson
<i>M. sinensis</i> 'yakushmanum'	63	T. Hodkinson
<i>M. sinensis</i>	2	T. Hodkinson
<i>M. sinensis</i> 'Variegatus'	1	T. Hodkinson
<i>M. sinensis</i> 'Grobe rontane'	31	T. Hodkinson
<i>M. sinensis</i> 'Gracillimus'	28	T. Hodkinson
<i>M. x giganteus</i> clone 1	-	T. Hodkinson
<i>M. x giganteus</i> clone 2	-	T. Hodkinson
<i>M. sacchariflorus</i> 'purpurescens'	61	T. Hodkinson
<i>M. nepalensis</i>	25	T. Hodkinson
<i>Miscanthidium junceus</i>	88	T. Hodkinson
<i>Miscanthidium junceus</i>	89	T. Hodkinson
<i>Miscanthidium fuscus</i>	83	T. Hodkinson
<i>Miscanthidium ecklonii</i>	86	T. Hodkinson
<i>Saccharum officinarum</i>	104	T. Hodkinson
<i>Saccharum spontaneum</i>	119	T. Hodkinson
<i>Andropogon gerardii</i>	75	T. Hodkinson

5.3 RESULTS

5.3.1 Maximum parsimony analysis of *Miscanthus* ITS data.

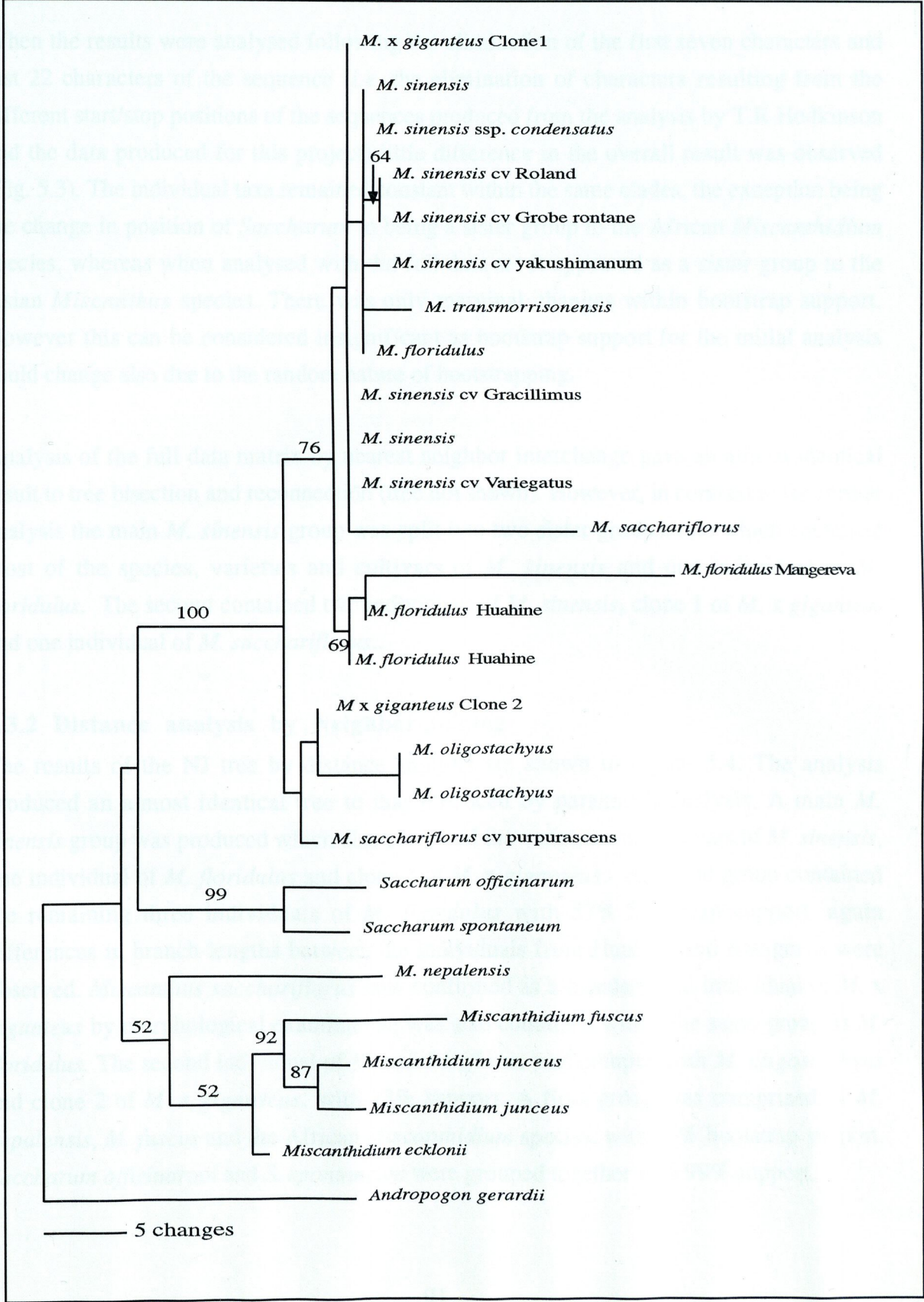
The results obtained from the heuristic search in 1000 replicates of random addition sequence, with tree bisection and reconstruction are shown in Fig. 5.2. Of the total of 632 characters, 517 were constant, 70 were variable and parsimony uninformative and 45 were parsimony informative, The consistency index was 0.8767, the retention index was 0.8939 and the tree length was 146.

The results showed a main *Miscanthus* group with 100% bootstrap support. This group comprised all *Miscanthus* species as defined by Lee (1964), with the exception of *M. nepalensis*. Branching from this clade are a main *M. sinensis* clade with bootstrap support of 76%. This clade also contains *M. transmorrisonensis*, which is now generally considered to be *M. sinensis* or a closely related sister taxon. It also contains clone 1 of *M. x giganteus*. This clone is one of the copy types produced from the amplification of the ITS region of one *M. x giganteus* individual extracted by Dr. T.R. Hodgkinson at the Royal Botanic Garden, Kew. Variation between the ITS repeats can lead to two different amplification products from the same individual, especially if, as in this case, the individual is a hybrid. The second clone of this individual was grouped within the clade containing *M. sacchariflorus*. One individual of *M. sacchariflorus* also grouped within the main *M. sinensis* clade and has subsequently been verified as a misidentified individual of *M. x giganteus*.

Three individuals of *M. floridulus*, two from the island of Huahine and one from the island of Mangareva in the southern Pacific Ocean formed a clear monophyletic group which is a sister group to the main *M. sinensis* clade. Differences in branch lengths, representing the number of steps taken within the analysis is observed between the two individuals from Huahine and the individual from Mangareva. The forth individual of *M. floridulus* is grouped within the main *M. sinensis* clade, this individual, sampled at the Royal Botanic Garden Kew is of unknown origin and may have been misidentified. A second sister group to *M. sinensis* contains the second clone of *M. x giganteus*, *M. sacchariflorus* and two individuals of *M. oligostachyus*, which together formed a monophyletic group, but with no bootstrap support. The two species of *Saccharum* formed a monophyletic group with 99% bootstrap support which is separate from all other *Miscanthus* species. All of the African species of *Miscanthus* and *M. fuscus* are grouped together with 52% bootstrap support. *Miscanthus nepalensis* is sister to this group also with 52% bootstrap support.

Fig. 5.2 One of 62 maximally parsimonious trees of all ITS DNA sequence characters, using heuristic search options with tree bisection and reconnection. Figures above branches indicate percentage bootstrap support.

CI = 0.8767, RI = 0.8939, tree length = 146



A consensus tree of the full character set (Appendix 6) was produced by a 50% majority consensus of 1000 trees. This tree demonstrates how part of the maximally parsimonious tree collapses but also indicates that much of the structure of the tree is retained. This is particularly evident in the division between *Miscanthus* and *Miscanthidium* and also with the retention of the *M. floridulus* grouping.

When the results were analysed following the elimination of the first seven characters and last 22 characters of the sequence (*i.e.* the elimination of characters resulting from the different start/stop positions of the sequences produced from the analysis by T.R. Hodgkinson and the data produced for this project) little difference in the overall result was observed (Fig. 5.3). The individual taxa remained constant within the same clades, the exception being the change in position of *Saccharum* to being a sister group to the African *Miscanthidium* species, whereas when analysed with the full data set it appeared as a sister group to the Asian *Miscanthus* species. There was only marginal changes within bootstrap support. However this can be considered insignificant as bootstrap support for the initial analysis could change also due to the random nature of bootstrapping.

Analysis of the full data matrix by nearest neighbor interchange gave an almost identical result to tree bisection and reconnection (tree not shown). However, in contrast to the former analysis the main *M. sinensis* group was split into two sister groups, one which contained most of the species, varieties and cultivars of *M. sinensis* and one individual of *M. floridulus*. The second contained two individuals of *M. sinensis*, clone 1 of *M. x giganteus* and one individual of *M. sacchariflorus*.

5.3.2 Distance analysis by Neighbor-Joining

The results of the NJ tree by distance analysis are shown in Figure 5.4. The analysis produced an almost identical tree to that produced by parsimony analysis. A main *M. sinensis* group was produced which also contained the varieties and cultivars of *M. sinensis*, one individual of *M. floridulus* and clone 1 of *M. x giganteus*. A second group contained the remaining three individuals of *M. floridulus* with 57% bootstrap support, again differences in branch lengths between the individuals from Huahine and Mangerva were observed. *Miscanthus sacchariflorus* now confirmed as a misidentified individual of *M. x giganteus* by morphological examination, was also contained within the same group as *M. floridulus*. The second individual of *M. sacchariflorus* was grouped with *M. oligostachyus* and clone 2 of *M. x giganteus*, with 83% support. A final group was comprised of *M. nepalensis*, *M. fuscus* and the African *Miscanthidium* species, with 96% bootstrap support. *Saccharum officinarum* and *S. spontaneum* were grouped together with 99% support.

Fig. 5.3 One of 60 maximally parsimonious trees of ITS DNA sequence reduced characters set, using heuristic search options with tree bisection and reconnection. Figures above branches indicate percentage bootstrap support. CI = 0.8732, RI = 0.8935, tree length = 142

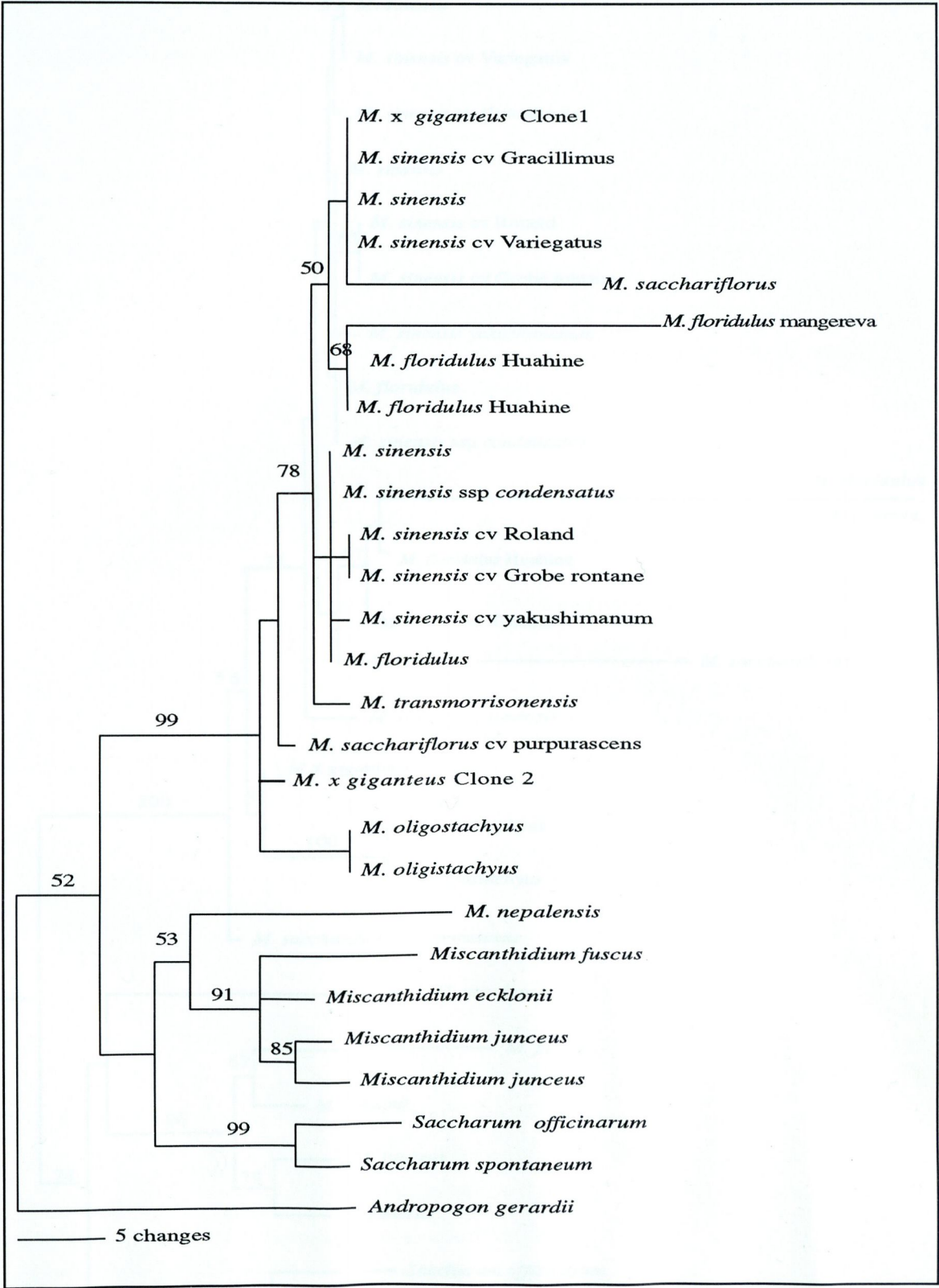
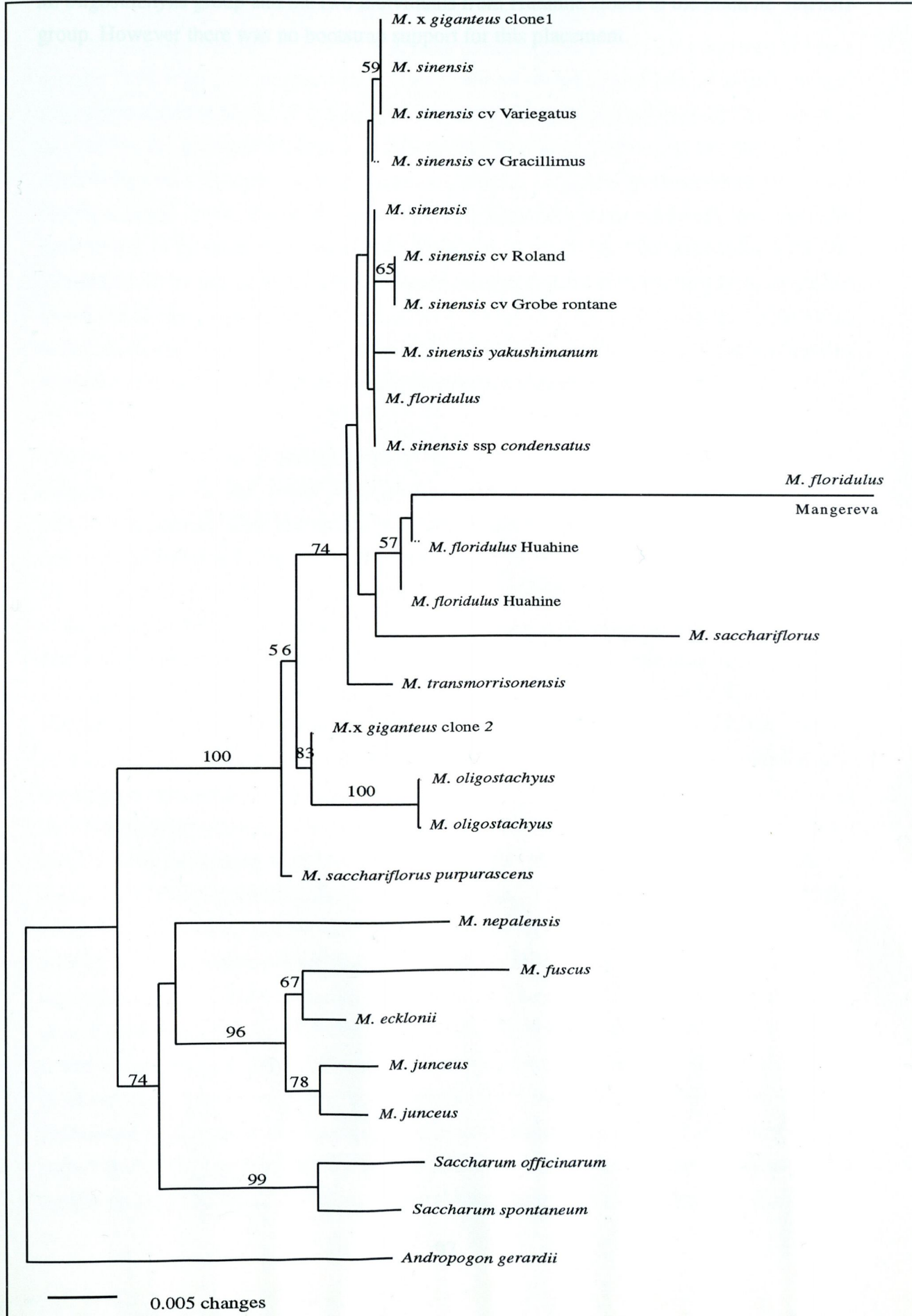


Fig 5.4 Distance analysis of all ITS DNA sequence characters, using Neighbor joining. Figures above branches indicate percentage bootstrap support.



5.4 Discussion

The analysis of the Internal Transcribed Spacer (ITS) region of *Miscanthus* ribosomal DNA was very successful. The sequence data was easily assembled by eye within the Sequencher software package and aligned in PAUP as most of the sequence was well conserved between species. ITS regions have previously been found to be conserved among closely related angiosperms (Hershkovitz & Zimmer, 1996). It has been indicated that the ITS region is involved in the maturation of nuclear rRNAs (Baldwin *et al.*, 1995) and this theory would indicate that the ITS region is under some evolutionary constraint in structure and sequence (Soltis & Soltis, 1998). The ITS region in grasses, especially at the subfamily level, has also been found to be moderately conserved by Hsiao *et al.* (1999). Initially some data was eliminated from the analysis due to primer start/stop positions varying between those sequences carried out at Trinity College, Dublin and the data matrix to which they were being added from the Royal Botanic Garden, Kew. In addition, there was some ambiguous sequence data, again mainly at the start and end of the sequence.

Observation of the data produced by Hodkinson *et al.* (1997) showed a clear monophyletic grouping of all of the Asian *Miscanthus* species. Included in this group was *M. transmorrisonensis*. This individual was first described by Hayata (Hayata, 1911), and is described as being very similar to *M. sinensis* var. *formosanus* Hack., but differing in its nearly glabrous leaf sheath and much less-branched panicles. This species is now considered to be included within *M. sinensis*, as to split it into a separate species based on such ambiguous characters is unrealistic (Clayton & Renvoize, 1986). *Miscanthus floridulus* is also included within this group, thereby supporting section *Miscanthus* of Lee (1964). A second clade, a sister to the *M. sinensis* clade, comprised *M. sacchariflorus* and *M. oligostachyus*. These two species represent sections *Triarrhena* and *Kariyasua* respectively, but no clear distinction between these two sections was observed. Here *M. oligostachyus* is shown to be monophyletic. The final clade was represented by the African *Miscanthidium* species, with the inclusion of *Miscanthus fuscus* and *Miscanthus nepalensis*. *Miscanthus fuscus* had previously clouded the distinction between the Asian *Miscanthus* species and the African *Miscanthidium* species, the African species being placed in a separate section based on their elongate inflorescence and short racemes. *Miscanthus fuscus* also possesses the same characteristics and this analysis has placed *M. fuscus* firmly within the *Miscanthidium* group. Hodkinson *et al.* (1997) have suggested that further work be carried out on *M. fuscus* to confirm its position. *Miscanthus nepalensis* is a representative of section *Diandra* of *Miscanthus*, but appears here to be separate to the rest of the sections of *Miscanthus*. Hodkinson *et al.* (1997) produced a strict consensus tree which included additional genera, including *Eulalia*, *Imperata* and *Spodiopogon* (within the Saccharinae) and *Sorghum* (within Sorghinae). In this analysis, *M. nepalensis* was placed as a sister group to *Eulalia*. In

addition, the African *Miscanthidium* species, which are considered to be similar to *Miscanthus* based on their morphology by Clayton and Renvoize (1986), were clearly separate from the core *Miscanthus* group based on their ITS sequences. Within the consensus tree of Hodkinson *et al.* (1997) the African species and *M. fuscus* form a monophyletic group.

This study added an additional five individuals to the data of Hodkinson *et al.* (1997) in an attempt to further resolve some of the questions relating to certain sections of *Miscanthus*. The addition of a second individual of *M. sacchariflorus* to this data set originally caused a certain amount of confusion, as this individual was grouped within the *M. sinensis* clade. However it is also possible that this individual is related to *M. x giganteus*. It is possible that this individual represents an allotetraploid with genomes from both *M. sinensis* and *M. sacchariflorus*. ITS copy genes can become homogenised by the process of concerted evolution and this could have caused this individual to appear to resemble *M. sinensis* more than *M. sacchariflorus*. The process of concerted evolution can occur in nuclear DNA sequences because of the high copy number of the tandemly repeated sequences. If some of these tandem repeats evolve independently, more than one copy type can occur. Concerted evolution can favour one copy type over another. In this case of a hybrid which has undergone subsequent cycles of reproduction the copy type can become homogenised. It is however not an exact match (as indicated by the longer branch length of this individual, see Fig. 5.2) which may suggest that recombination has occurred. A chromosome count on this individual to establish whether or not it is an allotetraploid is required to confirm this hypothesis. The unusual grouping of this individual was also reflected in AFLP analysis.

Miscanthus x giganteus was also sequenced at the Royal Botanic garden Kew but excluded from their original analysis due to the hybrid nature of this plant. ITS sequences which occur in tandem repeat can vary in an individual of hybrid origin. Attempts to amplify the ITS sequence of *M. x giganteus* resulted in two separate amplification products. Cloning of these products was required in order to produce sufficient material for sequencing. It is assumed that the placement of one of these cloned products within the *M. sinensis* clade and the second within the *M. sacchariflorus* clade reflects the hybrid nature of this individual, one clone being derived from *M. sinensis* and the second from *M. sacchariflorus*. This would confirm the hypothesis of Adati & Shiotani, 1962 and Linde-Laursen, (1993) that *M. x giganteus* is a hybrid involving these two species.

Another species added to the Kew data matrix was *M. oligostachyus*. This individual was indistinguishable from the individual of *M. oligostachyus* already analysed, thereby confirming the position of these two accessions. Here they are clearly shown to form a monophyletic group closely related to *M. sacchariflorus*.

The final species added to the Kew matrix was *M. floridulus*. The original matrix only had one individual of *M. floridulus* which was of unknown origin. This individual grouped with *M. sinensis* in the original analysis. However, the three individuals added to the matrix were of known origin, two from the island of Huahine and one from Mangareva in the southern Pacific Ocean. All of these individuals formed a monophyletic group which was a sister group to *M. sinensis*. Genetic differences between *M. floridulus* from Huahine and Mangareva, as indicated by varying branch lengths, demonstrated that *M. floridulus* from Mangareva contains a lot of unique genetic variation. The *M. floridulus* individual sequenced at Kew was grouped in the same clade as *M. sinensis* but the origin of this individual is uncertain. *Miscanthus floridulus* has a wider geographic range than *M. sinensis*, extending into remote islands of Polynesia, so it is not surprising that this species, especially those collected from these islands would show a certain degree of genetic distance to *M. sinensis*. It is worth noting here that although hybrids between *M. sinensis* and *M. floridulus* are common where their distribution overlaps, this is not the case in the eastern Polynesian islands such as Mangareva where only *M. floridulus* occurs.

The elimination of the ambiguous characters produced from varying stop/start points of the sequence did not produce a significantly different tree to that produced by the full character set, thus allowing a higher degree of confidence in the original data set and proving that the elimination of ambiguous characters was not, in this case, necessary. Examination of the tree statistics indicated a consistency index of 0.8767, this figure, while indicating a certain amount of homoplasy was not excessive. The retention index figure of 0.8935 indicates that most of the changes actually occurred on the internal nodes of the tree. When the bootstrap support is examined for these nodes it can be seen that most of these changes were likely to have been confined to the nodes leading to and within the *M. sinensis* clades, as bootstrap support for these branches is below 50%. This does not have any real bearing on the overall classification, as this grouping contains a majority of *M. sinensis* species, varieties and cultivars, which have been found to be highly variable from morphological, RAPD and AFLP data produced in this thesis.

In general the sequence analysis of *Miscanthus* has indicated that the genus comprises a monophyletic group distinct from other closely related genera, including *Miscanthidium*, *Saccharum* and *Eulalia*. Within the genus, section *Miscanthus*, comprising the Asian species of *Miscanthus*, forms a largely distinct monophyletic group. Included within this group are all of the cultivars of *Miscanthus*, indicating that these individuals are closely related to *M. sinensis*. *Miscanthus sacchariflorus* representing section *Triarrhena* is also supported as sister to the core *Miscanthus* group, although only one individual was available for analysis. Section *Kariyasua*, represented by two individuals of *M. oligostachyus* formed a monophyletic group which was distinct but sister to section *Triarrhena*. Section *Kariyasua* is

considered to be more closely related to *Diandra* than *Triarrhena* by Lee (1964a), it is also confined to mountainous regions of Japan whereas *M. sacchariflorus*, the sole representative of section *Triarrhena* is found in the wet lowlands of Japan, Korea and northern China, where the geographic distribution of these two sections does not overlap. However, DNA sequence data would indicate a closer relationship of section *Kariyasua* to *Triarrhena* than *Diandra*. *Miscanthus x giganteus* is clearly shown to be a hybrid between *M. sinensis* and *M. sacchariflorus* supporting the theory of Greef *et al.*, (1997) based on AFLP data. However, section *Diandra* is not supported as a section within the genus *Miscanthus*, and is more closely grouped with *Eualia* based on the data of Hodkinson *et al.* (1997). The African group of *Miscanthidium* and *M. fuscus* are also paraphyletic in relation to *Miscanthus*, and although Clayton and Renvoize (1986) place them morphologically closer to *Miscanthus*, ITS sequence data would indicate that they should remain separate.

When the results of the ITS sequence were analysed by distance analysis a dendrogram with very similar topology to the parsimony tree was produced. This indicates that the data is robust and that there is a strong phylogenetic signal in the data. Using different methods of phylogenetic reconstruction does not significantly influence the topology of the resulting trees. Comparison of distance analysis of the sequence data by NJ with the AFLP data by NJ is largely congruent and this aspect of the analysis is discussed further in chapter six.

Hodkinson *et al.* (1997) had suggested the use of molecular data to examine the taxonomy and phylogeny of the genus. Prior to this, the use of key characters for the identification of the species was the extent, but it failed to identify and take account of the range of variation within the individual species. To assess this variation a morphological analysis of the genus was carried out. This involved a comparison of the broader relationships of the members to other genera within the family, the *Miscanthus* complex and the relationships of the Asian and African species of the genus. As previously mentioned, no prior assumptions had been made as to the classification system was suggested and for this reason characters were analysed in terms of their utility of classification system to be assessed. However, it did not prove to be a useful tool to bi-state morphological characters in *Miscanthus* due to allow a classification to be taken. For this reason, only systems based on phenetic analysis of the characters in relation to the morphology of the genus.

Multivariate analysis indicated that a morphological assessment of the genus was a powerful and useful tool in elucidating the taxonomy of *Miscanthus*. The classification of *Miscanthus* (Honda, 1933; Lee, 1964a & c; Ohwi, 1965) based on the whole plant have provided classifications of the genus, the multivariate approach is taken. A key to the five species of *Miscanthus* is available.

Chapter 6

General Discussion

Species status and morphology

What actually comprises a species is possibly one of the most difficult and much-debated issues in systematics. In general there are five major species concepts, as stated in chapter one. Which of these systems is used, is according to Sluys (1991), a case of “pluralism and pragmatism” and ultimately depends on the field of investigation of the researcher. This study set out to answer two related but fundamentally different questions. One was to provide a classification of the genus *Miscanthus*, because previous classifications were confused. The second was to provide a practical means of differentiating between the many species and varieties of *Miscanthus* being assessed for their biomass potential. In attempting to answer the first question no prior assumptions, as to which type of species concept was superior, were made. Therefore a range of different methods were examined, each of which could, in theory, be used to formulate a classification based on a particular concept.

In the case of *Miscanthus* the classification had to this point, been based on the descriptive analysis of a few key characters; although more recent work by Greef *et al.* (1997) and Hodkinson *et al.* (1997) had investigated the use of molecular data to examine the taxonomy and phylogeny of the genus. Prior to this, the use of key characters did allow the identification of the species to some extent, but it failed to identify and take into account the range of variation within the individual species. To assess this variation, a multivariate morphological assessment of the genus was carried out. This investigation also examined the broader relationships of *Miscanthus* to other genera within the *Saccharum/Miscanthus* complex and the relationship of the Asian and African species of *Miscanthus* to each other. As previously mentioned, no prior assumptions had been made as to which classification system was superior, and for this reason characters were sought which would allow a range of classification systems to be assessed. However, it did not prove possible to find enough bi-state morphological characters in *Miscanthus* to allow a cladistic or phylogenetic approach to be taken. For this reason, only systems based on phenetic analysis could be examined in relation to the morphology of the genus.

Multivariate analysis indicated that a morphological assessment alone could not provide a powerful enough tool to elucidate the taxonomy of *Miscanthus*. While previous classifications (Honda, 1930; Lee, 1964a & c; Ohwi, 1965) based on descriptive analysis of the whole plant have provided classifications of the genus, these break down when a multivariate approach is taken. A key to twelve species of *Miscanthus* is given in table 6.1.

Table 6.1 Key to twelve species of *Miscanthus* (modified from Hodkinson *et al.* 1997)

- 1. Rhizome elongate, stems spaced; glumes ciliate; lemmas awnless;.....*M. sacchariflorus*
- 1. Rhizome very short, stems forming a dense clump, glumes glabrous or hairy, lemmas awned or awnless:
 - 2. Spikelets 2-3 mm long, exceeded by long tawny hairs from the callus, anthers 2;*M. nepalensis*
 - 2. Spikelets 2.5-10 mm long, callus hairs short or long, white or pink, anthers 2 or 3:
 - 3. Inflorescence axis 3/4 -4/5 the length of the inflorescence
 - 4. Callus hairs shorter than the spikelet; lemma awnless;....*M. fuscus*
 - 4. Callus hairs as long as or longer than the spikelet, rarely shorter; lemma awned:
 - 5. Leaf blades terete;*M. junceus*
 - 5. Leaf blades linear, narrow or broad:
 - 6. Racemes 8-28 cm long;*M. floridulus*
 - 6. Racemes 5-10 cm long:
 - 7. Leaf sheaths villous;*M. ecklonii*
 - 7. Leaf sheaths pilose or glabrous:
 - 8. Callus hairs 1/4-1/2 the length of the spikelet;*M. sorghum*
 - 8. Callus hairs 1/2 to as long as the length of the spikelet;*M. violaceus*
 - 3. Inflorescence axis 1/5-1/2 the length of the inflorescence:
 - 9. Callus hairs longer than the spikelet; lemma awned;*M. sinensis*
 - 9. Callus hairs equal to or shorter than the spikelet;
 - 10. Spikelets 3.5-5.0 mm long, anthers 2;*M. nudipes*
 - 10. Spikelets 5.5-10 mm long, anthers 3:
 - 11. Awn 10-15 mm long;*M. oligostachyus*
 - 11. Awn 1.5-5 mm long or lemma awnless;*M. tinctorius*

The wide range of variation within certain species (*M. sinensis* and *M. floridulus*) and the widespread hybridisation within the genus and between *Miscanthus* and other related genera makes the above key unworkable. This widespread variation within *M. sinensis* is an area not investigated by previous workers. This indicates that previous morphological classifications are not workable as they do not take into account the extremely wide range of

variation observed in *M. sinensis*. *Miscanthus sinensis*, along with *M. floridulus*, is the most widespread species in the genus and the range of variation demonstrated here by morphological multivariate analysis has shown that the range of variation in *M. sinensis* obscures the boundaries of most other species within the genus, with the exception of *M. sacchariflorus*. *Miscanthus floridulus* did not exhibit as wide a spread of variation as *M. sinensis*. However this species is more geographically isolated than *M. sinensis* and has not been exploited by the horticultural community to the same extent as *M. sinensis*, two factors which may account for its narrower range of variation. The wide-spread of variation of *M. sinensis* which obscures the species boundaries within *Miscanthus* also leads to the breakdown of the sections as defined by Lee (1964c). The classification of *Miscanthus* according to Lee (1964c) is shown in table 6.2.

Table 6.2 Classification of *Miscanthus* according to Lee (1964c).

Section	Species
<i>Triarrhena</i>	<i>M. sacchariflorus</i>
<i>Miscanthus</i>	<i>M. sinensis</i> , <i>M. floridulus</i> , <i>M. condensatus</i>
<i>Kariyasua</i>	<i>M. oligostachyus</i> , <i>M. changii</i> , <i>M. tinctorius</i>
<i>Diandra</i>	<i>M. eulalioides</i> , <i>M. szechuanensis</i> , <i>M. nudipes</i> , <i>M. brevipilus</i> , <i>M. nepalensis</i>

Miscanthus nepalensis and *M. nudipes*, are, in this study, within the range of variation shown in *M. sinensis*, indicating that they are not morphologically distinct enough to warrant them being placed in a different section. This situation would lead to these species being included in the *M. sinensis* complex. The other species examined within the genus are supported by a multivariate morphometric approach. The fact that *M. floridulus*, *M. nepalensis* and *M. nudipes* are within the range of variation of *M. sinensis*, of section *Miscanthus*, would lead to a breakdown of the current sections of the genus and leave support for sections *Triarrhena*, *Kariyasua*, and *Miscanthus* (with the inclusion of *M. nudipes* and *M. nepalensis* in section *Miscanthus*), but would not support section *Diandra*. In relation to the other genera within the *Saccharum* complex, the results showed that multivariate analysis of *Miscanthus* and *Saccharum* supports the conclusions of Clayton & Renvoize (1986) that the distinction between these two genera is rather artificial and is only upheld for economic reasons. *Erianthus*, on the other hand appeared to be more clearly resolved than *Saccharum*, even though this is considered to be a sub-genus of *Saccharum*. The relationship between the Asian *Miscanthus* and African *Miscanthidium* species showed them to be clearly distinct, even though others (Clayton & Renvoize, 1986), believe that the splitting of these two groups is unwarranted as the characters on which this split is based

(elongate inflorescence axis and short racemes) are also a feature of the Asian species *M. fuscus*.

Intraspecific variation.

The second question to be answered was whether morphology could be used at the intraspecific level to differentiate between the various cultivars of *Miscanthus*, based on multivariate analysis. Again, this did not prove possible. The most likely explanation for this was that most of the cultivars were simply selections of *M. sinensis*. Therefore the range of variation within *M. sinensis* demonstrated by the multivariate analysis included these individuals. The analysis has, however, been useful in demonstrating the closer relationship of these individuals to *M. sinensis* more than to any other species within the genus. Rather than revise the classification of *Miscanthus* based on the above findings, it was decided to examine the finding of the RAPD, AFLP and sequence data before a final decision was made.

Random Amplified Polymorphic DNA

RAPD analysis was the first molecular approach investigated and it was anticipated that this technique would further clarify the results of the morphometric assessment and provide a practical method of identifying many of the cultivars of *Miscanthus* being assessed for their potential role in biomass production. The results of the RAPD analysis contrasted that of the morphological analysis in respect of *M. nepalensis*. This species was clearly separate from all others by RAPD analysis, although unresolvable from *M. sinensis* by morphological analysis. However, RAPD analysis was not capable of delimiting any of the other species within the genus. RAPD analysis was thus shown to be unsuitable for the resolution of the species within the genus. This finding was not particularly surprising as it has already been suggested that RAPD analysis is generally more suited to investigation of lower taxonomic levels (Karp *et al.*, 1996). It appears from this study that RAPD became too variable at higher taxonomic levels to be of use in species identification. Others, Catalan *et al.*, (1995), have found RAPD useful at the species level and have successfully utilised RAPD to produce a phylogeny for the grass genus *Brachypodium*. However, this was achieved only after sufficient polymorphism was detected among the various species within the genus and hybridisation probes against blotted amplified fragments confirmed the homology of the bands. In this analysis of *Miscanthus* however, confirmation of the band homology was not worthwhile as the bands revealed by RAPD were not suitable for the delimitation of the species. It is interesting to note that RAPD bands have been shown to be non-homologous at the tribal level in grasses (Catalan *et al* 1995), indicating the caution which should be applied to the analysis of RAPD data.

The real strength and utility of RAPD was in its ability to produce markers which could be used in cultivar identification. In this respect the system was successful in allowing the differentiation of all of the cultivar accessions housed at Trinity College Botanic Garden. The production of hybridisation probes against these markers could be the next step in providing a simple and reproducible identification system for the various genotypes being assessed for their biomass potential. In addition, the degree of genetic relatedness of the various cultivars to the two species most likely to be involved in their ancestry was also demonstrated, the distance measures between *Miscanthus x giganteus* and *M. sinensis* was greater than the distance measure between *Miscanthus x giganteus* and *M. sacchariflorus*. This could be due to the possibility that the *M. sacchariflorus* accessions used were themselves hybrids between *M. sinensis* and *M. sacchariflorus*.

Amplified Fragment Length Polymorphism.

Amplified Fragment Length Polymorphism proved to be much more suitable for the investigation of *Miscanthus* at the species level than RAPD. Like the morphological analysis, AFLP also demonstrated the wide range of variation within *M. sinensis* and showed this species to encompass the range of variation of most other species. AFLP also showed *M. sacchariflorus* to be distinct from *M. sinensis* and all other species. *Miscanthus floridulus* was also shown to be distinct from *M. sinensis*, yet morphologically this species had been unresolvable from *M. sinensis*. Both *M. nepalensis* and *M. nudipes* were grouped together and shown to be distinct by AFLP analysis, although multivariate morphological analysis had not demonstrated this distinction. The results of AFLP analysis would therefore confirm those of morphological analysis in supporting sections *Triarrhena* and *Miscanthus* Section *Diandra*, while not supported by morphological evidence, would however, also be supported by AFLP analysis. The single member of section *Kariysua*, *M. oligostachyus* which had been supported by morphological assessment was not available for analysis by AFLP.

AFLP analysis has also allowed characterisation of the cultivars of *Miscanthus*. Hodkinson *et al.* (1997) have also used AFLP data to aid the characterisation of their *Miscanthus* collection. However, as RAPD was equally capable of providing sufficient data to characterise the collection, it is suggested that RAPD be used for this purpose. RAPD is a simpler and less expensive technique and results can be obtained more rapidly. In relation to cultivars of *Miscanthus*, genetic distance measures showed *M. x giganteus* to be intermediate between *M. sacchariflorus* and *M. sinensis*, providing further confirmation that *M. x giganteus* is a hybrid involving these two species. All of the other cultivars showed closer distance measures to *M. sinensis*, a situation also revealed by RAPD analysis. The cultivar 'Gracillimus' which was shown to be the most genetically distant to *M. sinensis* by RAPD was also shown to be the most genetically distant to this species by AFLP. These results would further support the hypothesis that most of the cultivars are derived from *M. sinensis*.

DNA sequences and phylogeny.

DNA sequence information agrees with the morphological evidence in that *M. sinensis* is again shown to have a broad spread of variation. But as opposed to the morphological evidence *M. sinensis* is shown to be clearly distinct from *M. floridulus*. Previous sequence information by Hodkinson *et al.* (1997) indicated that *M. floridulus* was part of a monophyletic group, which included *M. sinensis*. However, the results of the sequence analysis conducted here indicate that *M. floridulus* forms a monophyletic group which is a sister group to *M. sinensis*. These findings are based on three individuals of *M. floridulus* collected from two islands in the south Pacific, where *M. sinensis* does not occur, and therefore represents *M. floridulus* from a fairly isolated gene pool. It is possible that *M. floridulus* from Asia has hybridised with *M. sinensis* causing its genetic and morphological profile to overlap with *M. sinensis*.

Sequence analysis supports morphological and AFLP analysis in respect to *M. sacchariflorus*, again showing it to be clearly distinct from all other species. *Miscanthus oligostachyus*, which was shown to be distinct from all other species by morphological evidence, was also supported on molecular grounds. The species *M. nepalensis*, representing section *Diandra*, which had not been supported by morphological analysis was shown to be very clearly distinct from all other species within the genus; a finding also reported by Hodkinson *et al.* (1997). This species appears to be a sister group to the monophyletic group formed by the African *Miscanthidium* species. *Miscanthus nepalensis* had also been shown to be distinct from all other species within *Miscanthus* by AFLP analysis and this clear distinction of the African species of *Miscanthus* supports the findings of the morphological analysis, that these species are clearly separate from the Asian species, even though Clayton & Renvoize, (1986) disagreed with the initial splitting of this group which was based solely on two key characters (elongate inflorescence and short racemes).

The results of the sequence analysis have allowed the phylogeny of the genus to be investigated. The results indicating that the Asian species of the genus are a monophyletic group. Within this group, all of the sections of *Miscanthus* are supported, with the exception of the inclusion of *M. floridulus* within section *Miscanthus*. This would agree with the findings of Hodkinson *et al.* (1997), with the exception of the inclusion of *M. floridulus* within the section *Miscanthus*.

The contrasting evidence of the sequence and AFLP data appears to serve as a method of exploring some of the ambiguities found in the morphological data set, rather than refuting the results of the analysis. The sequence and AFLP data has served to resolve some of the confusion which resulted from the morphological analysis. It has shown that *M. floridulus* is a distinct species but closely related to *M. sinensis*. It has also confirmed that *M. nepalensis*

is distinct from all other species within the genus and closer to the African species. Its positioning in section *Diandra* is still, however, unresolved due to the difficulty of obtaining material of the other members of this group for comparison. *Miscanthus nepalensis* has not, however, been considered for new combination status within the African *Miscanthidium* group. Bootstrap support for this combination by AFLP or DNA sequence analysis is not particularly high and as the other members of *Diandra* were not available for consideration such a combination is unwarranted. However, the suggestion of Clayton & Renvoize (1986) that the entire *Miscanthidium* genus should be placed within *Miscanthus* is rejected. The bootstrap support for *Miscanthidium* by DNA sequence analysis using distance measures has indicated that both *Miscanthidium* and *Saccharum* are clearly distinct from *Miscanthus*. Greef *et al.* (1997), used AFLP data to examine the relationship of *M. sinensis*, *M. sacchariflorus* and *M. x giganteus* and their results also indicated that these two species formed two distinct groups with *M. x giganteus* falling within the *M. sacchariflorus* group, however no other species within the genus were examined.

Parsimony analysis of the AFLP data was not entirely congruent with the sequence data. *Miscanthus floridulus* formed a separate clade to all other species and appeared to be more genetically distant to *M. sinensis* than had been shown by DNA sequence analysis. The remainder of the species within the analysis all formed one major clade with 54% bootstrap support. Again, this was much more poorly resolved than by DNA sequence analysis. Although *M. nepalensis* did appear more distant to the rest of the species within the main clade it still formed a monophyletic group, whereas sequence analysis had shown it to be paraphyletic to the rest of *Miscanthus*. As was previously mentioned, AFLP analysis is often not considered suitable for phylogeny reconstruction and the results of this analysis have confused rather than clarified the relative positions of the various species within the sections of Lee (1964).

Conclusions.

This research has been successful in resolving the confusion surrounding the classification of *Miscanthus*, in that it has provided methods of identifying genotypes being investigated for their potential as biomass crops. RAPD and AFLP analyses have provided marker systems for these genotypes and this will be valuable to breeders in the future. In relation to the general classification of the genus, *Miscanthus* comprises a group of closely related species. Multivariate morphological analysis has shown the boundaries of the individual species is obscured, probably by hybridisation. The extent of hybridisation within the genus should however be further investigated by controlled breeding experiments, even though this has already been attempted, both within this project and by Greef and Deuter (1997). The lack of success with these experiments has been due to different flowering times between the various species of the genus. However, this problem should be resolved by growing the

species in a controlled environment in order to regulate flowering. It has also demonstrated that the section *Diandra* and *Kariyasua* as defined by Lee (1964), are unworkable. AFLP data has confirmed the assessment of the genus by morphological analysis and clarified the position of *M. floridulus*, showing it to be a distinct species and a sister group to *M. sinensis*. AFLP analysis has also shown *M. nepalensis* and *M. nudipes* to be distinct from any other species within the genus. DNA sequence data confirmed the results of AFLP analysis and has further resolved the position of *M. nepalensis* as being separate from the main *Miscanthus* core group. *Miscanthus nepalensis* appears more closely allied to the African *Miscanthidium* species. Further analysis of the other members of section *Diandra* to confirm the phylogenetic position of this section is required. The related genera, *Saccharum* and *Erianthus*, are upheld by DNA sequence data. However, although ordination techniques support *Erianthus* as a separate genus, *Saccharum* is not clearly distinguished from *Miscanthus*.

In summary, this study agrees with Lees classification of sections *Miscanthus* and *Triarrhena*. Section *Diandra* is not supported and on the evidence of this study the species *M. nepalensis* and *M. nudipes* should be placed within the genus *Miscanthidium*. In addition this study does not support section *Kariyasua*, however as only one species of this section was available for analysis a final decision on the position of this section can not be made. The study agrees with the placement of the species *M. sinensis*, *M. condensatus* and *M. floridulus* within section *Miscanthus*. *Miscanthus sacchariflorus* representing section *Triarrhena* is also supported. The species *M. oligostachyus* is also supported, but the section in which it should be placed in is still unresolved.

This project has demonstrated the merits of examining different morphometric and molecular approaches in the elucidation of the genus *Miscanthus*. It has shown that while morphology is an important tool for the identification of individuals in the field, its use for the classification of the genus is limited. This will apply to any genus exhibiting a wide spread of variation. DNA fingerprinting techniques have been shown to be highly suited to the identification of cultivars. Both RAPD and AFLP will be useful in future work and have already allowed us to find markers for two of the cultivars (*M. 'Posidon'* and *M. 'Goliath'*) which have been shown to be more cold tolerant than the other cultivars examined from the living collection at Trinity College Botanic Garden. These two cultivars, which are indistinguishable morphologically, had performed well in cold tolerance experiments Clifton-Brown (1997) and were originally thought to be the same genotype. However both RAPD and AFLP have shown these two cultivars to be clearly distinct. This work now needs to be developed so that genome maps can be developed to help identify quantitative trait loci affecting agronomically important traits such as cold tolerance.

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Appendix 1: List of *Miscanthus* accessions housed at Trinity College Botanic Garden.

Abbreviations: R indicates plant material received as rhizomes, P indicates material received as a living plant and S indicates material received as seed.

Species	Source	Origin	Accompanying accession number	Trinity <i>Miscanthus</i> collection number
<i>M. sinensis</i> 'Goliath'	Liege University Botanic Garden	Mijanuro Dake, Yakushima, Japan	none	TCDS1
<i>M. sinensis</i> 'purpurescence'	Hortus Botanicus, Vacratet, Hungary	Unknown	2620 (1992)	TCDS2
<i>M. sinensis</i>	Hortus Botanicus, Vacratet, Hungary	Unknown	2316 (1993)	TCDS3
<i>M. sacchariflorus</i>	Hortus Botanicus, Vacratet, Hungary	Unknown	2314(1993)	TCDS4
<i>M. sinensis</i>	Orto Botanico, Dell University, Italy	Unknown	none	TCDS5
<i>M. sinensis</i>	Jardin Botanique de Bordeaux, France	Unknown	E1:0040/68	TCDS6
<i>M. sinensis</i>	Jardin Botanique de Bordeaux, France	Unknown	E1:0047/68	TCDS7
<i>M. sinensis</i>	University of Oxford Botanic Garden	Unknown	none	TCDS8
<i>M. sacchariflorus</i>	J. Costin Nursery, Co. Kildare, Ireland	Mountain top, Apolo, Hakkaido, Japan	CL37	TCDS9
<i>M. sinensis</i>	J. Costin Nursery, Co. Kildare, Ireland	Shoreline, Hakkaido, Japan	CL19	TCDS10
<i>M. sinensis</i>	J. Costin Nursery, Co. Kildare, Ireland	Wild collected, Japan	CL143	TCDS11
<i>M. sinensis</i>	J. Costin Nursery, Co. Kildare, Ireland	Magnolia gardens, Nagoya, Japan	CL225	TCDS12
<i>M. sinensis</i> 'Silverfeather'	Glasnevin Botanic Garden	Unknown	none	TCDR14

Species	Source	Origin	Accompanying accession number	Trinity <i>Miscanthus</i> collection number
<i>M. sacchariflorus</i>	Glasnevin Botanic Garden	Unknown	none	TCDR15
<i>M. sinensis</i> 'Variagatus'	Glasnevin Botanic Garden	Unknown	none	TCDP16
<i>M. floridulus</i>	Glasnevin Botanic Garden	Unknown	none	TCDP17
<i>M. sinensis</i> 'gracillimus'	Peter Stam nursery, Cappaquin, Co. Wexford, Ireland	Unknown	none	TCDP18
<i>M. sinensis</i>	Peter Stam nursery, Cappaquin, Co. Wexford, Ireland	Unknown	none	TCDR19
<i>M. sinensis</i> 'Zebrinus'	Newlands Garden centre, Dublin, Ireland	Unknown	none	TCDP20
<i>M. sinensis</i> 'Zebrinus'	Edinburgh Botanic Garden	Unknown	803204	TCDR21
<i>M. sacchariflorus</i>	Edinburgh Botanic Garden	Unknown	19271037	TCDP22
<i>M. floridulus</i>	College of Agriculture, University of Philippines	Mountains of Cavinti, Luguna, Philipines.	620-712	TCDR23
<i>M. sinensis</i> x <i>giganteus</i>	Marburg Botanic Garden, Germany	Unknown	none	TCDR24
<i>M. sinensis</i>	Marburg Botanic Garden, Germany	Unknown	none	TCDR25
<i>Miscanthus</i> hybrid	Vitro Plant, Germany	Hybrid 1.1 (Cross information not relaeased)	93M0007	TCDR26
<i>Miscanthus</i> hybrid	Vitro Plant, Germany	Hybrid 1.2 (Cross information not relaeased)	93M0008	TCDR27
<i>Miscanthus</i> hybrid	Vitro Plant, Germany	Hybrid 2.1 (Cross between <i>M. sinensis</i> 'variagatus' & <i>M. sinensis</i> 'Zebrinus')	92M0110	TCDR28
<i>Miscanthus</i> hybrid	Vitro Plant, Germany	Hybrid 2.2 (Cross between two <i>M.</i> <i>sinensis</i> forms with high biomass yield)	92M0179	TCDR29
<i>M. sinensis</i> 'Grossefontane'	Piccoplant, Germany	Cultivated	none	TCDR30

Species	Source	Origin	Accompanying accession number	Trinity <i>Miscanthus</i> collection number
<i>M. sinensis</i> 'Variagatus'	Norfolk Lavander Garden Centre	Unknown	none	TCDR33
<i>M. sinensis</i> x <i>giganteus</i>	Danish Institute of Landscape plants	Unknown	none	TCDR34
<i>M. sinensis</i> x <i>giganteus</i>	Piccoplant, Germany	Micropropegated from culture	none	TCDR36
<i>M. sinensis</i>	Rosewarne Gardens, Cornwall, England	Unknown	RW1-1	TCDP49
<i>M. sinensis</i>	Rosewarne Gardens, Cornwall, England	Unknown	RW1-2	TCDP50
<i>M. sinensis</i> x <i>giganteus</i>	Piccoplant, Germany	Micropropegated	none	TCDP37
<i>M. sinensis</i>	University of Palermo, Italy	Unknown	none	TCDP38
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Morioka, Iwate, Japan	R2	TCDR39
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Nasudake, Tochigi, Japan	R4	TCDR40
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Nasudake, Tochigi, Japan	R6	TCDR41
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Nasudake, Tochigi, Japan	R7	TCDR42
<i>M. sinensis</i> 'Silverfeather'	Potsdam Botanic Garden, Germany	Unknown	none	TCDR43
<i>M. sinensis</i>	Potsdam Botanic Garden, Germany	Unknown	none	TCDR44
<i>M. sacchariflorus</i>	Potsdam Botanic Garden, Germany	Unknown	none	TCDR45
<i>M. sinensis</i>	Palermo Botanic Gardens, Italy	Unknown	none	TCDR46
<i>M. sinensis</i>	Palermo Botanic Gardens, Italy	Unknown	none	TCDR47

Species	Source	Origin	Accompanying accession number	Trinity <i>Miscanthus</i> collection number
<i>M. sinensis</i>	Danish Institute of Plant Science, Hornum, Denmark	Unknown	none	TCDS48
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW51	TCDP51
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW5-2	TCDP52
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW5-3	TCDP53
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW8-1	TCDP54
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW8-2	TCDP55
<i>M. sacchariflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW12-1	TCDP56
<i>M. Ssacchariflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW14-1	TCDP57
<i>M. sacchariflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW14-2	TCDP58
<i>M. sacchariflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW14-3	TCDP59
<i>M. sacchariflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW14-4	TCDP60
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW24-1	TCDP61
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW24-2	TCDP62
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW30-1	TCDP63
<i>M. sinensis</i>	Rosewarne gardens, Cornwall, England	Unknown	RW30-2	TCDP64

Species	Source	Origin	Accompanying accession number	Trinity <i>Miscanthus</i> collection number
<i>M. saccharflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW129-1	TCDP65
<i>M. saccharflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW129-8	TCDP66
<i>M. saccharflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW130-1	TCDP67
<i>M. saccharflorus</i>	Rosewarne gardens, Cornwall, England	Unknown	RW130-2	TCDP68
<i>M. sinensis</i>	Danish Institute of Plant Science	Unknown	MS90-1	TCDP69
<i>M. sinensis</i>	Danish Institute of Plant Science	Unknown	MS90-3	TCDP70
<i>M. sinensis</i>	Danish Institute of Plant Science	Unknown	MS90-6	TCDP71
<i>M. sinensis</i>	Danish Institute of Plant Science	Unknown	MS90-8	TCDP72
<i>M. sinensis</i>	Danish Institute of Plant Science	Unknown	MS90-11	TCDP73
<i>M. sinensis</i>	Danish Institute of Plant Science	Unknown	MS88-110	TCDP74
<i>M. sinensis</i>	Danish Institute of Plant Science	Unknown	MS88-111	TCDP75
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Aso, Kumamoto, Japan	R1	TCDR76
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Morioka, Iwate	R2	TCDR77
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Senbonmatso, Tochigi, Japan	R3	TCDR78
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Nasudake, Tochigi, Japan	R4	TCDR79

Species	Source	Origin	Accompanying accession number	Trinity <i>Miscanthus</i> collection number
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Shiobara, Tochigi, Japan	R5	TCDR80
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Ohda, Shimane, Japan	R6	TCDR81
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Sapporo, Hokkaido, Japan	R7	TCDR82
<i>M. sinensis</i>	Faculty of Agriculture, Miyazaki University, Japan	Narugo, Miyagi, Japan	R8	TCDR83
<i>M. sacchariflorus</i>	Glasnevin Botanic Garden	Unknown	none	TCDP84
<i>M. sinensis</i>	Glasnevin Botanic Garden	Unknown	none	TCDP85
<i>M. sinensis</i> x <i>giganteus</i>	Glasnevin Botanic Garden	Unknown	none	TCDP86
<i>M. sacchariflorus</i>	Glasnevin Botanic Garden	Unknown	none	TCDP87
<i>M. sacchariflorus</i>	Glasnevin Botanic Garden	Unknown	none	TCDP88
<i>M. sinensis</i> 'Silverfeather'	Glasnevin Botanic Garden	Unknown	none	TCDP89
<i>M. sinensis</i> 'Variagatus'	Glasnevin Botanic Garden	Unknown	none	TCDP90
<i>M. sacchariflorus</i>	Glasnevin Botanic Garden	Unknown	none	TCDP91
<i>M. sinensis</i> 'Goliath'	Piccoplant, Germany	Cultivated	none	TCDP92
<i>M. sacchariflorus</i>	Royal Botanic Garden, Kew, England	Unknown	1969-19097	TCDP93
<i>M. condensatus</i>	Royal Botanic Garden, Kew, England	Unknown	1969-19091	TCDP94
<i>M. sinensis</i> 'Silverfeather'	Royal Botanic Garden, Kew, England	Unknown	1969-19093	TCDP95
<i>M. sinensis</i> 'Zebrinus'	Royal Botanic Garden, Kew, England	Unknown	1969-19096	TCDP96
<i>M. sinensis</i>	Royal Botanic Garden, Kew, England	Unknown	1978-1389	TCDP97

Species	Source	Origin	Accompanying accession number	Trinity <i>Miscanthus</i> collection number
<i>M. sinensis</i> 'Gracillimus'	Royal Botanic Garden, Kew, England	Unknown	1969-19098	TCDP98
<i>M. sinensis</i> 'Rotsilber'	Royal Botanic Garden, Kew, England	Unknown	none	TCDP99
<i>M. sinensis</i> 'Undine'	Royal Botanic Garden, Kew, England	Unknown	1988-2516	TCDP100
<i>M. floridulus</i>	Royal Botanic Garden, Kew, England	Unknown	1978-1387	TCDP101
<i>M. sinensis</i> 'Silberspine'	Royal Botanic Garden, Kew, England	Unknown	1988-2519	TCDP102
<i>M. sinensis</i> Variagatus'	Royal Botanic Garden, Kew, England	Unknown	1969-19093	TCDP103
<i>M. sinensis</i>	Royal Botanic Garden, Kew, England	Unknown	1976-108	TCDP104
<i>M. transmorrisonensis</i>	Royal Botanic Garden, Kew, England	Taiwan, Tai Chung County	1990-2748	TCDp105

Appendix 2. List of all herbarium material examined by morphological analysis

No.	Specimen label	Herbarium	Sheet	Country	Collector	Date collected.	Determinavit
1.01	<i>M. sacchariflorus</i>	Edinburgh	102/92-27	Honshu, Japan	M. Togashi	27/10/1984	Hackel
1.02	<i>M. sacchariflorus</i>	Edinburgh	102/92-	China	Hackel		Hackel
1.03	<i>M. sacchariflorus</i>	Edinburgh	102/92-40	China			Hackel
1.04	<i>M. sacchariflorus</i>	Edinburgh	102/92-19		Hackel		Hackel
1.05	<i>M. sacchariflorus</i>	Edinburgh	102/92-6	Honshu, Japan	M. Togashi	10/10/1973	
1.06	<i>M. sacchariflorus</i>	Edinburgh	102/92-55	Tokyo, Japan	T. Makino	1/10/1936	J. Ohwi
1.07	<i>M. sacchariflorus</i>	Edinburgh	102/92-61	China			Hackel
1.08	<i>M. sacchariflorus</i>	Edinburgh	102-92-17				
1.09	<i>M. sacchariflorus</i>	Edinburgh	102/92-28	Japan		13/9/1910	
1.10	<i>M. sacchariflorus</i>	Kew	H1999/00/00				
1.11	<i>M. sacchariflorus</i>	Edinburgh	102/92-22	Japan			
2.01	<i>M. sinensis</i>	Edinburgh	102/92-21	Swatow, HongKong			
3.01	<i>M. sinensis</i>	Edinburgh	102/92-22	Taiwan		15/11/1932	
3.02	<i>M. sinensis</i>	Edinburgh	102/92-19	Korea		3/10/1908	
3.03	<i>M. sinensis</i>	Edinburgh	102/92-16	Korea		3/10/1908	
3.04	<i>M. sinensis</i>	Edinburgh	102/92-24	Korea		17/08/1908	
3.05	<i>M. sinensis</i>	Edinburgh	102/92-29	Hwei-yang, China	W.T. Tsang	1/10/1935	E.D. Merrill
3.06	<i>M. sinensis</i>	Edinburgh	102/92-3				
3.07	<i>M. sinensis</i>	Edinburgh	102/92-31	Korea			
3.08	<i>M. sinensis</i>	Edinburgh	102/92-12	Kiangsu, China	D'argy		Hackel
3.09	<i>M. sinensis</i>	Edinburgh	102/92-7	Honshu, Japan		13/9/1963	G. Murta
3.10	<i>M. sinensis</i>	Edinburgh	102/92-13			7/9/1906	
3.11	<i>M. sinensis</i>	Edinburgh	102/92-23				
3.12	<i>M. sinensis</i>	Edinburgh	102/92-27				
3.13	<i>M. sinensis</i>	Edinburgh	102/92-15	Hwei-yang, China	W.T. Tsang	11/8/1935	E.D. Merrill
3.14	<i>M. sinensis</i>	Edinburgh	102/92-	Kweichow, China	A.N. Steward	10/7/1931	

No.	Specimen label	Herbarium	Sheet	Country	Collector	Date collected.	Determinavit
3.15	<i>M. sinensis</i>	Edinburgh	102/92-4	Kiangsi, China	H.C.Cheo	23/9/1932	
3.16	<i>M. sinensis</i>	Edinburgh	102/92-30	Kwantung, China	T.O.Kang	22/5/1924	
3.17	<i>M. sinensis</i>	Edinburgh	102/92-19	Chekiang, China	C.Y. Chiao	11/8/1927	E.D. Merrill
3.18	<i>M. sinensis</i>	Edinburgh	102/92-26	Honshu, Japan	N. Oya	12/10/1972	M. Tamurn
4.01	<i>M. nudipes</i>	Edinburgh	102/92- 23	Szechuan	K.L.Chu	17/9/1936	
5.01	<i>M. sinensis var condensatus</i>	Edinburgh	102/92-34	Oshima, Japan	M. Mizushima	22/9/1922	
5.02	<i>M. sinensis</i>	Edinburgh	102/92-36	Tokyo, Japan		9/9/1908	
5.03	<i>M. sinensis</i>	Edinburgh	102/92-35	Tokyo, Japan		9/1907	
5.04	<i>M. sinensis</i>	Edinburgh	109/92-17	Chihli, China	M.S. Clemens	13/10/1913	
6.01	<i>M. sinensis</i>	Edinburgh	109/92-30	N.W. Himalayas		28/8/1890	
6.02	<i>M. sinensis</i>	Edinburgh	109/92-32	India			Hackel
6.03	<i>M. sinensis</i>	Edinburgh	109/92-3	N.W. Himalayas		27/8/1890	
6.04	<i>M. sinensis</i>	Edinburgh	102/92-38				
7.01	<i>M. japonicus</i>	Paris	95/72	Riou-Kiou, Japan	R.P. Ferrie	16/6/1900	
7.02	<i>M. japonicus</i>	Paris	95/72-	Japan	R.P. Ferrie		
7.03	<i>M. japonicus</i>	Paris	95/72(1763)	Tonkin			
7.04	<i>M. japonicus</i>	Paris	95/72	Tai hoku, Japan		11/1914	
7.05	<i>M. japonicus</i>	Paris	95/72	Hokuto, Japan		9/1914	
7.06	<i>M. japonicus</i>	Paris	95/72 (492)			11/1885	
7.07	<i>M. japonicus</i>	Paris	95/72 (5062)		M. Petlot	9/1927	
7.08	<i>M. japonicus</i>	Paris	95/72 (2222)	Chuang, IndoChina		7/1/1916	
7.09	<i>M. japonicus</i>	Paris	95/72 (2099)	Konha Kou, Laos	Caifane	14/10/1920	
7.10	<i>M. japonicus</i>	Paris	95/72			5/1909	
7.11	<i>M. japonicus</i>	Paris	95/72 (999)	Hong Kong	M. l'Abbe Faurie		
7.12	<i>M. sinensis</i>	TCD	93				
8.01	<i>Saccharum sp</i>	TCD					
8.02	<i>Saccharum sp</i>	TCD					
8.03	<i>M. sacchariflorus</i>	Paris	95/72 (3008)	Hien yang, China		30/10/1916	
8.04	<i>M. sacchariflorus</i>	Paris	95/72 (5583)	Japan	M. l'Abbe Faurie	10/1904	
9.01	<i>M. sinensis</i>	Paris	95/72 (3565)	Phu Luang, Thailand		9/1/1966	
9.02	<i>M. sinensis</i>	Paris	95/72 (4696)	Montagnes, Japan	M. l'Abbe Faurie	3/1/1890	

No.	Specimen label	Herbarium	Sheet	Country	Collector	Date collected.	Determinavit
9.03	<i>M. condensatus</i>	Paris	95/72 (911)	Tchen-keou-tin China	M. l'Abbe Faurie		
9.04	<i>M. sinensis</i>	Paris	95/72 (130)	Ming valley, China		2/11/1908	
9.05	<i>M. sinensis</i>	Paris	95/72 (1501)	Japan	M. l'Abbe Faurie	24/3/1887	
10.01	<i>M. formosanus</i>	Paris	95/72 (704)	Formosa (Taiwan)		2/1904	
11.01	<i>M. sinensis var condensatus</i>	Paris	95/72 (1601)	Honshu, Japan	Maruyama	29/10/1957	
12.01	<i>M. purpurescens</i>	Paris	95/72 (6642)	Fujiyama, Japan	M. l'Abbe Faurie	30/12/1890	
12.02	<i>M. purpurescens</i>	Paris	95/72	Japan	M. l'Abbe Faurie	17/11/1887	
12.03	<i>M. purpurescens</i>	Paris	95/72 (1730)	Japan	M. l'Abbe Faurie	1898	
12.04	<i>M. purpurescens</i>	Paris	95/72	Korea	M. l'Abbe Faurie	28/9/1901	
12.05	<i>M. purpurescens</i>	Paris	95/72 (1582)	Kiaoshan, China		24/8/1922	Handel-Mezzetti
13.01	<i>M. japonicus</i>	Paris	95/72 (1008)	Japan		1887	A. Camus
13.02	<i>M. japonicus</i>	Paris	95/72 (13202)	Japan		5/9/1834	
13.03	<i>M. japonicus</i>	Paris	95/72 (1763)	Japan		8/10/1888	
13.04	<i>M. japonicus</i>	Paris	95/72 (147)	Japan		10/1914	
14.01	<i>S. officinarum</i>	TCD	66	Martinique		1884	
14.02	<i>S. officinarum</i>	TCD	173	Savanna Mts Mauritius	Parnell,Cronc/WJ	30/6/85	J Gehu
14.03	<i>S. officinarum</i>	TCD	(8134)	Pak TongChai, Korea	AFG Kerr		
14.04	<i>S. spontaneum</i>	TCD	(2158)	Chiangmai	AFG Kerr		
14.05	<i>S. arundinaceum</i>	TCD	(900)		AFG Kerr	5/12/1909	
14.06	<i>Erianthus sp</i>	TCD	(2148)			1854	
15.01	<i>M. floridulus</i>	Kew	H1999/00/00 21	New Guinea	L.J. Brass	22/10/1959	
15.02	<i>M. floridulus</i>	Kew	H1999/00/00 20	Central Prov. Sulawesi	S.Soenarko		
15.03	<i>M. floridulus</i>	Kew	H1999/00/00 19	South Sulawesi	M.J. Sands	10/11/69	S. Renvoize
16.01	<i>M. tinctorius</i>	Kew	H1999/00/00 5	Shimojo, Honshu, Japan	M. Muramatsu	13/10/1957	
17.01	<i>Miscanthidium ecklonii</i>	Kew	H1999/00/00 11	Natal, S. Africa	E.R. Robinson	16/2/67	J.J.Moll
17.02	<i>Miscanthidium grossweilii</i>	Kew	H1999/00/00 18	N. Rhodesia			
17.03	<i>Miscanthidium tetrafolium</i>	Kew	H1999/00/00 9	N. Rhodesia	M. Richards	28/3/1957	
17.04	<i>Miscanthidium junceum</i>	Kew	H1999/00/00 15	Transvaal, S. Africa	G. Hemm	3/3/78	
18.01	<i>M. nepalensis</i>	Kew	H1999/00/00 17				
18.02	<i>M. nepalensis</i>	Kew	H1999/00/00 17				
18.03	<i>M. nepalensis</i>	Edinburgh					

No.	Specimen label	Herbarium	Sheet	Country	Collector	Date collected.	Detminavit.
18.04	<i>M. nepalensis</i>	Edinburgh					
18.05	<i>M. nepalensis</i>	Edinburgh					
19.01	<i>M. 'Rotsilber'</i>	TCD	TCDP99	RBG Kew			
19.02	<i>M. 'Silverfeather'</i>	TCD	TCDP89	NBG Glasnevin			
19.03	<i>M. 'Silberspine'</i>	TCD	TCDP102	NBG Glasnevin			
19.04	<i>M. 'Posidon'</i>	TCD	TCDR32	NBG Glasnevin			
19.05	<i>M. 'Zebrinus'</i>	TCD	TCD21	RBG Edinburgh			
19.06	<i>M. x giganteus</i>	TCD	TCDP34	Piccoplant, Germany			
19.07	<i>M. x giganteus</i>	TCD	TCDP34(2)	Piccoplant, Germany			
19.08	<i>M. x giganteus</i>	TCD	TCDP37	Piccoplant, Germany			
20.01	<i>Erianthus sp</i>	TCD	TCD8000	India	J.D.H		
20.02	<i>Erianthus sp</i>	TCD	TCD8000	India	J.D.H		
20.03	<i>Erianthus sp.</i>	TCD	TCD8000	India			

Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
<i>M. sacchariflorus</i>	1.01	0	0.0	9.5	0.0	2.5	5.2	1	1	3.4	8.5	4.7	5.0	1.2	1.0	3.9	5.0	2.9
<i>M. sacchariflorus</i>	1.02	0	0.0	8.0	0.0	2.5	5.0	0	1	0.0	8.5	4.7	5.0	1.2	1.0	3.9	5.0	2.5
<i>M. sacchariflorus</i>	1.03	0	0.0	8.0	0.0	2.5	5.0	0	1	0.0	9.5	5.2	5.0	1.3	1.2	4.0	4.2	2.0
<i>M. sacchariflorus</i>	1.04	0	0.0	8.2	0.0	2.8	4.8	1	1	3.0	8.0	4.5	5.1	1.0	1.0	4.5	5.1	2.6
<i>M. sacchariflorus</i>	1.05	0	0.0	9.5	0.0	2.5	5.0	1	1	3.0	6.0	5.0	4.8	1.0	1.1	5.0	4.4	2.5
<i>M. sacchariflorus</i>	1.06	0	0.0	11.0	0.0	5.5	8.5	1	1	3.0	6.0	4.2	5.1	1.1	1.3	3.8	3.9	2.6
<i>M. sacchariflorus</i>	1.07	0	0.0	10.0	0.0	2.5	5.0	0	1	0.0	7.5	4.2	5.0	0.9	1.2	4.7	4.2	2.7
<i>M. sacchariflorus</i>	1.08	0	0.0	9.4	0.0	2.4	5.3	0	1	0.0	6.5	4.3	4.1	1.3	0.8	3.3	5.1	2.5
<i>M. sacchariflorus</i>	1.09	0	0.0	8.5	0.0	2.8	4.2	1	1	8.0	8.0	5.0	5.0	0.8	1.0	6.3	5.0	3.0
<i>M. sacchariflorus</i>	1.10	0	0.0	12.0	0.0	3.5	5.5	1	1	8.0	8.0	5.0	4.4	0.7	0.6	7.1	7.3	2.2
<i>M. sinensis</i>	2.01	0	0.0	3.0	5.5	2.5	3.2	1	1	5.0	5.5	4.0	4.0	1.2	1.0	3.3	4.0	3.5
<i>M. sinensis</i> var <i>formosanus</i>	3.01	1	7.5	6.0	0.0	1.0	2.0	0	0	0.0	0.0	3.5	3.5	1.2	1.2	2.9	2.9	2.5
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	3.02	1	9.2	6.7	0.0	2.0	4.0	1	1	1.8	3.5	5.6	5.6	1.5	1.7	3.7	3.3	4.5
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	3.03	1	12.0	7.3	0.0	3.0	6.0	1	1	1.7	3.0	5.5	5.7	1.2	1.3	4.6	4.4	4.0
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	3.04	1	8.8	6.0	0.0	1.0	3.0	1	1	1.0	2.1	5.7	5.4	1.5	1.4	3.8	3.9	3.0
<i>M. sinensis</i>	3.05	1	8.7	5.7	0.0	1.5	3.5	1	1	0.5	2.2	4.2	4.5	1.1	1.1	3.8	4.1	3.0
<i>M. sinensis</i>	3.06	1	9.0	6.0	0.0	1.7	7.0	0	0	0.0	0.0	4.5	5.0	1.2	1.0	3.8	5.0	4.1
<i>M. sinensis</i>	3.07	1	9.0	4.1	0.0	1.5	3.5	1	1	1.2	1.2	5.3	5.0	1.3	1.4	4.1	3.6	3.3
<i>M. sinensis</i>	3.08	1	9.7	6.7	0.0	1.5	3.5	0	0	0.0	0.0	4.9	5.2	1.4	1.3	3.5	4.0	3.8
<i>M. sinensis</i>	3.09	1	7.8	5.0	0.0	1.5	3.5	0	1	0.0	1.0	4.1	4.0	1.1	1.0	3.7	4.0	3.0
<i>M. sinensis</i>	3.10	1	7.2	7.0	0.0	1.5	3.5	1	0	1.5	0.0	4.5	4.5	1.4	1.2	3.2	3.8	3.5
<i>M. sinensis</i>	3.11	1	11.1	7.4	0.0	2.5	7.5	1	1	3.0	3.8	6.5	6.6	1.2	1.5	5.4	4.4	5.0
<i>M. sinensis</i>	3.12	1	7.0	6.5	0.0	1.8	3.5	0	0	0.0	0.0	3.0	3.4	1.3	1.2	2.3	2.8	2.3
<i>M. sinensis</i>	3.13	1	6.5	5.3	0.0	1.0	3.0	1	1	0.5	1.5	4.2	4.5	1.1	1.3	3.8	3.5	4.3
<i>M. sinensis</i>	3.14	1	5.5	5.2	0.0	1.0	3.5	0	0	0.0	0.0	3.5	3.7	1.2	1.1	2.9	3.4	2.5
<i>M. sinensis</i>	3.15	1	9.0	5.2	0.0	1.5	4.5	1	1	0.9	1.1	5.1	5.0	1.2	1.5	4.3	3.3	3.7
<i>M. sinensis</i>	3.16	1	6.2	3.6	0.0	1.4	4.5	0	0	0.0	0.0	2.2	2.0	0.7	0.6	3.1	3.3	1.5
<i>M. sinensis</i>	3.17	1	6.5	5.0	0.0	1.2	2.7	0	0	0.0	0.0	2.8	3.4	0.9	1.1	3.1	3.1	2.2
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	3.18	1	7.5	7.0	0.0	0.8	2.0	1	1	1.5	1.5	5.0	4.9	1.2	1.2	4.2	4.1	2.9
<i>M. nudipes</i> (Szechuan)	4.01	1	9.0	8.0	0.0	1.5	2.5	1	1	8.0	8.0	2.2	2.0	0.9	0.9	2.4	2.2	1.9
<i>M. sinensis</i> (<i>M. condensatus</i>)	5.01	1	13.0	5.5	0.0	1.0	3.5	1	1	3.0	3.0	5.0	5.3	2.0	1.5	2.5	3.5	3.0
<i>M. sinensis</i> (<i>M. condensatus</i>)	5.02	1	13.5	7.0	0.0	1.0	3.0	1	1	1.5	2.0	4.2	4.0	1.1	1.0	3.8	4.0	3.5
<i>M. sinensis</i> (<i>M. condensatus</i>)	5.03	1	11.0	6.5	0.0	1.0	3.0	1	1	2.0	2.0	4.0	4.2	1.2	1.2	3.3	3.5	3.2
<i>M. sinensis</i> (<i>M. condensatus</i>)	5.04	1	8.5	5.5	0.0	3.0	7.4	1	1	1.7	1.5	5.2	5.4	1.1	1.0	4.7	5.4	4.0
<i>Saccharum</i> (India)	6.01	1	9.7	8.0	3.0	0.0	2.4	0	1	0.0	2.3	3.1	3.0	1.0	1.1	3.1	2.7	3.5
<i>Saccharum</i> (India)	6.02	1	10.0	9.7	2.0	0.0	2.5	0	1	0.0	2.0	2.9	3.0	0.9	1.0	3.2	3.0	3.5
<i>Saccharum</i> (India)	6.03	1	10.0	10.0	7.0	0.0	2.4	0	1	0.0	1.8	3.0	3.0	1.2	1.3	2.5	2.3	3.5
<i>Saccharum</i> (India)	6.04	1	9.0	9.0	2.6	0.0	2.5	0	1	0.0	2.0	3.0	2.8	1.0	0.9	3.0	3.1	2.7

Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.01	1	8.7	4.0	0.0	1.5	3.5	0	0	0.0	0.0	3.5	3.0	1.0	1.0	3.5	3.0	2.5
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.02	1	9.4	6.0	0.0	1.0	2.5	0	0	0.0	0.0	3.4	3.4	1.0	1.0	3.4	3.4	2.6
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.04	1	7.3	5.3	0.0	1.0	3.0	0	0	0.0	0.0	3.2	3.5	1.0	1.0	3.2	3.5	2.3
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.05	1	14.0	7.0	0.0	1.0	2.5	1	1	2.0	2.5	4.7	5.0	1.2	1.5	3.9	3.3	4.0
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.06	1	8.5	4.5	0.0	2.0	4.5	0	0	0.0	0.0	3.5	3.5	1.0	1.2	3.5	2.9	3.0
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.07	1	7.5	7.5	0.0	2.0	4.0	0	0	0.0	0.0	4.0	4.4	1.3	1.4	3.1	3.1	3.2
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.08	1	6.0	3.7	0.0	2.0	4.0	0	0	0.0	0.0	5.1	4.5	0.7	0.8	7.3	5.6	2.7
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.09	1	5.0	4.5	0.0	1.5	3.0	0	0	0.0	0.0	3.7	3.7	1.0	1.1	3.7	3.4	3.0
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.10	1	7.8	5.0	0.0	1.0	4.0	0	0	0.0	0.0	3.7	4.0	1.1	1.0	3.4	4.0	2.7
<i>M. sinensis</i> (<i>M. japonicus</i>)	7.11	1	10.5	6.0	0.0	1.0	3.0	0	0	0.0	0.0	2.8	3.4	1.0	1.0	2.8	3.4	1.9
<i>M. sacchariflorus</i>	8.01	0	0.0	12.5	0.0	1.5	4.0	1	1	3.0	5.0	5.0	5.5	1.0	0.9	5.0	6.1	3.0
<i>M. sacchariflorus</i>	8.02	0	0.0	10.0	0.0	1.5	3.0	1	1	2.0	9.0	5.0	5.0	1.0	0.9	5.0	5.6	3.4
<i>M. sacchariflorus</i>	8.03	0	0.0	10.0	0.0	2.0	5.0	1	1	2.0	9.0	3.5	3.5	0.9	0.9	3.9	3.9	3.4
<i>M. sacchariflorus</i>	8.04	0	0.0	12.0	0.0	2.0	4.5	1	1	2.5	9.0	4.5	3.9	1.1	1.2	4.1	3.3	3.2
<i>Saccharum</i>	9.01	1	19.0	7.5	0.0	0.0	4.7	0	0	0.0	0.0	4.0	4.0	1.0	1.0	4.0	4.0	3.3
<i>M. sinensis</i>	9.02	1	7.5	6.3	0.0	1.0	2.5	0	1	0.0	1.5	4.2	4.2	1.0	1.0	4.2	4.2	3.2
<i>M. sinensis</i> (<i>variagatus</i>)	9.03	1	8.9	5.5	0.0	1.5	3.5	0	0	0.0	0.0	5.4	5.4	1.2	1.3	4.5	4.2	4.0
<i>M. sinensis</i>	9.04	1	7.5	5.4	0.0	1.0	3.0	0	0	0.0	0.0	4.5	4.0	1.2	1.2	3.8	3.3	4.0
<i>M. sinensis</i>	9.05	1	7.6	7.0	0.0	1.5	2.0	1	1	1.5	1.5	4.0	3.9	1.1	1.3	3.6	3.0	2.2
<i>M. sinensis</i> (<i>M. formosanus</i>)	10.01	1	4.0	4.0	0.0	0.5	1.4	0	0	0.0	0.0	2.6	2.4	1.0	0.9	2.6	2.7	1.9
<i>M. condensatus</i>	11.01	1	11.5	5.5	0.0	0.5	2.5	1	1	2.0	2.0	4.0	4.2	1.2	1.2	3.3	3.5	3.0
<i>M. sinensis</i> (<i>M. purpurescens</i>)	12.01	1	10.0	5.0	0.5	2.2	4.2	1	1	3.5	3.5	5.5	6.0	1.7	1.5	3.2	4.0	5.0
<i>M. sinensis</i> (<i>M. purpurescens</i>)	12.02	1	6.8	5.0	0.0	1.7	4.0	0	0	0.0	0.0	3.7	3.6	1.2	1.2	3.1	3.0	2.3
<i>M. sinensis</i> (<i>M. purpurescens</i>)	12.03	1	6.0	3.0	0.3	2.0	4.0	1	1	2.5	2.5	5.7	5.7	2.1	2.2	2.7	2.6	3.0
<i>M. sinensis</i> (<i>M. purpurescens</i>)	12.04	1	15.0	6.5	0.0	3.0	5.0	1	1	2.0	3.5	5.0	4.8	1.2	1.3	4.2	3.7	3.9
<i>M. sinensis</i> (<i>M. purpurescens</i>)	12.05	1	13.0	7.5	0.0	2.0	5.0	1	1	4.5	5.0	4.6	4.8	1.5	1.5	3.1	3.2	4.0
<i>M. sinensis</i> (<i>M. japonicus</i>)	13.01	1	6.6	4.5	0.0	1.3	3.0	0	0	0.0	0.0	4.0	3.7	1.0	1.2	4.0	3.1	3.4
<i>M. sinensis</i> (<i>M. japonicus</i>)	13.02	1	7.8	7.5	0.0	0.8	2.5	0	0	0.0	0.0	3.7	3.6	1.1	1.3	3.4	2.8	3.0
<i>M. sinensis</i> (<i>M. japonicus</i>)	13.03	1	8.0	6.0	0.0	2.0	4.0	0	0	0.0	0.0	4.2	4.4	1.2	1.2	3.5	3.7	2.6
<i>M. sinensis</i> (<i>M. japonicus</i>)	13.04	1	7.5	6.0	0.0	0.4	2.4	0	0	0.0	0.0	3.1	3.7	1.0	1.2	3.1	3.1	1.7
<i>S. officinarum</i>	14.01	0	0.0	8.5	8.5	0.0	4.0	0	0	0.0	0.0	2.1	2.2	1.1	1.2	1.9	1.8	2.5
<i>S. officinarum</i>	14.02	0	0.0	11.0	0.0	0.0	3.3	0	0	0.0	0.0	3.9	3.9	1.1	1.2	3.5	3.3	3.9
<i>S. narenga</i>	14.03	0	0.0	2.5	1.0	0.0	3.5	0	1	0.0	1.5	3.0	3.3	1.0	1.1	3.0	3.0	3.0
<i>S. spontaneum</i>	14.04	0	0.0	14.0	11.5	0.0	4.6	0	0	0.0	0.0	3.6	3.9	0.9	1.0	4.0	3.9	2.0
<i>S. arundinaceum</i>	14.05	0	0.0	5.0	4.5	0.0	6.2	1	1	5.0	5.0	3.2	3.2	0.9	0.9	3.6	3.6	2.9
<i>S. infirmum</i>	14.06	0	0.0	8.0	0.0	0.0	4.7	0	0	0.0	0.0	3.0	2.9	1.0	0.9	3.0	3.2	2.8
<i>M. x giganteus</i> P37	15.02	1	1.5	11.0	0.0	1.0	3.8	1	1	3.0	9.0	4.5	5.0	1.2	1.2	3.8	4.2	2.5
<i>M. x giganteus</i> P37	15.03	1	1.5	11.5	0.0	1.5	3.5	1	1	4.5	4.5	4.7	5.0	1.4	1.3	3.4	3.8	3.4

Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	plant	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
<i>M. x giganteus P34</i>	15.04	0	0.0	8.0	0.0	1.5	3.5	0	1	0.0	8.0	5.0	4.0	1.0	1.0	5.0	4.0	3.0
<i>M. 'Zebrinus'</i>	16.01	1	9.0	5.0	0.0	1.5	4.0	0	0	0.0	0.0	4.8	5.0	1.2	1.2	4.0	4.2	3.8
<i>M. 'Silverfeather'</i>	16.02	1	8.3	8.0	0.0	1.2	4.0	1	1	1.5	2.3	5.1	5.0	1.5	1.2	3.4	4.2	3.4
<i>M. 'Rotsilber'</i>	16.03	1	9.0	7.8	0.0	1.1	3.9	1	1	1.8	4.2	5.2	5.5	1.1	1.8	4.7	3.1	3.9
<i>M. 'Posidon'</i>	16.04	1	9.5	7.0	0.0	2.0	4.0	0	1	0.0	3.5	5.4	6.0	1.2	1.8	4.5	3.3	3.5
<i>M. 'Silverfeather'</i>	16.05	1	9.4	8.0	0.0	0.9	3.0	1	1	1.0	1.5	5.0	4.9	1.3	1.4	3.8	3.5	3.0
<i>M. 'silberspine'</i>	16.06	1	8.5	5.0	0.0	2.0	5.0	1	1	2.0	4.0	5.0	4.7	1.2	1.4	4.2	3.4	3.0
<i>Miscanthidium junceum</i>	17.01	1	4.0	2.7	0.0	2.5	4.0	1	1	2.0	2.0	5.0	4.8	1.2	1.1	4.2	4.4	3.5
<i>Miscanthidium grossweilein</i>	17.02	1	4.0	2.5	0.0	2.0	4.0	1	1	2.0	2.0	4.0	4.0	1.0	1.0	4.0	4.0	3.5
<i>Miscanthidium tetrefolium</i>	17.03	1	3.5	3.5	0.0	3.0	8.0	1	1	3.5	3.5	5.0	4.8	1.2	1.3	4.2	3.7	4.5
<i>Miscanthidium. ecklonii</i>	18.01	1	6.0	2.0	0.0	3.0	5.5	1	1	2.0	2.0	3.5	2.8	1.0	1.0	3.5	2.8	2.5
<i>M. floridulus</i>	19.01	1	8.3	7.0	0.0	2.0	4.0	0	0	0.0	0.0	3.4	3.1	0.9	0.9	3.8	3.4	3.0
<i>M. floridulus</i>	19.02	1	8.0	6.5	0.0	2.5	4.0	0	0	0.0	0.0	3.5	3.0	1.0	0.9	3.5	3.3	2.9
<i>M. floridulus</i>	19.03	1	9.0	5.8	0.0	2.5	4.0	0	0	0.0	0.0	2.5	1.5	1.0	0.5	2.5	3.0	2.4
<i>M. oligostachys</i>	20.01	1	12.0	8.0	0.0	2.0	5.5	1	1	3.0	3.0	6.7	6.7	1.6	1.6	4.2	4.2	4.2
<i>M. oligostachys</i>	20.02	1	10.0	7.0	0.0	2.0	5.5	1	1	3.0	3.0	7.2	7.2	1.8	1.8	4.0	4.0	4.5
<i>M. nudipes</i>	21.01	1	5.0	4.0	0.0	1.0	3.0	1	1	2.5	2.5	5.0	4.2	1.2	1.2	4.2	3.5	4.2
<i>M. tinctorius</i>	22.01	0	0.0	2.0	0.0	1.5	2.0	1	1	1.0	1.0	6.0	5.5	1.8	1.6	3.3	3.4	4.7
<i>M. nepalensis</i>	23.01	1	14.0	8.5	0.0	2.0	4.5	0	1	0.0	2.5	2.0	2.0	0.8	0.8	2.5	2.5	2.0
<i>M. nepalensis</i>	23.02	1	16.0	8.0	0.0	2.0	3.2	0	1	0.0	2.2	2.5	2.5	0.6	0.9	4.2	2.8	2.2
<i>M. nepalensis</i>	23.03	1	17.0	8.5	0.0	2.5	4.2	0	1	0.0	2.5	2.8	2.6	0.8	0.8	3.5	3.3	2.4
<i>M. nepalensis</i>	23.04	1	13.0	8.0	0.0	3.0	4.5	0	1	0.0	4.0	2.7	2.6	0.9	0.8	3.0	3.3	2.4
<i>M. nepalensis</i>	23.05	1	12.0	8.0	0.0	1.6	3.1	0	1	0.0	3.0	2.8	2.8	1.0	1.0	2.8	2.8	2.7
<i>M. nudipes</i>	24.01	1	4.0	1.2	0.0	1.8	3.5	1	1	3.0	3.0	4.2	4.2	1.0	1.0	4.2	4.2	4.2

Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
<i>M. sacchariflorus</i>	0.5	5.8	3.4	0.6	5.7	2.0	0.3	6.7	0.4	0.3	1.3	21.0	0.5	42.0	14	0.0	1	1
<i>M. sacchariflorus</i>	0.4	6.3	3.0	0.5	6.0	1.0	0.3	3.3	0.4	0.2	2.0	12.5	0.2	62.5	22	0.0	0	0
<i>M. sacchariflorus</i>	0.5	4.0	3.6	0.5	7.2	1.1	0.3	3.7	0.4	0.3	1.3	10.0	0.3	33.3	25	0.0	0	1
<i>M. sacchariflorus</i>	0.4	6.5	2.2	0.6	3.7	0.7	0.2	3.5	0.6	0.5	1.2	7.0	1.0	7.0	17	0.0	1	1
<i>M. sacchariflorus</i>	0.3	8.3	2.7	0.5	5.4	1.3	0.4	3.3	0.5	0.3	1.7	12.0	0.5	24.0	18	0.0	1	1
<i>M. sacchariflorus</i>	0.4	6.5	2.5	0.6	4.2	1.3	0.3	4.3	0.5	0.2	2.5	8.0	0.5	16.0	19	0.0	0	0
<i>M. sacchariflorus</i>	0.4	6.8	2.5	0.5	5.0	1.5	0.3	5.0	0.5	0.3	1.7	9.0	0.4	22.5	16	0.0	0	0
<i>M. sacchariflorus</i>	0.4	6.3	3.0	0.5	6.0	0.4	0.3	1.3	0.5	0.2	2.5	15.0	0.7	21.4	24	0.0	0	1
<i>M. sacchariflorus</i>	0.6	5.0	3.0	0.5	6.0	1.0	0.5	2.0	0.7	0.4	1.8	19.0	1.0	19.0	26	0.0	0	1
<i>M. sacchariflorus</i>	0.5	4.4	2.0	0.4	5.0	0.6	0.2	3.0	0.5	0.4	1.3	17.0	1.0	17.0	11	0.0	0	0
<i>M. sinensis</i>	0.7	5.0	3.5	1.0	3.5	1.8	0.5	3.6	0.7	0.4	1.8	4.0	0.4	10.0	15	0.0	0	1
<i>M. sinensis</i> var <i>formosanus</i>	0.7	3.6	3.1	0.8	3.9	1.3	0.3	4.3	0.2	0.2	1.0	7.0	1.8	3.9	0	0.0	0	1
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	0.8	5.6	4.4	1.1	4.0	0.9	0.5	1.8	0.7	0.5	1.4	6.5	0.3	21.7	20	0.0	1	1
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	0.8	5.0	4.4	0.8	5.5	0.9	0.4	2.3	0.6	0.3	2.0	7.0	1.5	4.7	17	0.0	1	1
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	0.7	4.3	3.5	0.8	4.4	0.8	0.6	1.3	0.4	0.2	2.0	8.0	0.8	10.0	44	0.0	0	1
<i>M. sinensis</i>	0.9	3.3	3.3	0.7	4.7	1.6	0.3	5.3	0.6	0.3	2.0	7.0	0.5	14.0	34	0.0	0	1
<i>M. sinensis</i>	0.6	6.8	4.3	0.5	8.6	0.6	0.2	3.0	0.4	0.2	2.0	7.0	0.5	14.0	30	0.0	0	1
<i>M. sinensis</i>	0.6	5.5	3.6	0.6	6.0	1.7	0.3	5.7	0.6	0.3	2.0	5.0	1.0	5.0	36	0.0	1	1
<i>M. sinensis</i>	0.8	4.8	4.0	0.9	4.4	2.0	0.5	4.0	0.5	0.3	1.7	3.5	0.4	8.8	46	0.0	1	1
<i>M. sinensis</i>	0.6	5.0	3.4	1.1	3.1	1.0	0.4	2.5	0.6	0.3	2.0	6.0	1.5	4.0	16	0.0	0	1
<i>M. sinensis</i>	0.9	3.9	3.5	0.8	4.4	1.0	0.3	3.3	0.5	0.5	1.0	10.0	1.0	10.0	21	0.0	0	1
<i>M. sinensis</i>	0.7	7.1	5.1	0.8	6.4	1.0	0.3	3.3	0.7	0.6	1.2	9.0	1.0	9.0	22	0.0	1	1
<i>M. sinensis</i>	0.6	3.8	2.6	0.8	3.3	0.9	0.2	4.5	0.4	0.2	2.0	5.5	1.0	5.5	34	0.0	1	0
<i>M. sinensis</i>	0.6	7.2	2.4	0.9	2.7	0.6	0.3	2.0	0.5	0.2	2.5	10.0	1.2	8.3	17	0.0	1	1
<i>M. sinensis</i>	0.9	2.8	3.0	0.7	4.3	0.9	0.4	2.3	0.5	0.3	1.7	9.0	1.5	6.0	21	0.0	1	0
<i>M. sinensis</i>	1.0	3.7	4.0	0.9	4.4	1.0	0.5	2.0	0.6	0.5	1.2	9.0	1.0	9.0	20	0.0	0	0
<i>M. sinensis</i>	0.7	2.1	2.0	0.7	2.9	1.1	0.3	3.7	0.4	0.2	2.0	7.0	0.8	8.8	13	0.0	0	1
<i>M. sinensis</i>	0.5	4.4	2.6	0.7	3.7	0.6	0.2	3.0	0.3	0.3	1.0	11.0	2.0	5.5	20	0.0	0	1
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	0.7	4.1	4.0	0.8	5.0	0.5	0.3	1.7	0.3	0.3	1.0	6.5	0.6	10.8	21	0.0	1	1
<i>M. nudipes</i> (Szechuan)	0.8	2.4	1.9	0.6	3.2	1.0	0.4	2.5	0.3	0.3	1.0	6.0	0.5	12.0	50	0.0	0	1
<i>M. sinensis</i> (<i>M. condensatus</i>)	0.6	5.0	3.9	1.2	3.3	3.0	0.4	7.5	1.0	0.6	1.7	17.0	1.0	17.0	8	0.0	0	1
<i>M. sinensis</i> (<i>M. condensatus</i>)	0.6	5.8	3.0	0.9	3.3	0.8	0.4	2.0	0.6	0.4	1.5	8.0	0.4	20.0	13	0.0	0	1
<i>M. sinensis</i> (<i>M. condensatus</i>)	0.6	5.3	3.6	1.0	3.6	2.0	0.4	5.0	0.9	0.4	2.3	6.0	0.4	15.0	22	0.0	0	1
<i>M. sinensis</i> (<i>M. condensatus</i>)	0.7	5.7	3.9	0.9	4.3	1.0	0.5	2.0	0.5	0.6	0.8	8.0	2.0	4.0	23	1.0	1	1
<i>Saccharum</i> (India)	0.2	17.5	3.5	1.0	3.5	0.4	0.2	2.0	0.3	0.2	1.5	12.0	0.5	24.0	25	0.0	0	1
<i>Saccharum</i> (India)	0.2	17.5	3.5	1.0	3.5	0.6	0.2	3.0	0.4	0.2	2.0	10.0	0.6	16.7	29	1.0	0	1
<i>Saccharum</i> (India)	0.2	17.5	3.0	0.5	6.0	0.4	0.2	2.0	0.5	0.4	1.3	12.0	0.5	24.0	16	0.0	0	1
<i>Saccharum</i> (India)	0.1	27.0	2.8	0.4	7.0	0.8	0.5	1.6	0.5	0.5	1.0	10.0	0.3	33.3	20	0.0	0	1

Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
<i>M. sinensis (M. japonicus)</i>	0.8	3.1	2.5	0.8	3.1	1.0	0.3	3.3	0.4	0.3	1.3	15.0	2.0	7.5	11	0.0	0	1
<i>M. sinensis (M. japonicus)</i>	0.8	3.3	2.5	0.7	3.6	1.5	0.3	5.0	0.5	0.4	1.3	16.0	2.0	8.0	14	0.0	0	1
<i>M. sinensis (M. japonicus)</i>	0.5	4.6	2.8	0.6	4.7	0.3	0.2	1.5	0.4	0.2	2.0	18.0	0.4	45.0	10	0.0	0	1
<i>M. sinensis (M. japonicus)</i>	1.0	4.0	4.0	1.1	3.6	2.0	0.3	6.7	0.5	0.3	1.7	10.0	1.5	6.7	14	0.0	0	0
<i>M. sinensis (M. japonicus)</i>	0.6	5.0	3.0	0.9	3.3	1.6	0.3	5.3	0.4	0.3	1.3	7.0	1.5	4.7	14	0.0	1	1
<i>M. sinensis (M. japonicus)</i>	1.0	3.2	3.7	0.9	4.1	2.0	0.6	3.3	0.4	0.3	1.3	16.0	2.0	8.0	14	0.0	0	0
<i>M. sinensis (M. japonicus)</i>	0.6	4.5	3.7	0.6	6.2	1.5	0.4	3.8	0.4	0.3	1.3	11.5	1.5	7.7	16	0.0	0	1
<i>M. sinensis (M. japonicus)</i>	0.6	5.0	2.8	0.8	3.5	2.5	0.3	8.3	0.4	0.3	1.3	5.5	1.0	5.5	39	0.0	1	1
<i>M. sinensis (M. japonicus)</i>	0.7	3.9	3.0	0.8	3.8	1.0	0.4	2.5	0.4	0.3	1.3	8.0	1.5	5.3	14	0.0	1	1
<i>M. sinensis (M. japonicus)</i>	0.6	3.2	2.2	0.7	3.1	0.5	0.2	2.5	0.4	0.3	1.3	14.0	2.0	7.0	0	0.0	0	0
<i>M. sacchariflorus</i>	0.5	6.0	4.1	0.5	8.2	1.8	0.2	9.0	0.5	0.3	1.7	6.3	1.0	6.3	28	0.0	0	1
<i>M. sacchariflorus</i>	0.5	6.8	3.8	0.6	6.3	2.0	0.3	6.7	0.5	0.2	2.5	10.0	0.3	33.3	19	0.0	0	1
<i>M. sacchariflorus</i>	0.6	5.7	3.4	0.6	5.7	1.5	0.3	5.0	0.3	0.2	1.5	17.0	0.2	85.0	15	0.0	0	1
<i>M. sacchariflorus</i>	0.4	8.0	3.5	0.7	5.0	2.2	0.2	11.0	0.5	0.3	1.7	13.0	0.8	16.3	22	0.0	0	1
<i>Saccharum</i>	0.8	4.1	3.8	0.7	5.4	1.8	0.5	3.6	0.5	0.2	2.5	14.0	0.3	46.7	11	0.0	0	1
<i>M. sinensis</i>	0.6	5.3	3.9	0.9	4.3	2.7	0.4	6.8	0.4	0.5	0.8	7.0	1.5	4.7	28	0.0	1	1
<i>M. sinensis (variagatus)</i>	0.9	4.4	4.1	1.0	4.1	2.4	0.6	4.0	0.4	0.4	1.0	8.0	2.0	4.0	20	0.0	0	1
<i>M. sinensis</i>	0.6	6.7	3.8	0.8	4.8	0.9	0.3	3.0	0.3	0.3	1.0	4.0	0.3	13.3	44	0.0	1	1
<i>M. sinensis</i>	1.0	2.2	2.2	1.1	2.0	0.7	0.6	1.2	0.5	0.3	1.7	18.0	0.5	36.0	30	0.0	1	0
<i>M. sinensis (M. formosanus)</i>	0.4	4.8	2.0	0.5	4.0	0.7	0.5	0.0	0.0	0.0	0.0	17.0	1.2	14.2	17	0.0	0	0
<i>M. condensatus</i>	0.7	4.3	3.0	0.6	5.0	2.9	0.3	9.7	0.5	0.4	1.3	12.0	1.0	12.0	18	0.0	0	0
<i>M. sinensis (M. purpurescens)</i>	1.2	4.2	5.0	1.0	5.0	3.9	0.6	6.5	0.4	0.5	0.8	8.0	0.1	80.0	40	0.0	0	1
<i>M. sinensis (M. purpurescens)</i>	0.8	2.9	2.7	0.6	4.5	1.5	0.5	3.0	0.4	0.4	1.0	18.0	1.0	18.0	34	0.0	1	1
<i>M. sinensis (M. purpurescens)</i>	1.0	3.0	4.7	2.0	2.4	3.5	0.6	5.8	0.7	0.5	1.4	15.0	0.4	37.5	70	1.0	1	1
<i>M. sinensis (M. purpurescens)</i>	0.9	4.3	4.5	0.8	5.6	1.6	0.4	4.0	0.4	0.4	1.0	4.0	0.2	20.0	24	0.0	1	1
<i>M. sinensis (M. purpurescens)</i>	0.9	4.4	4.3	1.5	2.9	1.8	0.3	6.0	0.4	0.4	1.0	7.5	1.0	7.5	20	0.0	1	1
<i>M. sinensis (M. japonicus)</i>	0.5	6.8	3.4	0.8	4.3	1.4	0.4	3.5	0.4	0.3	1.3	10.0	0.8	12.5	28	0.0	0	1
<i>M. sinensis (M. japonicus)</i>	0.6	5.0	2.5	0.8	3.1	1.8	0.8	2.3	0.3	0.3	1.0	16.0	1.0	16.0	32	0.0	0	1
<i>M. sinensis (M. japonicus)</i>	0.6	4.3	3.0	0.8	3.8	1.0	0.3	3.3	0.3	0.1	3.0	22.0	2.0	11.0	22	0.0	0	1
<i>M. sinensis (M. japonicus)</i>	0.7	2.4	2.7	0.7	3.9	1.0	0.2	5.0	4.0	2.0	2.0	16.0	0.5	32.0	11	0.0	0	0
<i>S. officinarum</i>	0.8	3.1	2.6	0.7	3.7	1.9	0.1	19.0	0.5	0.3	1.7	23.0	1.0	23.0	16	0.0	0	1
<i>S. officinarum</i>	0.4	9.8	2.8	0.7	4.0	0.6	0.3	2.0	0.5	0.4	1.3							
<i>S. narenga</i>	0.5	6.0	2.6	0.6	4.3	1.6	0.5	3.2	0.5	0.3	1.7	6.0	0.5	12.0	13	0.0	0	1
<i>S. spontaneum</i>	0.2	10.0	2.6	0.8	3.3	0.8	0.4	2.0	0.4	0.2	2.0	6.0	0.5	12.0	12	0.0	0	1
<i>S. arundinaceum</i>	0.8	3.6	2.6	0.9	2.9	1.7	0.5	3.4	0.5	0.4	1.3	19.0	1.0	19.0	15	0.0	0	1
<i>S. infirmum</i>	1.0	2.8	2.6	0.8	3.3	1.3	0.3	4.3	0.4	0.4	1.0	10.0	0.5	20.0	16	0.0	0	1
<i>M. x giganteus P37</i>	0.5	5.0	3.5	0.6	5.8	2.0	0.5	4.0	4.0	4.0	1.0	5.0	0.5	10.0	18	0.0	1	1
<i>M. x giganteus P37</i>	0.9	3.8	3.4	1.0	3.4	1.5	0.5	3.0	0.6	0.4	1.5	6.5	0.5	13.0	22	0.0	0	1

Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
<i>M. x giganteus P34</i>	0.8	3.8	3.2	0.5	6.4	1.8	0.5	3.6	0.3	0.2	1.5	8.0	1.0	8.0	16	0.0	0	1
<i>M. 'Zebrinus'</i>	0.9	4.2	4.0	0.7	5.7	1.0	0.4	2.5	0.4	0.4	1.0	9.0	1.0	9.0	32	0.0	1	1
<i>M. 'Silverfeather'</i>	1.2	2.8	4.5	1.2	3.8	1.7	0.6	2.8	0.4	0.5	0.8	11.0	1.0	11.0	35	0.0	1	0
<i>M. 'Rotsilber'</i>	0.8	4.9	3.9	0.8	4.9	1.5	0.4	3.8	0.3	0.3	1.0	7.5	1.5	5.0	21	0	1	1
<i>M. 'Posidon'</i>	0.8	4.4	4.0	0.9	4.4	1.1	0.4	2.8	0.5	0.3	1.7	4.0	0.3	13.3	27	0.0	1	1
<i>M. 'Silverfeather'</i>	1.0	3.0	5.5	0.8	6.9	1.4	0.5	2.8	0.4	0.3	1.3	7.0	1.5	4.7	20	0.0	1	0
<i>M. 'silberspine'</i>	0.8	3.8	4.5	1.3	3.5	2.7	0.4	6.8	0.3	0.3	1.0	2.5	1.0	2.5	20	0.0	0	1
<i>Miscanthidium junceum</i>	0.6	5.8	3.5	0.6	5.8	2.5	0.4	6.3	0.3	0.3	1.0	10.0	0.5	20.0	33	0.0	1	1
<i>Miscanthidium grossweilein</i>	0.5	7.0	3.7	0.6	6.2	2.0	0.3	6.7	0.2	0.2	1.0	12.0	0.5	24.0	30	0.0	1	1
<i>Miscanthidium tetrefolium</i>	0.6	7.5	4.3	0.5	8.6	1.6	0.3	5.3	0.3	0.2	1.5	7.0	1.0	7.0	27	0.0	1	1
<i>Miscanthidium. ecklonii</i>	0.5	5.0	2.3	0.5	4.6	1.8	0.5	3.6	0.3	0.2	1.5	7.0	0.6	11.7	22	0.0	1	1
<i>M. floridulus</i>	0.4	7.5	2.8	0.5	5.6	0.3	0.2	1.5	0.2	0.2	1.0	23.0	1.0	23.0	42	0.0	0	1
<i>M. floridulus</i>	0.5	5.8	2.5	0.5	5.0	0.3	0.3	1.0	0.2	0.2	1.0	13.0	1.8	7.2	39	0.0	0	1
<i>M. floridulus</i>	1.0	2.4	2.4	0.6	4.0	0.3	0.2	1.5	0.2	0.2	1.0	17.0	3.0	5.7	15	0.0	0	1
<i>M. oligostachys</i>	0.8	5.3	6.5	1.0	6.5	3.4	0.5	6.8	0.4	0.3	1.3	9.0	0.2	45.0	57	0.0	0	1
<i>M. oligostachys</i>	0.8	5.6	6.8	1.0	6.8	3.8	0.5	7.6	0.4	0.3	1.3	9.0	0.2	45.0	60	0.0	0	1
<i>M. nudipes</i>	0.6	7.0	4.4	0.8	5.5	4.2	0.7	6.0	0.2	0.2	1.0	4.0	0.5	8.0	50	1.0	1	1
<i>M. tinctorius</i>	1.5	3.1	4.4	1.4	3.1	4.0	0.6	6.7	0.2	0.2	1.0	10.0	1.0	10.0	56	0.0	0	1
<i>M. nepalensis</i>	0.7	2.9	2.0	0.8	2.5	1.0	0.5	2.0	0.2	0.2	1.0	12.0	1.0	12.0	20	0.0	0	1
<i>M. nepalensis</i>	0.8	2.8	2.2	0.8	2.8	1.4	0.6	2.3	0.3	0.2	1.5	10.0	0.8	12.5	33	0.0	0	1
<i>M. nepalensis</i>	0.7	3.4	2.4	0.7	3.4	1.0	0.4	2.5	0.3	0.2	1.5	10.0	1.0	10.0	25	0.0	0	1
<i>M. nepalensis</i>	0.8	3.0	2.5	0.6	4.2	1.0	0.5	2.0	0.3	0.2	1.5	6.0	1.0	6.0	39	0.0	1	1
<i>M. nepalensis</i>	0.7	3.9	2.7	0.8	3.4	1.2	0.4	3.0	0.3	0.2	1.5	8.0	0.8	10.0	30	0.0	0	1
<i>M. nudipes</i>	1.0	4.2	4.2	1.0	4.2	2.3	0.7	3.3	0.2	0.2	1.0	4.0	0.4	10.0	43	0.0	0	1

Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	36	37	38	39	40	41	42
<i>M. sacchariflorus</i>	2.5	0	70.0	250.0	180.0	0.72	3
<i>M. sacchariflorus</i>	0.0	0	110.0	310.0	200.0	0.65	3
<i>M. sacchariflorus</i>	2.8	0	55.0	210.0	155.0	0.74	3
<i>M. sacchariflorus</i>	1.2	0	105.0	290.0	185.0	0.64	3
<i>M. sacchariflorus</i>	2.0	0	90.0	310.0	220.0	0.71	3
<i>M. sacchariflorus</i>	0.0	0	100.0	350.0	250.0	0.71	3
<i>M. sacchariflorus</i>	0.0	0	130.0	325.0	195.0	0.60	3
<i>M. sacchariflorus</i>	2.5	0	95.0	250.0	155.0	0.62	3
<i>M. sacchariflorus</i>	2.5	0	120.0	225.0	105.0	0.47	3
<i>M. sacchariflorus</i>	0.0	0	50.0	290.0	240.0	0.83	3
<i>M. sinensis</i>	2.0	0	210.0	300.0	90.0	0.30	3
<i>M. sinensis</i> var <i>formosanus</i>	1.5	0	60.0	250.0	190.0	0.76	3
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	2.0	0	0.0	170.0	170.0	1.00	3
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	4.5	0	0.0	175.0	175.0	1.00	5
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	2.2	0	0.0	100.0	100.0	1.00	5
<i>M. sinensis</i>	1.6	0	65.0	245.0	180.0	0.73	3
<i>M. sinensis</i>	8.0	1	350.0	570.0	220.0	0.39	3
<i>M. sinensis</i>	0.8	0	30.0	130.0	100.0	0.77	3
<i>M. sinensis</i>	4.5	0	50.0	270.0	220.0	0.81	3
<i>M. sinensis</i>	5.5	0	90.0	260.0	170.0	0.65	3
<i>M. sinensis</i>	4.0	0	60.0	230.0	170.0	0.74	3
<i>M. sinensis</i>	2.5	0	120.0	270.0	150.0	0.56	3
<i>M. sinensis</i>	0.0	0	180.0	315.0	135.0	0.43	3
<i>M. sinensis</i>	2.0	0	50.0	165.0	115.0	0.70	3
<i>M. sinensis</i>	0.0	0	115.0	270.0	155.0	0.57	3
<i>M. sinensis</i>	0.0	0	30.0	210.0	180.0	0.86	3
<i>M. sinensis</i>	2.0	0	140.0	280.0	140.0	0.50	3
<i>M. sinensis</i>	1.0	0	340.0	390.0	50.0	0.13	3
<i>M. oligostachyus</i> (<i>M. sinensis</i>)	4.0	0	0.0	160.0	160.0	1.00	5
<i>M. nudipes</i> (Szechuan)	1.5	0	20.0	95.0	75.0	0.79	3
<i>M. sinensis</i> (<i>M. condensatus</i>)	0.5	0	90.0	250.0	160.0	0.64	3
<i>M. sinensis</i> (<i>M. condensatus</i>)		1	100.0	300.0	200.0	0.67	3
<i>M. sinensis</i> (<i>M. condensatus</i>)	2.0	0	50.0	215.0	165.0	0.77	3
<i>M. sinensis</i> (<i>M. condensatus</i>)	1.3	0	15.0	170.0	155.0	0.91	3
<i>Saccharum</i> (India)	3.5	1	290.0	290.0	0.0	0.00	3
<i>Saccharum</i> (India)	3.0	1	260.0	260.0	0.0	0.00	3
<i>Saccharum</i> (India)	3.5	1	240.0	240.0	0.0	0.00	3
<i>Saccharum</i> (India)	1.5	1	100.0	180.0	80.0	0.44	3

Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	36	37	38	39	40	41	42
<i>M. sinensis</i> (<i>M. japonicus</i>)	1.5	0	240.0	280.0	40.0	0.14	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	1.5	0	300.0	395.0	95.0	0.24	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	2.0	0	290.0	360.0	70.0	0.19	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	0.0	0	310.0	430.0	120.0	0.28	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	4.0	1	300.0	420.0	120.0	0.29	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	0.0	0	240.0	350.0	110.0	0.31	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	3.0	0	160.0	300.0	140.0	0.47	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	1.5	1	190.0	330.0	140.0	0.42	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	2.5	0	310.0	390.0	80.0	0.21	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	0.0	0	320.0	430.0	110.0	0.26	3
<i>M. sacchariflorus</i>	4.0	0	25.0	185.0	160.0	0.86	3
<i>M. sacchariflorus</i>	3.5	0	30.5	230.0	199.5	0.87	3
<i>M. sacchariflorus</i>	0.8	0	15.0	380.0	365.0	0.96	3
<i>M. sacchariflorus</i>	1.5	0	0.0	170.0	170.0	1.00	3
<i>Saccharum</i>	1.0	0	0.0	190.0	190.0	1.00	3
<i>M. sinensis</i>	3.8	0	20.0	170.0	150.0	0.88	3
<i>M. sinensis</i> (<i>variagatus</i>)	5.0	0	90.0	230.0	140.0	0.61	3
<i>M. sinensis</i>	2.0	1	120.0	220.0	100.0	0.45	3
<i>M. sinensis</i>	0.0	0	70.0	140.0	70.0	0.50	3
<i>M. sinensis</i> (<i>M. formosanus</i>)	0.0	0	280.0	370.0	90.0	0.24	3
<i>M. condensatus</i>	0.0	0	80.0	200.0	120.0	0.60	5
<i>M. sinensis</i> (<i>M. purpurescens</i>)	1.5	0	0.0	75.0	75.0	1.00	3
<i>M. sinensis</i> (<i>M. purpurescens</i>)	5.0	0	70.0	210.0	140.0	0.67	3
<i>M. sinensis</i> (<i>M. purpurescens</i>)	1.5	0	0.0	90.0	90.0	1.00	3
<i>M. sinensis</i> (<i>M. purpurescens</i>)	5.0	0	30.0	200.0	170.0	0.85	3
<i>M. sinensis</i> (<i>M. purpurescens</i>)	3.5	0	50.0	220.0	170.0	0.77	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	2.0	0	155.0	230.0	75.0	0.33	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	3.0	0	65.0	135.0	70.0	0.52	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	2.0	0	70.0	210.0	140.0	0.67	3
<i>M. sinensis</i> (<i>M. japonicus</i>)	0.0	0	210.0	360.0	150.0	0.42	3
<i>S. officinarum</i>	3.0	0	430.0	430.0	0.0	0.00	3
<i>S. officinarum</i>		0	111.0	250.0	139.0	0.56	3
<i>S. narenga</i>	4.5	1	350.0	350.0	0.0	0.00	3
<i>S. spontaneum</i>	5.0	1	440.0	440.0	0.0	0.00	3
<i>S. arundinaceum</i>	4.0	0	340.0	340.0	0.0	0.00	3
<i>S. infirmum</i>	7.0	0	300.0	300.0	0.0	0.00	3
<i>M. x giganteus</i> P37	2.0	0	70.0	260.0	190.0	0.73	3
<i>M. x giganteus</i> P37	5.0	0	70.0	290.0	220.0	0.76	3

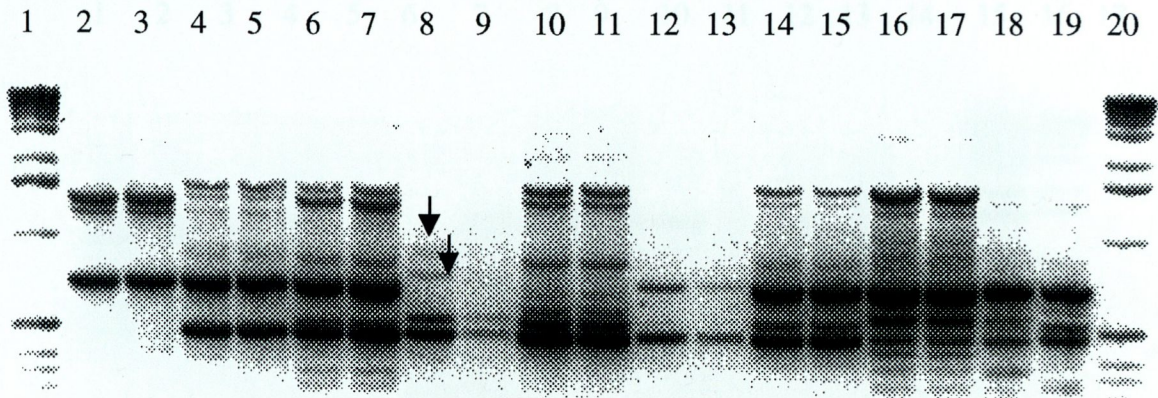
Appendix 3: Data matrix for all characters scored for morphological analysis. Key to character codes is given on last page of appendix.

species	36	37	38	39	40	41	42
<i>M. x giganteus</i> P34	2.5	0	70.0	220.0	150.0	0.68	3
<i>M. 'Zebrinus'</i>	3.5	0	90.0	280.0	190.0	0.68	3
<i>M. 'Silverfeather'</i>	0.0	0	40.0	210.0	170.0	0.81	3
<i>M. 'Rotsilber'</i>	2.0	0	180	300	120.0	0.40	3
<i>M. 'Posidon'</i>	5.0	0	60.0	180.0	120.0	0.67	3
<i>M. 'Silverfeather'</i>	0.0	0	50.0	240.0	190.0	0.79	3
<i>M. 'silberspine'</i>	3.0	0	50.0	200.0	150.0	0.75	3
<i>Miscanthidium junceum</i>	2.5	0	40.0	40.0	0.0	0.00	3
<i>Miscanthidium grosswelein</i>	2.0	0	60.0	60.0	0.0	0.00	3
<i>Miscanthidium tetrefolium</i>	2.0	0	40.0	40.0	0.0	0.00	3
<i>Miscanthidium. ecklonii</i>	2.5	1	40.0	40.0	0.0	0.00	3
<i>M. floridulus</i>	2.5	0	150.0	270.0	120.0	0.44	3
<i>M. floridulus</i>	3.0	0	150.0	240.0	90.0	0.38	3
<i>M. floridulus</i>	3.0	0	35.0	340.0	305.0	0.90	3
<i>M. oligostachys</i>	3.0	0	0.0	150.0	150.0	1.00	5
<i>M. oligostachys</i>	3.0	0	0.0	85.0	85.0	1.00	5
<i>M. nudipes</i>	3.5	1	20.0	90.0	70.0	0.78	6
<i>M. tinctorius</i>	3.0	0	0.0	18.0	18.0	1.00	4
<i>M. nepalensis</i>	2.6	1	90.0	220.0	130.0	0.59	3
<i>M. nepalensis</i>	2.8	1	70.0	200.0	130.0	0.65	3
<i>M. nepalensis</i>	2.3	1	60.0	210.0	150.0	0.71	3
<i>M. nepalensis</i>	2.6	1	90.0	190.0	100.0	0.53	3
<i>M. nepalensis</i>	2.0	1	50.0	170.0	120.0	0.71	3
<i>M. nudipes</i>	3.4	0	120.0	230.0	110.0	0.48	6

Key to character codes used in appendix 3

1. Awn present or absent
2. Awn length
3. Callus hair length
4. Pedicel; hair length
5. Short pedicel length
6. Long pedicel length
7. Upper glume hair present or absent
8. Lower glume hair present or absent
9. Upper glume hair length
10. Lower glume hair length
11. Upper glume length
12. Lower glume length
13. Upper glume width
14. Lower glume width
15. Ratio upper glume length to width
16. Ratio Lower glume length to width
17. Upper lemma length
18. Upper lemma width
19. Ratio upper lemma length to width
20. Lower lemma length
21. Lower lemma width
22. Ratio lower lemma length to width
23. Palea length
24. Palea width
25. Ratio of palea length to width
26. Lodicule Length
27. Lodicule width
28. Ratio lodicule length to width
29. Leaf width
30. Leaf mid vein width
31. Ratio leaf width to mid vein width
32. Leaf margin spines per 10mm of margin
33. Abaxial leaf hairs present or absent
34. Adaxial leaf hairs present or absent
35. Ligule hairs present or absent
36. Ligule hair length
37. Inflorescence hairs present or absent
38. Inflorescence axis length
39. Inflorescence total length
40. Ratio inflorescence axis length to total length
41. Character 39/axis total length
42. Number of nerves on Upper glume

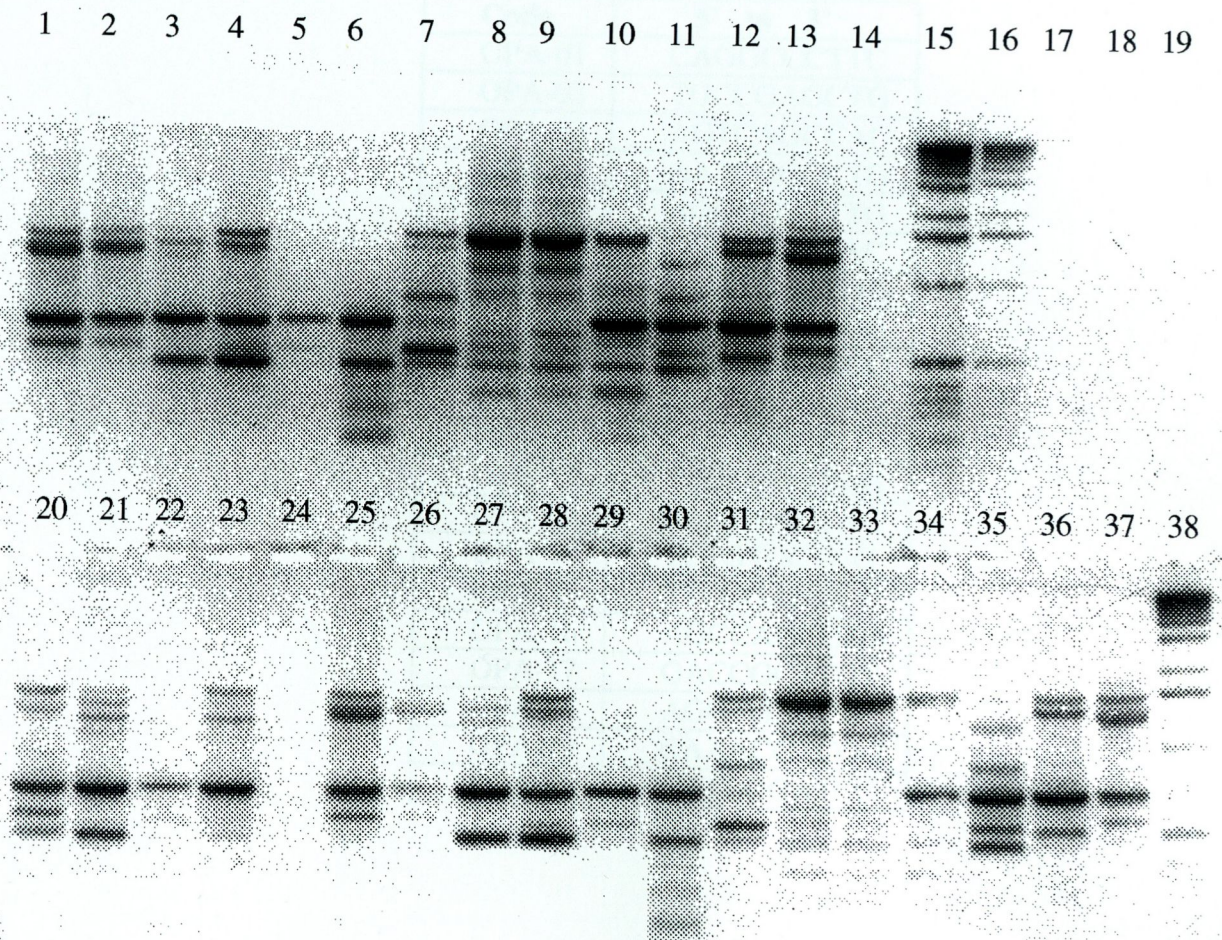
Appendix 4a. Example of RAPD banding patterns obtained using primer OPA-16
 Lane 8 shows an example of weak bands (indicated by arrows) which would have been ignored
 As they are absent in the replicate sample in lane 9.



Key to lanes:

- Lane 1 1KB DNA size ladder
- Lane 2 & 3 *M. transmorrisonensis*
- Lane 4 & 5 *M. sinensis*
- Lane 6 & 7 *M. ‘Gracillimus’*
- Lane 8 & 9 *M. ‘Zebrinus’*
- Lane 10 & 11 *M. ‘Posidon’*
- Lane 12 & 13 *M. ‘Rotsilber’*
- Lane 14 & 15 *M. ‘Roland’*
- Lane 16 & 17 *M. sacchariflorus*
- Lane 18 & 19 *M. nudipes*
- Lane 20 1 KB DNA size ladder

Appendix 4b. Example of RAPD banding patterns obtained with primer OPA 20



Lane 1 *M. x giganteus* (88)
 Lane 2 *M. x giganteus* (88)
 Lane 3 *M. 'Silberspine'*
 Lane 4 *M. 'Goliath'*
 Lane 5 *M. 'Undine'*
 Lane 6 *M. 'Silverfeather'*
 Lane 7 *M. 'Grossefontane'*
 Lane 8 *M. nepalensis*
 Lane 9 *M. nepalensis*
 Lane 10 *M. oligostachyus*
 Lane 11 *M. condensatus*
 Lane 12 *M. sinensis*
 Lane 13 *M. sinensis*
 Lane 14 Negative control
 Lane 15 1 KB DNA size ladder
 Lane 16 1 KB DNA size ladder

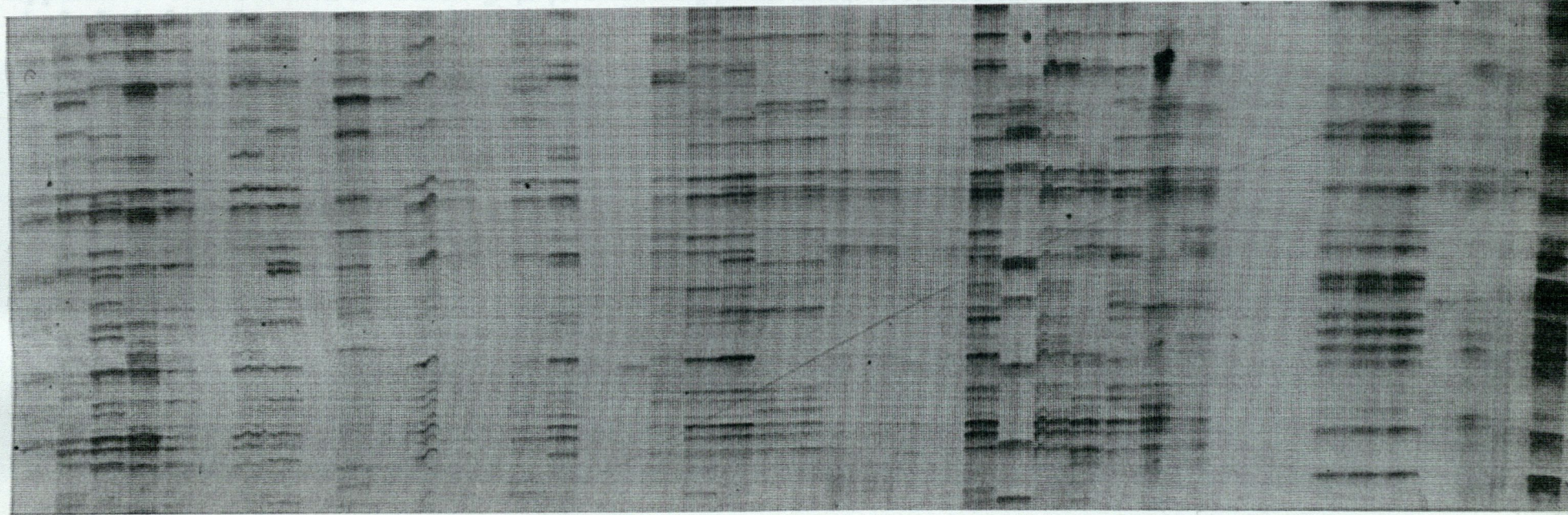
Lane 20 *M. x giganteus* (37)
 Lane 21 *M. x giganteus* (37)
 Lane 22 *M. 'Silberspine'*
 Lane 23 *M. 'Silberspine'*
 Lane 24 Failed sample
 Lane 25 *M. 'Grossefontane'*
 Lane 26 *M. 'Grossefontane'*
 Lane 27 *M. sinensis*
 Lane 28 *M. sinensis*
 Lane 29 *M. condensatus*
 Lane 30 *M. condensatus*
 Lane 31 *M. 'Rotsilber'*
 Lane 32 *M. sacchariflorus*
 Lane 33 *M. sacchariflorus*
 Lane 34 failed lane
 Lane 35 positive control
 Lane 36 *M. sinensis*
 Lane 37 *M. sacchariflorus*
 Lane 38 1 KB DNA size ladder

Appendix 4c. Sequence of primers tested from Operon Technologies, Inc.
primer Kit A.

Code	5' to 3'
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-12	TCGGCGATAG
OPA-13	CAGCACCCAC
OPA-14	TCTGTGCTGG
OPA-15	TTCCGAACCC
OPA-16	AGCCAGCGAA
OPA-17	GACCGCTTGT
OPA-18	AGGTGACCGT
OPA-19	CAAACGTCGG
OPA-20	GTTGCGATCC

Appendix 5a. Portion of an AFLP autoradiograph produced using primer *EcoRI*ACC/*MseI*CAT.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37

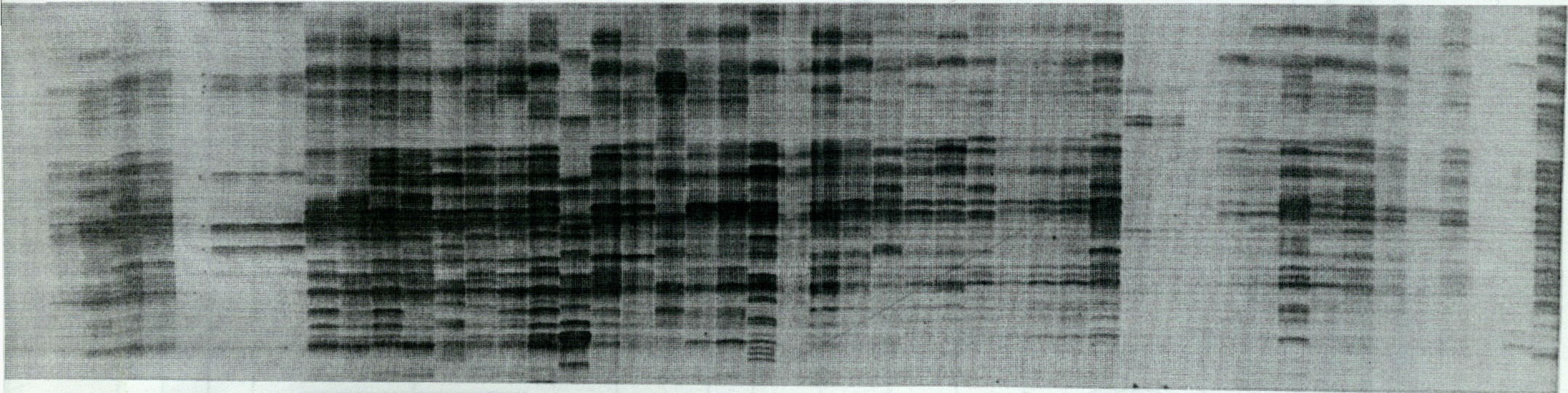


Key to lanes

- | | | |
|----------------------------------|-------------------------------------|----------------------------------|
| 1 <i>M. sinensis</i> | 10 <i>M. sacchariflorus</i> | 20 & 21 <i>M. sinensis</i> |
| 2 <i>M. sacchariflorus</i> | 11 weak lane | 22 & 23 <i>M. oligostachyus</i> |
| 3 <i>M. sacchariflorus</i> | 12 failed lane | 24 & 25 <i>M. condensatus</i> |
| 4 <i>M. oligostachyus</i> | 13 & 14 failed lanes | 26 & 27 weak lanes |
| 5 <i>M. oligostachyus</i> | 15 <i>M. floridulus</i> (Mangerava) | 28 <i>M. nepalensis</i> |
| 6 Blank lane | 16 <i>M. floridulus</i> (Mangerava) | 29 <i>M. x giganteus</i> |
| 7 <i>M. floridulus</i> (Huahine) | 17 & 18 Blank lanes | 30 & 31 <i>M. sacchariflorus</i> |
| 8 <i>M. floridulus</i> (Huahine) | 19 <i>M. transmorrisonensis</i> | 32-34 <i>M. sinensis</i> |
| 9 Blank lane | | 35-37 Tomatoe control |

Appendix 5b. Portion of an AFLP autoradiograph produced using primer *EcoRIACA/MseICAC*.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40



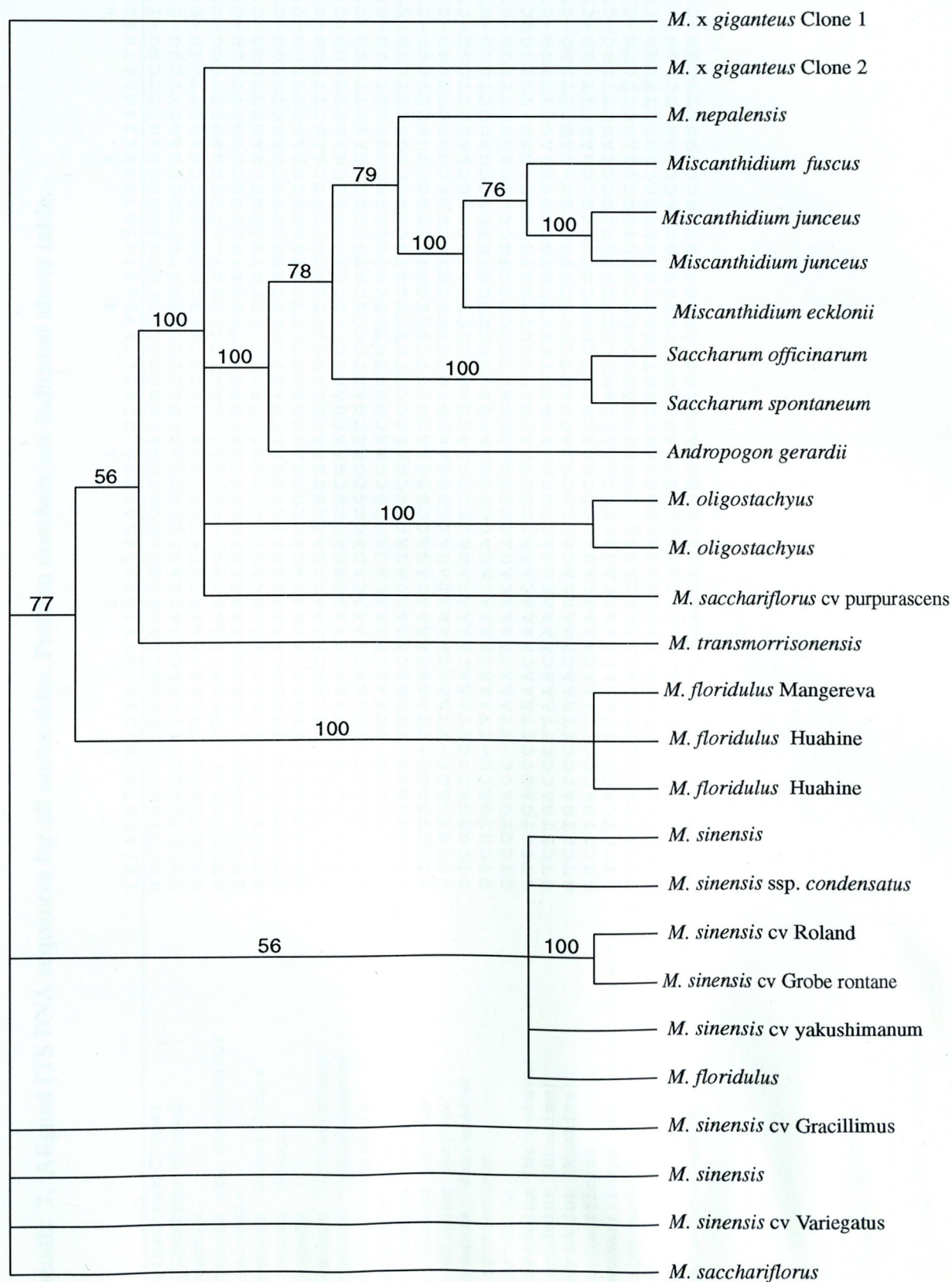
Key to lanes

1 - 3 Tomatoe control
 4 - 7 *M. sacchariflorus*
 8 - 11 *M. sinensis*
 12 *M. nepalensis*
 13 & 14 *M. oligostachyus*
 15 *M. nudipes*
 16 & 17 *M. floridulus* (Huahine)
 18 *M. condensatus*
 19 failed lane

20 & 21 *M. floridulus* (Mangerava)
 22 *M. sinensis*
 23-25 *M. x giganteus*
 26 failed lane
 27 & 28 *M. 'Silverfeather'*
 29 *M. 'Posidon'*
 30-32 failed lanes
 33 & 34 weak lanes
 35. *M. 'Posidon'*

36 & 37 *M. 'Rotsilber'*
 38-40 *M. 'Goliath'*

Using heuristic search options with tree bisection and reconnection. Figures above branches represent frequencies of all observed bipartitions.



Appendix 7. Aligned ITS DNA sequences for all nucleotides. Position numbers are indicated above table.

	1										2										3										4										5										6										7																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
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Appendix 7 (continued).[illegible]

Appendix 7 (continued).

	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
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Appendix 7 (continued).

	2	2	3	3	3	3	3	3	3
	8	9	0	1	2	3	4	5	
	1	2	3	4	5	6	7	8	9
<i>M. X giganteus</i> Clone1	T	G	G	T	G	T	G	A	A
<i>M. X giganteus</i> Clone2	T	G	G	T	G	T	G	A	A
<i>M. sinensis</i>	T	G	G	T	G	T	G	A	A
<i>M. sinensis</i> ssp <i>condensatus</i>	T	G	G	T	G	T	G	A	A
<i>M. nepalensis</i>	T	G	G	T	G	T	G	A	A
<i>M. sinensis</i> 'Gracillimus'	T	G	G	T	G	T	G	A	A
<i>M. sinensis</i> 'Roland'	T	G	G	T	G	T	G	A	A
<i>M. sinensis</i>	T	G	G	T	G	T	G	A	A
<i>M. sinensis</i> 'Großfontane'	T	G	G	T	G	T	G	A	A
<i>M. sinensis</i> 'yakushmanum'	T	G	G	T	G	T	G	A	A
<i>M. transmorrisonensis</i>	T	G	G	T	G	T	G	A	A
<i>Miscanthidium fuscus</i>	T	G	G	T	G	T	G	A	A
<i>Miscanthidium ecklonii</i>	T	G	G	T	G	T	G	A	A
<i>Miscanthidium junceus</i>	T	G	G	T	G	T	G	A	A
<i>Miscanthidium junceus</i>	T	G	G	T	G	T	G	A	A
<i>M. sinensis</i> 'Variegatus'	T	G	G	T	G	T	G	A	A
<i>M. oligostachyus</i>	T	G	G	T	G	T	G	A	A
<i>M. floridulus</i>	T	G	G	T	G	T	G	A	A
<i>M. floridulus</i> (Mangerava)	T	G	G	T	G	T	G	A	A
<i>M. floridulus</i> (Huahine)	T	G	G	T	G	T	G	A	A
<i>M. floridulus</i> (Huahine)	T	G	G	T	G	T	G	A	A
<i>M. sacchariflorus</i>	T	G	G	T	G	T	G	A	A
<i>M. sacchariflorus</i> var. <i>purpurascens</i> T	G	G	T	G	T	G	A	A	T
<i>M. oligostachyus</i>	T	G	G	T	G	T	G	A	A
<i>Saccharum officinarum</i>	T	G	G	T	G	T	G	A	A
<i>Andropogon gerardii</i>	T	G	G	T	G	T	G	A	A
<i>Saccharum spontaneum</i>	T	G	G	T	G	T	G	A	A

Appendix 7 (continued).

	3	3	3	3	3	4	4	4
	5	6	7	8	9	0	1	2
	1	2	3	4	5	6	7	8
<i>M. X giganteus</i> Clone1	G	A	G	G	G	C	A	C
<i>M. X giganteus</i> Clone2	G	A	G	G	G	C	A	C
<i>M. sinensis</i>	G	A	G	G	G	C	A	C
<i>M. sinensis</i> ssp <i>condensatus</i>	K	M	G	G	G	C	A	C
<i>M. nepalensis</i>	G	A	G	G	G	C	A	C
<i>M. sinensis</i> 'Gracillimus'	G	A	G	G	G	C	A	C
<i>M. sinensis</i> 'Roland'	G	A	G	G	G	C	A	C
<i>M. sinensis</i>	G	A	G	G	G	C	A	C
<i>M. sinensis</i> 'Großfontane'	G	A	G	G	G	C	A	C
<i>M. sinensis</i> 'yakushmanum'	G	A	G	G	G	C	A	C
<i>M. transmorrisonensis</i>	G	A	G	G	G	C	A	C
<i>Miscanthidium fuscus</i>	G	A	G	G	G	C	A	C
<i>Miscanthidium ecklonii</i>	G	A	G	G	G	C	A	C
<i>Miscanthidium junceus</i>	G	A	G	G	G	C	A	C
<i>Miscanthidium junceus</i>	G	A	G	G	G	C	A	C
<i>M. sinensis</i> 'Variegatus'	G	A	G	G	G	C	A	C
<i>M. oligostachyus</i>	G	A	G	G	G	C	A	C
<i>M. floridulus</i>	G	A	G	G	G	C	A	C
<i>M. floridulus</i> (Mangerava)	A	A	G	G	G	C	A	C
<i>M. floridulus</i> (Huahine)	G	A	G	G	G	C	A	C
<i>M. floridulus</i> (Huahine)	G	A	G	G	G	C	A	C
<i>M. sacchariflorus</i>	G	A	A	G	G	C	A	C
<i>M. sacchariflorus</i> var. <i>purpurascens</i>	G	A	G	G	G	C	A	C
<i>M. oligostachyus</i>	G	A	G	G	G	C	A	C
<i>Saccharum officinarum</i>	G	A	G	G	G	C	A	C
<i>Andropogon gerardii</i>	G	A	G	G	G	C	A	C
<i>Saccharum spontaneum</i>	G	A	G	G	G	C	A	C

Appendix 7 (continued).

	4	4	4	4	4	4	4	4	4
	2	3	4	5	6	7	8	9	
	1	2	3	4	5	6	7	8	9
<i>M. X giganteus</i> Clone1	G	T	G	T	T	T	G	G	C
<i>M. X giganteus</i> Clone2	G	T	G	T	T	T	G	G	C
<i>M. sinensis</i>	G	T	G	T	T	T	G	G	C
<i>M. sinensis</i> ssp <i>condensatus</i>	G	T	G	T	T	T	G	G	C
<i>M. nepalensis</i>	G	T	G	T	T	T	G	G	C
<i>M. sinensis</i> 'Gracillimus'	G	T	G	T	T	T	G	G	C
<i>M. sinensis</i> 'Roland'	G	T	G	T	T	T	G	G	C
<i>M. sinensis</i>	G	T	G	T	T	T	G	G	C
<i>M. sinensis</i> 'Großfontane'	G	T	G	T	T	T	G	G	C
<i>M. sinensis</i> 'yakushmanum'	G	T	G	T	T	T	G	G	C
<i>M. transmorrissonensis</i>	G	T	G	T	T	T	G	G	C
<i>Miscanthidium fuscus</i>	G	T	G	T	T	T	G	G	C
<i>Miscanthidium ecklonii</i>	G	T	G	T	T	T	G	G	C
<i>Miscanthidium junceus</i>	G	T	G	T	T	T	G	G	C
<i>Miscanthidium junceus</i>	G	T	G	T	T	T	G	G	C
<i>M. sinensis</i> 'Variegatus'	G	T	G	T	T	T	G	G	C
<i>M. oligostachyus</i>	G	T	G	T	T	T	G	G	C
<i>M. floridulus</i>	G	T	G	T	T	T	G	G	C
<i>M. floridulus</i> (Mangerava)	G	T	G	T	T	T	G	G	C
<i>M. floridulus</i> (Huahine)	G	T	G	T	T	T	G	G	C
<i>M. floridulus</i> (Huahine)	G	T	G	T	T	T	G	G	C
<i>M. sacchariflorus</i>	G	T	G	T	T	T	G	G	C
<i>M. sacchariflorus</i> var. <i>purpurascens</i>	G	T	G	T	T	T	G	G	C
<i>M. oligostachyus</i>	G	T	G	T	T	T	G	G	C
<i>Saccharum officinarum</i>	G	T	G	T	T	T	G	G	C
<i>Andropogon gerardii</i>	G	T	G	T	T	T	G	G	C
<i>Saccharum spontaneum</i>	G	T	G	T	T	T	G	G	C

Appendix 7 (continued).[illegible]

Appendix 7 (continued).

	5	5	5	5	6	6
	6	7	8	9	0	1
	1	2	3	4	5	6
<i>M. X giganteus</i> Clone1	T	A	A	G	A	C
<i>M. X giganteus</i> Clone2	T	A	A	G	A	C
<i>M. sinensis</i>	T	A	A	G	A	C
<i>M. sinensis</i> ssp <i>condensatus</i>	T	A	A	G	A	C
<i>M. nepalensis</i>	T	A	A	G	A	C
<i>M. sinensis</i> 'Gracillimus'	T	A	A	G	A	C
<i>M. sinensis</i> 'Roland' [	T	A	A	G	A	C
<i>M. sinensis</i>	T	A	A	G	A	C
<i>M. sinensis</i> 'Großfontane'	T	A	A	G	A	C
<i>M. sinensis</i> 'yakushmanum'	T	A	A	G	A	C
<i>M. transmorrisonensis</i>	T	A	A	G	A	C
<i>Miscanthidium fuscus</i>	T	A	A	G	A	C
<i>Miscanthidium ecklonii</i>	T	A	A	G	A	C
<i>Miscanthidium junceus</i>	T	T	A	G	A	C
<i>Miscanthidium junceus</i>	T	A	A	G	A	C
<i>M. sinensis</i> 'Variegatus'	T	A	A	G	A	C
<i>M. oligostachyus</i>	T	A	A	G	A	C
<i>M. floridulus</i>	T	A	A	G	A	C
<i>M. floridulus</i> (Mangerava)	-	-	-	-	-	-
<i>M. floridulus</i> (Huahine)	-	-	-	-	-	-
<i>M. floridulus</i> (Huahine)	-	-	-	-	-	-
<i>M. sacchariflorus</i>	-	-	-	-	-	-
<i>M. sacchariflorus</i> var. <i>purpurascens</i>	-	-	-	-	-	-
<i>M. oligostachyus</i>	T	A	A	G	A	C
<i>Saccharum officinarum</i>	T	A	A	G	A	C
<i>Andropogon gerardii</i>	T	T	A	G	A	C
<i>Saccharum spontaneum</i>	T	A	A	G	A	C