# An integrated approach to the taxonomy of some mitosporic fungi of the *Bipolaris* complex.

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**A thesis submitted in partial fulfilment of the requirements for the degree of**

**PHILOSOPHIAE DOCTOR (BOTANY)**

**in the**

**FACULTY OF NATURAL AND AGRICULTURAL SCIENCES**

**UNIVERSITY OF PRETORIA**

**October 2001**

**An integrated approach to the taxonomy of some mitosporic fungi of the *Bipolaris* complex.**

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PhD

Previous taxonomic studies culminated in the current differentiation of the mitosporic fungal genera *Drechslera*, *Curvularia*, *Bipolaris* and *Exserohilum*. The circumscription of *Drechslera* and *Exserohilum* are generally accepted and supported by the association with the teleomorphic ascomycetous genera *Pyrenophora* and *Setosphaeria* respectively. The separation of *Curvularia* and *Bipolaris*, both anamorphs of *Cochliobolus*, has been disputed. Many highly similar species have been described that share features of more than one genus. In this study 45 strains representing 14 species of *Bipolaris*, *Curvularia* and *Exserohilum* were characterised with regard to spore morphology, growth characteristics in culture and host preferences. Nucleic acid-based methods were used to evaluate the status of current morphological species. Integration of all this information represents a new approach to the taxonomy of this group. A list of *Bipolaris, Curvularia* and *Exserohilum* species known to occur in South Africa is provided and confirmed their host relations presently known from the literature and includes eight new records. The stability of various spore dimensions and physiological reactions of cultures was analysed by using *First Order Inductive Logic* (FOIL) and a *Decision Tree* (DT) algorithms. Both tools proved spore shape to be more consistent than dimensions, and that colony diameter on days three to five, provided measurements with the best diagnostic value. Colony growth rates can be diagnostic within the long-spored species (fusoid to cylindrical spores) but not between the short-spored (clavate to navicular spores) species of the *Bipolaris*-complex. Differences in colony diameters from five of 25 culture-based tests enabled FOIL to differentiate between species with 79% certainty. Partial sequencing of the 28S rRNA encoding gene differentiated between

*Exserohilum* species and a second group including *Bipolaris* and *Curvularia* species. The molecular marker type, Inter-simple sequence repeats (ISSR) using four anchored primers DBD-$(AC)_7$, BDB-$(CAC)_5$, DHB-$(CGA)_5$ and VHV-$(GT)_7G$, were used with the similarity coefficients of Dice and Pearson and the clustering method of Ward to reveal various levels of intra-specific variability. ISSR data indicated greater similarity between *Cochliobolus* anamorph species with three to four septate conidia than the species with multiseptated conidia. All short-spored species investigated are plurivorous while the long-spored species seem to have a definite graminicolous host preference. Based on these observations it is proposed that the genera *Curvularia* and *Bipolaris* be retained as distinct taxa but amended: plurivorous mitosporic *Cochliobolus* species with short, clavate or navicular spores should be assigned to *Curvularia* and mitosporic *Cochliobolus* species with fusoid to cylindrical spores with gramicolous host preferences should be assigned to *Bipolaris*. The study confirmed *Exserohilum* to be a well-defined genus. *Exserohilum inaequale* was found to be synonymous with *C. cymbopogonis*. Two species, separated only on spore length, *E. longirostratum* and *E. rostratum* should be retained since they are also distinct in physiology and ISSR patterns. Integration of physiological traits, spore dimensions, host relationships and ISSR fingerprint patterns can therefore enhance the taxonomy of the *Bipolaris* complex.

**'n Geïntegreerde benadering tot die taksonomie van enkele mitosporiese swamme van die *Bipolaris* kompleks.**

deur

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PhD

Vorige taksonomiese studies het gelei tot die huidige onderskeiding van die mitosporiese swamgenera *Drechslera*, *Curvularia*, *Bipolaris* en *Exserohilum*. Die omskrywing van *Drechslera* en *Exserohilum* word algemeen aanvaar en word bevestig deur hul assosiasie met die teleomorfiese askomiseet genera *Pyrenophora* en *Setosphaeria*. Die onderskeid tussen *Curvularia* en *Bipolaris*, beide anamorfe van *Cochliobolus*, word egter bevraagteken. Binne die *Bipolaris* swamkompleks is egter verskeie, baie soortgelyke spesies beskryf wat kenmerke van meer as een van die genera vertoon. Hierdie studie het die spoormorfologie, kultuur kenmerke in kunsmatige kultuur en gasheer voorkeure van 45 rasse wat 14 spesies van *Bipolaris*, *Curvularia* en *Exserohilum* insluit, bestudeer. Die geldigheid van huidige morfologiese spesies is geëvalueer deur die insluiting van nukleïensuur-gebaseerede metodes. Integrasie van al hierdie inligting verteenwoordig 'n nuwe benadering tot die taksonomie van die groep. Die gasheer voorkeure van die swamme is bevestig deur die samestelling van 'n lys van *Bipolaris*, *Curvularia* en *Exserohilum* spesies wat voorkom in Suid–Afrika. Hierdie lys bevestig gasheer inligting beskikbaar in die literatuur en dokumenteer agt nuwe spesies aanmeldings. Die bestendigheid van spoorafmetings en fisiologiese reaksies van kulture is ondersoek met behulp van die *First Order Inductive Logic* (FOIL) en *Decision Tree* (DT) algoritmes. Berekeninge met beide algoritmes het aangedui dat spoorvorme'n meer konstante kenmerk as spoorafmetings is. Deursnee van kulture onder gekontroleerde toestande op dag drie to vyf na inokulasie, was van groter diagnostiese waarde. In die *Biopolaris* kompleks swamme is groeitempo ook diagnosties in die langspoor spesies (spindelvormig tot silindries), maar nie in die spesies met kort spore (knotsvormig tot bootvorming) nie.

Met behulp van FOIL is statisties vasgestel dat verskille in kultuurdeursneë soos aangedui deur vyf van die 25 toetse, met 79% sekerheid tussen spesies kan onderskei. Met behulp van 'n gedeelte van die nukleïensuur-basis volgorde van 28S RNA geen, is onderskeid getref tussen *Exserohilum* spesies en 'n tweede groep spesies wat *Bipolaris* en *Curvularia* verteenwoordig. Die molekulêre merker tipe (tussen kort herhalende basis volgordes; *Inter-simple sequence repeats;* (ISSR) en vier geankerde primers DBD-$(AC)_7$, BDB-$(CAC)_5$, DHB-$(CGA)_5$ en VHV-$(GT)_7G$ is gebruik om unieke bandpatrone te amplifiseer. Groeperings is gedoen met behulp van Ward se algoritme en die gelyksoortigheidskoëfisiënte van Dice en Pearson. Die ISSR analiese het verskeie vlakke van intra-spesies variasie aangedui en getoon dat *Cochliobolus* anamorwe met drie to vier gesepteerde spore nader verwant is aan mekaar as aan die spesies met multigesepteerde spore. All kortspoor spesies ondersoek tydens die studie, het verskeie soorte gashere in teenstelling met die langspoor spesies wat almal 'n duidelike voorkeur vir grasagtige gashere het. Gebaseer op al bogenoemde inligting word aanbeveel dat *Curvularia* en *Bipolaris* behou moet word as aparte genera, maar met aanpassing van die genus omskrywings. Die mitosporiese *Cochliobolus* spesies met geen gasheer voorkeure, kort knotsvormig- tot bootvormige spore moet toegewys word aan *Curvularia.* In teenstelling moet die mitosporiese *Cochliobolus* spesies met spindelvormig-tot silindriese spore en 'n voorkeur vir grasagtige gashere, aan die genus *Bipolaris* toegewys word. Die studie het bevestig dat *Exserohilum* 'n goed omskryfde genus is en aangetoon dat *E. inaequale* 'n sinoniem van *C. cymbopogonis* is. *Exserohilum longirostratum* en *E. rostratum* voorheen slegs onderskeibaar in spoorlengtes, word behou as aparte spesies omdat verskille in fisiologiese en ISSR kenmerke, waarneembaar is. Integrasie van fisiologiese kenmerke, spoor afmetings, gasheer-verwantskappe en ISSR data kan gebruik word om die taksonomie van die *Bipolaris* kompleks te verbeter.

## ACKNOWLEDGEMENTS

I wish to express my appreciation to the following organisations and persons who made this thesis possible:

a) The ARC-Plant Protection Research Institute for the use of their facilities and for financial support.
b) The following persons are gratefully acknowledged for their assistance during the course of the study: Dr John Alcorn, Mr. Andre Botha, Miss Glynnis Cook, Miss Gerda Blom, Miss Beth Grobbelaar, Mr Allan Hall, Miss Grace Maudu, Mrs. Senzi Nkosi, Dr Louis Nel, Miss Oloff O'Brien, Miss Louise Patterton, Mrs. Cathy Reichardt, Dr Cecilé Roux, Mrs. Reinette Swanepoel and Mrs. Elna van der Linde.
c) Professor Albert Eicker, my supervisor and Dr Wilhelm Botha, my co-supervisor for their guidance and support.
d) Dr Gerhard Prinsloo for providing textural criticism.
e) My family, parents and especially my husband Ron, for their unwavering encouragement and support.

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# 1 INTRODUCTION

## 1.1 Background

Many species of the *Bipolaris* complex cause serious diseases in graminicolous crops worldwide. Because of their economic importance to man it is necessary to differentiate between species of this group. The key to knowledge about a fungus is in its name: important, information already available in literature, such as its nature and interactions with other organisms, becomes available only when correctly identified. Correct identification therefore enables evaluation of the significance of such an organism to man.

Circumscription of fungal species is mostly arbitrary and not comparable to the species concept as defined for more differentiated eukaryotes. Distinction between fungal species is conventionally based on their structural and developmental features with characters such as cultural characteristics, growth rate, colour and the assimilation of certain nutrients sometimes used in addition. The simple morphologies of asexual fungi present few differentiating characters and even though these characters are relatively easy to determine they are usually common to many fungi. A single feature can therefore not resolve the identity of a fungus. Within the *Bipolaris* complex certain closely similar but broadly defined species have been described with some only differentiated on a single morphological difference.

A preliminary investigation (Appendix A) using data from Sivanesan (1987) in a Canonical Variate Analysis, indicated species of *Exserohilum* as a distinct group separate from *Bipolaris* and *Curvularia*. The same matrix generated from this analysis however, could not separate the species of the last two mentioned genera to the same extent. On the contrary, placement of certain species of *Bipolaris* and *Curvularia* on this matrix, overlapped.

The inadequacies of taxonomic systems based primarily on morphological criteria have compelled many to explore alternative methods. At first association with a sexual stage where considered best to resolve the taxonomic status of a mitosporic fungus but as more anamorph-teleomorph associations have been established, it has become clear that the conidiogenesis of a mitosporic fungus cannot be used to predict its teleomorphic or therefore, its taxonomic position. Further, recent studies have proved that modes of conidiogenesis in mitosporic fungi are not distinct processes but should rather be interpreted as an overlapping continuum of processes.

Alternative taxonomic features must be defined to enable proper differentiation of the *Bipolaris*-like fungi. Isozyme analysis, for example, has proved useful for some mitosporic genera but comparison of nucleic acid based data is believed to facilitate reliable and accurate identification of a variety of organisms. Molecular studies however, necessitate the use of sophisticated techn d expensive. Molecular studies

therefore seem impractical for routine identifications and are not accessible to all. However, comparison of morphologically defined groupings can be verified by comparison with clusters indicated by molecular data. Consistent and reliable taxonomic features, more easily obtained than those from nucleic acids, can then be defined and subsequently applied.

## 1.2 Objectives

a) To compile a list of the *Bipolaris, Curvularia* and *Exserohilum* species known to occur in South Africa. An updated compilation of the three genera under investigation could indicate host relationships of the various species, and can serve as a reference of their current distribution. Such a list might provide insight in shortcomings of current knowledge or opportunities for further investigations.

b) To compare a selection of species from the genera *Bipolaris, Curvularia* and *Exserohilum* as they are currently circumscribed, with the groupings of the same taxa as indicated by molecular characteristics. This comparison should elucidate the value of the currently used taxonomic criteria.

c) To explore the value of characteristics additional to those currently used, to facilitate identification of the selected fungi. The usefulness of some current differentiating criteria will be evaluated.

## 1.3 Scope

All three genera selected for investigation contain numerous species, too numerous for all to be included within the timeframe of this study. The investigation will not attempt to prepare new circumscriptions of species and genera selected for the study, or a reclassification of the group since the relationship of the asexual forms to the sexual forms are well known. The study will endeavour to test the value of certain current, and potential new, taxonomic criteria for anamorphs of this group. This is imperative because sexual stages are rarely available for identification purposes and frequently fail to grow in artificial culture or have lost the ability to do so. The study will therefore not include any investigation of teleomorphs of the *Bipolaris* complex. By integrating all data accumulated, the current circumscription of species and genera can be evaluated and a recommendation put forward to enhance the definition of groups within the selected genera. This approach should clarify the value of taxonomic criteria investigated.

## 1.4 Methodology

The original intent of this study was to include only species of the study group reported in South Africa, but because of certain restrictions (i.e. availability of pure cultures, wide variety of species recorded here), the eventual selection included species not previously recorded in this country. Permission had to be obtained from the Directorate of Plant

Health and Quality of the National Department of Agriculture to import these cultures from various collections abroad. The selection of species had to represent the following:

a) The variation of features within the study group
b) Isolates of different geographical regions
c) A large gene pool
d) Species with uncertain affiliation within the current classification
e) Species that are well defined according to current morphological concepts.

The three sets of data obtained, were based on morphology of spores, radial growth on different artificial growth media (physiology) and nucleic acid sequences (molecular). Since systematic studies of fungi should strive to eventually reflect phylogeny, the validity of morphological and biochemical features will be tested against the groups defined by molecular data. It will be attempted to obtain as many measurements of the selected criteria as resources permits. The three sets of criteria will be integrated by using well-known computer assisted applications for analysis and clustering.

## 1.5 Economic importance

Maize, wheat and beef are the major agricultural products of South Africa (FAO 1997). Considering the immense importance of grain crops in South Africa, compared to other agricultural commodities in terms of gross monitory value and arable land covered, the management of diseases is a significant factor in the protection of these crops (Abstract of Agricultural Statistics 1993; FAO report 1997). One group of pathogens, the helminthosporia, is particularly important since it include many fungi that cause considerable losses to different cereal crops, such as barley, maize, rice, oats, wheat and sorghum (Alcorn 1982a, b; Drechsler 1923; Kwasna 1995; Sivanesan 1987). These particular fungal parasites have over the years posed many problems to agriculture worldwide and include fungi that produce some of the most powerful phytotoxins known (Chełkowski 1995; Luttrell 1978; Shurtleff 1980; Smiley 1983, Ullstrup 1972).

The pathogenic species of *Bipolaris*, *Curvularia* and *Exserohilum* can cause diseases that include leaf spots, seedling blights, foot and root rot, blotches (Kwasna 1995), necrosis and chlorosis (due to host specific toxins) mostly of graminicolous hosts (Paul, Kent & Thomas 1995) (see list of South African records section 5.1). Serious diseases caused by these *Bipolaris*-like fungi are brown spot of rice (*B. oryzae* (Breda de Haan) Shoemaker), brown stripe of sugarcane (*B. stenospila* (Drechsler) Shoemaker), Northern leaf blight (*E. turcicum* (Pass.) Leonard & Suggs) and Southern leave blight (*B. maydis* (Nisik. & Miyake) Shoemaker) of maize (Luttrell 1978). The latter disease has been of great concern to maize farmers in South Africa when first reported in 1974 (Steyn 1978; Van der Westhuizen 1975). Losses due to this disease amounted to about one billion dollars to the United States of America during the 1970 epidemic. Crop losses between 50 - 100% were sustained and in some area[illegible]e that farm workers suffered

respiratory difficulties and skin irritation (Ullstrup 1972). According to Ullstrup (1972), it could have led to widespread malnutrition, if a similar epidemic were to have occurred in another country with a lesser economy or weaker infrastructure. The impact of such an epidemic would be comparable to the potato blight epidemic in Ireland during 1845 and 1846.

The true impact of helminthosporic diseases on forage and crops in South Africa has never been assessed. This would be difficult since other factors such as agricultural practices, fertilization, season and the plant's resistance, all of which influences the occurrence of disease, must be considered (De Jager & Le Roux 1979). However, the numerous reports of these pathogens are an indication of their wide occurrence (Putterill 1954; Van der Bijl 1916; Kemp & Van Jaarsveldt 1990; Van der Westhuizen 1978). In this regard Van der Westhuizen (1978) reported *B. zeicola* (Stout) Shoemaker on sorghum and other grasses, while *B. sorokiniana* (Sacc.) Shoemaker, which causes spot blotch in both wheat and barley, was reported in the Free State by Kemp & Van Jaarsveldt (1990). Currently, *B. sorokiniana* is known in all wheat-producing areas of South Africa (Maas & Kotzé 1981), and also became a serious disease of wheat in a neighbouring country, Zimbabwe (Scott 1995).

Reported diseases caused by helminthosporia on forage, include a severe leaf blight disease of Sudan grass caused by *H. turcicum* Pass. in Natal (Van der Bijl 1916), *B. crustacea* (Hen.) Alcorn, in the western Cape and *B. ravenelli* (M.A. Curtis) Shoemaker countrywide on *Sporobolus* spp. (Alcorn 1983; Putterill 1954). *Exserohilum turcicum* can also be found on other grasses throughout South Africa (Van der Bijl 1916). Studies abroad do not indicate severe yield loss or damage to grassland, pastures or turfs by these fungi, but show that they rather affect the quality of forage and cause problems by being distributed via seeds. This last factor is important in the propagation of disease free seedlings (De Jager & Le Roux 1979). Various species of *Curvularia* were also reported as pathogens of Graminiae, and cause leaf spots on maize. These parasites can be seed borne and include *C. pallescens* Boedijn, *C. tuberculata* Jain, *C. clavata* Alcorn *and C. eragrostidis* (Henn.) J.A. Meyer (as *C. maculans* (Bancroft) Boedijn) (McGee 1988).

The 182 species reviewed by Sivanesan (1987) included *Bipolaris*, *Curvularia Drechslera*, *Exserohilum* and their teleomorphs, known as parasites of graminicolous hosts. Some species can also cause disease in other plants such as cucurbits (Vannacci 1995), beans (Tarr 1963), bamboo (Bhat *et al.* 1989), coffee, mango (Nattrass 1961), eucalypt trees (Mohanan & Sharma 1986) and various palms (Chase & Broschat 1991). They have been reported to stain, and cause soft rot of wood (Kachlicki 1995; Van der Westhuizen 1955) as well as spoilage of animal feeds, foods (Samson 1989) or paint (Nattrass 1961). Another concern is the effects of numerous metabolites that they produce. It has been reported those toxins produced by helminthosporia, caused diseases in animals i.e. cats,

horses and man (Kachlicki 1995). Lastly, the helminthosporia have a small benefit to man in producing substances with anti-microbial properties or the precursors necessary for the production of corticosteroids (Kachlicki 1995; Schneider *et al.* 1985; Rozman *et al.* 1996).

# 2 APPROACHES TO FUNGAL SYSTEMATICS

## 2.1 Taxonomic value of morphological characters

The classification of filamentous fungi has previously been based almost exclusively morphological and ontogenic characters (Hawksworth 1974; Kohn 1992; Pitt 1985; Weising *et al.* 1995). As far back as 1884, Saccardo based his classification of the fungi on morphological features when fully developed (Hawksworth 1974). Structures regarded as being of importance included, amongst others, fruit bodies, spore forms and colour, and the terminology developed by Saccardo, is still in use (Hawksworth 1974). Thus, anamorphs that share great similarity in their morphological and developmental features and which have similar habitat or host preferences are usually regarded as conspecific (Kendrick 1981). The simple morphology of fungi, however, presents taxonomists with relatively few characters for comparison, specifically in the anamorphic genera where no teleomorphs are known (Kohn 1992; Sutton & Cole 1983). The absence of teleomorphs prevents clarification of generic relationships through conventional means (Kohn 1992; Kurtzman *et al.* 1986). Therefore, in strong contrast to many other more advanced groups of organisms, delimitation of species and genera in fungi is mostly arbitrary (Kendrik 1981).

The practice of arbitrary species differentiation led Kendrick (1981) to postulate that there are too many genera of hyphomycetes: the characters used to delimit them are inadequate and poorly defined while separations are often based on a single variable character. Even though some taxonomists have been cautious by using a combination or a range of differentiating criteria (Sutton & Cole 1983) many anamorphic species have been described on a single feature such as the host on which they were found (Alexopoulus & Mims 1979). However, this practice is complicated by the inherent variability found in the genera of the hyphomycetes (Pitt 1985). For instance, in several isolates phenotypic differences have been reported under altered physical, chemical and biological environments. Also, reports of variation in reproductive and vegetative structures were observed when comparing isolates on natural substrates with the same isolate in artificial culture. Some physical factors influencing fungal growth are osmotic potential, light, photoperiod and temperature (Crous *et al.* 1992; Hunter & Barnett 1978; Kendrick 1981; Pitt 1985; Rodrigues & Yoder 1991; Subramanian 1983; Sutton 1981; Sutton & Cole 1983). These fungi, which are haploid in their vegetative state, are only known as asexual forms and subject to environmental selection for endless generations without genetic control (mostly an absence of sexuality except for some with parasexual genetic exchange) (Pitt 1985). According to Pitt (1985) variability in these fungi requires the examination of "hundreds" of isolates. He stated that some isolates of *Penicillium* which do not fit a known species could be sub-cultured for a second and third time and that the subsequent subtle

morphological and physiological character changes will fit a known species (Pitt 1985). In spite of this, few studies on the hyphomycetes have considered the stability of criteria under various environmental conditions before new taxa were created (Pitt 1985). Sutton & Cole (1983) even suggested that for identification purposes, two distinct systems are required for each genus - one based on features on natural substrates and the other on growth in culture. They conceded, however, that it is an idealistic solution to the problem.

A classification system based solely on structure has therefore long been regarded as being artificial and inadequate (Alexopoulus & Mims 1979). Delineation of fungal taxa is difficult because the phenomena of horizontal gene transfer (the donation and acquisition of genetic information between unrelated species), while homoplasy (traits can evolve convergent on more than one occasion in unrelated lineages) occurs frequently in fungi (Mitchell, Roberts & Moss 1995). Micales, Bonde and Petersen (1992) speculated that genetic variability in fungi will correlate with the physiological specialization of the fungus i.e., obligate parasites and highly specialized pathogens will exhibit low levels of genetic variability. Several authors have concluded that morphological and behavioural evolution might involve primarily changes in gene-regulation rather than changes in genetic loci (Soumpasis 1980).

## 2.2 Taxonomic value of ontogenic characters

There have been various reviews, which have focused on the ontogenic, rather than the morphological approach to fungal systematics (Kendrick 1981; Nag Raj 1981; Subramanian 1983; Sutton 1981; Sutton & Hennebert 1993). The structural development of fungi has received considerable attention ever since the work of Constantin in 1888 (Sutton & Hennebert 1993). Unfortunately, the wide interest in ontogenic processes has resulted in considerable confusion with regard to the terminology, even though an attempt was made at standardization, during the International Mycological Congress held in Kananaskis, Canada in 1974 (Kendrick 1979).

Subsequent to the Congress Minter and colleagues demonstrated the plasticity of conidiogenic events, giving a new perspective to conidiogenic processes (Minter, Kirk & Sutton 1982, 1983; Minter, Sutton & Brady 1983). Their findings are in strong contrast to the separately defined process previously considered correct, and highlighted the flaws in the classification system based on ontogenic processes as proposed by Hughes (Alexopoulus & Mims 1979). On its own, the ontogenic approach fails and does not provide the information required for the construction of a phylogenetic classification (Hennebert & Sutton 1994). It offers no consistent correlation between the taxonomies of the Ascomycota and of the mitosporic fungi at generic level (Hennebert & Sutton 1994). In addition to the inherent flaws, inaccurate descriptions and inadequate terminology have contributed to the present dissatisfaction with the ontogenic approach (Hennebert & Sutton

1994). Even so, conidiogenesis is still considered to be one of the most important sets of characters used to define species by (Hennebert & Sutton 1994).

Hennebert & Sutton (1994) have elaborated on three of the five processes described by Minter and colleagues (Minter *et al.* 1982, 1983; Minter, Sutton & Brady 1983). Hennebert & Sutton (1994) proposed a set of unitary parameters to describe ontogeny in the mitosporic fungi. These parameters pertain to maturation and germination of conidia but they still need to add morphogenesis of conidiophores and conidiomata. By using the unitary parameters of conidiogenesis, Sutton and Hennebert (1994) considered some fungal groupings as being homogenic or heterogenic and concluded that more descriptive studies are needed. Kendrick & Murase (1993) also demonstrated the existence of certain monophyletic groups within the hyphomycetes on the basis of developmental and morphological features, and some groups were even correlated with known teleomorph associations. Hennebert and Sutton (1994) extended this concept and believe that the use of unitary parameters is a necessary precursor in the integration of anamorphic and teleomorphic taxa in one taxonomic system. However, they agree that molecular studies will have to be integrated with these parameters since certain patterns of conidiogenous events are polyphyletic in origin and conidial fungi have the ability to evolve independent of meiotic states.

## 2.3 Taxonomic value of physiological characters

Morphological and ontogenic characteristics are relatively easy to determine, but as taxonomic limitations necessitated a search for additional characteristics (Towner & Cockayne 1993). This led to more attention being given to growth responses and biochemical aspects of fungi. The use of "chemical" characters, traditionally only used to differentiate bacteria and yeasts, were soon investigated for their value in other groups of fungi (Hawksworth 1974; Paterson & Bridge 1994).

The range of biochemical features to explore is considerable (Bridge & Hawksworth 1984; Fox 1993; Paterson & Bridge 1994). Being heterotrophic, fungi produce many metabolites such as mycotoxins and extracellular enzymes in order to utilise substrates as nutrients (Alexopoulus & Mims 1979; Sharma & Salunkhe 1991; Steyn 1980; Steyn & Vleggaar 1986). However, not all biochemical criteria have proved to be of taxonomic value. These include mycotoxins production (Bu'Lock (1980), vitamin deficiencies (Cochrane 1958), total protein patterns (Towner & Cockayne 1993), long chain fatty acid (16 to 18 carbon chain lengths) composition of cell walls and other membrane systems (Alexopoulus & Mims 1979; Kock, Wingfield & Erasmus 1993) as well as certain immunological techniques (Paterson & Bridge 1994).

Provided their limitations are taken into consideration, biochemical measurements can be a powerful taxonomic tool, especially at species level (Frisvad 1994; Pitt 1985). Fungi, as eukaryotes, are able to control the expression of genes and regulate enzymatic pathways

(Pitt 1985) and many factors, both genetic and non-genetic, can cause non-expression of genes (Bu'Lock 1980; Hawksworth & Mouchacca 1994). If a specific metabolite or enzyme is not found in a particular test, it does not prove that the enzyme or metabolite cannot be made by the isolate (Pitt 1985). It is therefore essential to repeat a particular test many times to prove its validity (Pitt 1985). Apart from rigorous control of environmental conditions when studying metabolic products in fungi, the potential for deterioration of cultures should also be kept in mind (Pitt 1985).

## 2.4 Growth responses

Many simple measurements on solid media in artificial culture provide useful characters to supplement morphological criteria. Such tests include stains to indicate the presence of a chemical substance (Seifert 1985), utilisation of nutrients, carbon and nitrogen sources (Paterson & Bridge 1994), the production of certain metabolites such as acids (Paterson & Bridge 1994), the ability to form pigments (Hawksworth 1974; Von Arx 1986) or odours (Hawksworth 1974), the reaction to fungicides or other inhibitory compounds and presumptive enzymatic reactions (Paterson & Bridge 1994).

Pitt (1985) examined the influence of basal media and additions to basal media as part of the overall assessment of test reproducibility. The production of both secondary metabolites and extracellular enzymes can be profoundly influenced by relative minor changes in basal medium (such as the addition of copper and zinc) or the manufacturer and type of organic ingredients (such as peptone).

### a) Enzyme kits

Easy to use and commercially available enzyme kits, such as the APIZYM™ and BIOLOG™ can provide standardised methods of testing for specific physiological properties with the advantage that specialised equipment and personnel are not required. The APIZYM™ system presents a semi-quantitative measurement of enzymatic activities by using 19 different substrates and has been used as an identification aid in bacteria with encouraging results (Bridge & Hawksworth 1984; Paterson & Bridge 1994). A chemically defined medium must be used for the culturing of test organisms. Bridge and Hawksworth (1984) believe these systems would also be useful to accumulating data on enzymatic properties of microfungi for use in biochemical systematics and biotechnology. They expect these systems to aid in the rapid identification of critical groups of filamentous fungi such as *Acremonium, Aspergillus, Gliocladium, Trichoderma* and *Verticillium*. The APIZYM™ system has already proved to be useful in the rapid preliminary screening of isolates of the genus *Penicillium*. The system could not be used to identify *Penicillium* isolates to species level, but predicted the sub-genus and could be used to confirm the identification of critical isolates where there has been some uncertainty based on morphological characters alone (Bridge & Hawksworth 1984). A different product, API

Rapid CH, containing various carbon sources was also used to distinguish between two isolates of *Beauveria bassiana* (Todarova *et al.* 1994).

### 2.4.1 Isozyme analysis

Isozyme analysis in the fungi has been reviewed by Micales, Bonde and Petersen (1986) and many procedures in this regard have been described by Paterson and Bridge (1991). These techniques include, amongst others, thin layer- or high performance liquid chromatography of secondary metabolites or isozymes and gel electrophoresis of proteins. Both publications reported these methods to be highly effective, yielding much taxonomically useful data.

Many enzymes are present in cells in more than one form, corresponding to proteins or bands of different molecular sizes, but showing the same enzymatic activity. These different molecular forms of the same enzyme are referred to as iso-enzymes or isozymes (Paterson & Bridge 1994). Isozymes arise from three different genetic and biochemical phenomena: firstly because of multiple allelism at a single locus, secondly multiple loci coding for a single enzyme or thirdly through post-translation processing (Micales *et al.* 1986).

Unstained starch-gels of electrophoretically separated fungal proteins can be incubated in specific enzyme-substrate reaction stains so that the protein bands corresponding to particular enzymes become visible (Paterson & Bridge 1994). Paterson and Bridge (1994) reported staining systems for esterases, catalases and phosphatases and successfully used them in intra-specific studies on the mitosporic genera *Beauveria, Fusarium, Metarhizium, Penicillium, Verticillium,* and the basidiomycete *Ganoderma.* Groups of species defined within the genus *Trichoderma* also differed in their ability to produce certain enzymes (Meyer *et al.* 1992). Isozyme characterisation proved effective in examples over most of the fungal kingdom (Micales *et al.* 1992) and enabled differentiation of taxa higher than species level (Bridge & Hawksworth 1984; Ciegler *et al.* 1973; Frisvad 1981; Hawksworth 1974). The technique can be used to study variation in fungal populations (Paterson & Bridge 1994; Micales *et al.* 1992) but its use in taxonomy will need much further development (Frisvad 1994).

Interpretation of electrophoretic banding patterns can produce large amounts of information about the nuclear condition and genetic make-up of the organism only after careful consideration of certain factors influencing repeatability (Micales *et al.* 1986). Before embarking on a systematic study, proteins initially extracted should be screened to reveal what enzymes are active in the taxa under investigation. This process should also elucidate which buffers would provide optimum resolution for each enzyme (Micales *et al.* 1986). These proteins can be extracted from any part of a fungus but inconsistent results may be obtained when a mixture of different tissues is used. Results are also dependent on growth conditions and different stages of fungal development (Micales *et al.* 1986).

### 2.4.2 Cell wall composition

Cell wall composition seemed a promising taxonomic tool since studies indicated differences between fungi (Alexopoulus & Mims 1979). Two distinct lineages in fungi were defined depending on the structural cell wall polysaccharides being either chitinous or cellulosic (Bartnicki-Garcia 1986). Most of the fungal groups from the cellulosic linage have since been disregarded as true fungi by Cavalier-Smith (1998). Taxonomic use of polysaccharides from fungal cell walls clearly separate the major classes of fungi but seems to have little value in differentiation below this level (Bartnicki-Garcia 1986; Lewis 1991). An exception is Kock *et al.* (1993) who reported certain cell wall proteins useful in indicating relationships between anamorph-teleomorph connections in the *Ceratocystis-Ophiostoma* group of fungi. Some studies indicated that the composition of cell walls in many fungal species vary depending on colony age, growth medium, pH and temperature. Such variation would severely limit its value in systematics (Alexopoulus & Mims 1979; Kock *et al.* 1993).

### 2.4.3 Immunological techniques

Advanced immunological or serological techniques in taxonomy have limited use and are mostly highly specific (Manicom *et al.* 1990). Monoclonal antibodies and enzyme linked immunosorbent assay (ELISA) or double diffusion techniques can be used to identify specific isolates (Paterson & Bridge 1994), and in the genera *Fusarium* and *Penicillium* tandem-crossed immuno-electrophoresis confirmed work based on their morphology (Frisvad 1994; Manicom *et al.* 1990). Novak and Kohn (1991) also used ELISA tests to differentiate groups within fungal genera that form sclerotia and stromata, but otherwise only limited success has been recorded.

## 2.5 Taxonomic value of nucleic acid-based characters

Notwithstanding the extensive exploration of isozyme and fatty acid composition of cell walls to assess infraspecific and interspecific variation in fungi, DNA based techniques have more or less replaced these biochemical methods (Nielsen *et al.* 2001). Many review articles can be found on molecular characterization of fungi and include, amongst others, those by Bruns, White and Taylor (1991), Klich and Mullaney (1992), Kurtzman (1985), Mills (1993), Mitchell *et al.* (1995) and Weising *et al.* (1991). From these publications it is clear that molecular markers offer the first reliable methods for making phylogenetic assessments of fungi, for testing the validity of a morphological taxonomic system, and for assisting in the correct identification of fungi to sub-specific levels. Even though molecular methods should not be expected to provide all taxonomic answers, they offer much more information than is available from conventional comparisons (Bruns *et al.* 1991; Pitt 1985). Selection of the region of the genome to be targeted, and the type of technique to use, are largely dependent on the nature of the investigation envisaged (Duncan *et al.* 1998).

### 2.5.1 Target DNA

The genome sizes of the filamentous fungi are estimated to range between 21-38 x $10^6$ basepairs (Griffin 1992) and contain several types of nucleic acids, which show differing degrees of sequence conservation (Kurtzman 1985). Because various regions of the genome are differently conserved, it is possible to resolve various levels of taxonomic hierarchy depending on the area targeted (White *et al.* 1990). No particular gene sequence can therefore determine all the taxonomic relationships of an organism (Mitchell *et al.* 1992).

**a) Mitochondrial DNA**

Mitochondrial DNA (mtDNA) encodes some of the proteins essential for the proper functioning of electrochemical processes of cells but large sections, however, have little or no coding function (Duncan *et al.* 1998). This part of the genome is maternally inherited without genetic recombination and mutates much faster than genomic DNA but at a relative constant rate across organisms (Duncan *et al.* 1998).

Mitochondrial DNA can be obtained by ultracentrifugation in a $CsCl_2$ gradient or by digesting whole DNA with the restriction enzyme *Msp*I (a four base pair GC-cutter) and then separated with electrophoresis on agarose gels (Duncan *et al.* 1998). The principle behind the latter stage is that genomic DNA is GC rich while mtDNA are AT-rich. The mtDNA fraction can then be digested with various restriction enzymes, amplified by using the polymerase chain reaction (PCR) and banding patterns obtained after electrophoresis (Duncan *et al.* 1998). Differentiation between mating types of closely related species or isolates within a species can be achieved by targeting mtDNA (Duncan *et al.* 1998). Two alternative approaches targeting mtDNA, namely complete sequencing and the use of probes derived form purified mtDNA, have been used successfully to differentiate various species of *Phytophthora* (Duncan *et al.* 1998).

**b) Ribosomal RNA (rRNA) and ribosomal DNA (rDNA)**

RNA genes (rDNA) encode the rRNA that is incorporated into ribosomes, the site of protein synthesis in the cell (Duncan *et al.* 1998). The genes are arranged in a cluster, which itself is tandemly repeated in an array that can contain as many as several hundred clusters. Each cluster encodes the 18S, 5.8S, 28S and, in some cases, 5S rRNA subunits (Duncan *et al.* 1998). These subunits are extremely conserved across all eukaryotes and have been used extensively to examine relationships between taxa, usually at a supra-genetic level from orders to kingdoms (Mitchell *et al.* 1995). According to Mitchell *et al.* (1995) the 28S rRNA gene can provide enough variation to separate species.

Between the subunits are spacer regions, which do not encode rRNA. One of these, the intergenic spacer (IGS), is never transcribed to RNA; the other three, the internally transcribed (ITS) spacers ITS1 and ITS2 and the externally transcribed spacer (ETS)

are excised from the pre-rRNA transcript leaving the subunits, which subsequently form the structural backbone of a ribosome (Duncan *et al.* 1998). The spacer regions are less conserved than the structural units and mutations in this region are seemingly neutral (Duncan *et al.* 1998). Such changes accumulate consistently over time and can be used to study relatedness among taxa below the level of orders, in particular genera, species and subspecies (Duncan *et al.* 1998; Mitchell *et al.* 1995). Although valuable to distinguish species, the ITS regions have limited value as markers for sexual recombination within a species (Duncan *et al.* 1998). In studies of *Phytophthora* it became clear that the ITS1 region is more variable between species than the ITS2 regions, with virtually no variation within the 5.8S region even among other genera related to *Phytophthora*. Universal primers do exist to amplify the whole or parts of the region spanning both the ITS and 5.8S regions (Duncan *et al.* 1998). This particular region is less than 1000 bp in length (Duncan *et al.* 1998). Once amplified the PCR products can be sequenced directly using any of a number of kits (Duncan *et al.* 1998).

**c) Total genomic DNA**

The most complete analysis for molecular gene maps can be obtained by using total DNA with some specific marker techniques such as restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP) or simple sequence repeats (SSR). Each of these multilocus-fingerprinting techniques has both advantages and disadvantages that will be discussed under separate headings.

### 2.5.2 Hybridisation-based fingerprinting

The technique recognizes multiple DNA loci simultaneously (Duncan *et al.* 1998). The process involves:

a) Cutting genomic DNA with restriction enzymes
b) Electrophoretic separation of fragments according to size
c) Denaturation of DNA into single stands
d) Blotting of the fragments from the gel onto a membrane
e) Hybridisation: the probe and the membrane-bound DNA are brought together under conditions, which favour hybridisation between similar sequences
f) Removal of excess probe by washing.

Bands so obtained can be viewed with various methods depending on whether the probes have previously being radioactively labelled or not (Klich & Mullaney 1992). In-gel hybridisation with labelled oligonucleotides is faster and more efficient that conventional blotting techniques since there is no need for DNA transfer (Weising *et al.* 1995).

Two different types of probes can be used: firstly cloned DNA fragments or oligonucleotides, which are complementary to so-called minisatellites, and secondly

tandem repeats of very short motifs mostly 1 to 5 base pairs (bp) called simple sequence repeats (SSR) (Weising *et al.* 1995). Minisatellites are tandem repeats of a basic motif of about 10 to 60 bp. A high degree of polymorphisms can usually be observed when using either one of the two types of probes (Weising *et al.* 1995). Apart from minisatellite detecting probes derived from living organisms, some probes can be designed relatively easily using synthetic tandem repeats of arbitrarily chosen sequence (usually 14-20 bp) for example $(GATA)_4$, $(GACA)_4$, $(CT)_8$ and $(GTG)_5$ (Weising *et al.* 1995). The cloned Jeffery's probes 33.15 and 33.6 and the M13 repeat probe are probably the most extensively used minisatellite probes to fingerprint a variety of organisms including humans, animals, plants, fungi, protozoa and bacteria (Weising *et al.* 1995). A multitude of highly informative simple sequence- and minisatellite-derived multi-locus probes are commercially available for plant and fungal DNA fingerprinting (Weising *et al.* 1995). Although the majority of probes are successful in revealing polymorphisms, the resolution level is highly dependent on the particular probe used (Weising *et al.* 1995).

The fingerprinting results are not influenced by the method of DNA extraction used (Bart-Delabesse *et al.* 1998) or subculturing of the isolates tested (Bretagne *et al.* 1997). Depending on the combination of species and oligonucleotide probe, species-, variety-, strain- or individual-specific "fingerprints" can be obtained in fungi (Field & Willis 1996; Weising *et al.* 1991) and used to detect diversity and relatedness of individuals in populations (Geistlinger *et al.* 1997; Groppe *et al* 1995). Discrimination between species of the filamentous fungal genera *Aspergillus*, *Penicillium* and *Trichoderma* have been achieved by hybridisation to $(CT)_8$, $(GTG)_5$, $(GATA)_4$, $(GACA)_4$ and the M13 (GAGGGTGGCGGTTCT) repeat (Schlick *et al.* 1994; Weising *et al.* 1991). In *Aschochyta rabici* an ascomycete (Weising *et al.* 1991), and *Heterobasidion annosum* a basidiomycete (DeScenzo & Harrington 1994), single spore derived mycelia, yielded clearly distinguishable fingerprint patterns.

**a) Restriction fragment length polymorphisms (RFLP)**

Restriction endonuclease enzymes recognise and cleave DNA at highly specific sequences, thus creating fragments of defined length (Bruns *et al.* 1991). Many different restriction enzymes are commercially available, each yielding a different pattern (Klich & Mullaney 1992). The fragments or recognition sequences can be separated according to molecular size by electrophoresis in acrylamide of agarose gels (Bruns *et al.* 1991; Klich & Mullaney 1992). The patterns formed on gels are subsequently revealed with dyes such as ethidium-bromide and viewed under UV light, by silver staining or by autoradiography if the fragments are first labelled with a radioactive tracer (Bruns *et al.* 1991)

The fragment patterns may be compared in essentially three ways: the fragment patterns themselves, mapping of the enzyme sites, or by probes of specific sequences of

the fragments (Bruns *et al.* 1991). In using the pattern of fragments, or the individual fragments themselves 10 to 40 isolates can be compared simultaneously (Bruns *et al.* 1991). Mapping of fragments enables deduction of a map of the enzyme sites, which then become the units of analysis (Bruns *et al.* 1991). Unfortunately, any length mutations (insertions and deletions) are included in mapmaking, making the method error prone (Bruns *et al.* 1991; Klich & Mullaney 1992). In fungi, this is very likely since length mutations occur at high frequency in mtDNA, and probably in nuclear DNA (Bruns *et al.* 1991; Kurtzman 1985; Taylor 1986). If a probe is not used, similarly sized non-homologous DNA fragments will appear to be identical, giving the impression of greater similarity between isolates than actually exists (Bruns *et al.* 1991; Kurtzman 1985). With the third method, fragments are probed with either random or defined radioactively labelled nucleic acid probes to minimise the effect of length mutations (Klich & Mullaney 1992; Duncan *et al.* 1998). DNA are transferred from the gel to either nylon or nitrocellulose membranes and hybridised to a radiolabelled gene i.e. rDNA (Mills 1993).

Different regions of the genome can be targeted for RFLP's (Bruns *et al.* 1991). The restricted digestion of total DNA is potentially the simplest of RFLP tests but this results in a complex pattern of many bright bands (fragments) against a smear of background DNA (Bruns *et al.* 1991; Klich & Mullaney 1992). Bright bands representing the different DNA fragments are difficult to distinguish from the background DNA (Bruns *et al.* 1991).

Several features contribute to the popularity of mtDNA as target to measure relatedness and have been widely used for evolutionary studies in fungi (Bruns *et al.* 1991; Kurtzman 1985). Mitochondrial DNA RFLP's investigate a small part of the genome (19 to 176 kilo base pairs), and thus produces more discrete bands (Bruns *et al.* 1991; Kurtzman 1985). Despite the relatively small size of mtDNA, it supplies enough characters for differentiation (Bruns *et al.* 1991). Further, no evidence exists for methylation (i.e. cytosine methylated to 5-methylcytosine) of bases, thus a potentially confounding factor of nuclear DNA, is avoided (Bruns *et al.* 1991). Morphological degenerate cultures can also be characterised with success (Klich & Mullaney 1992)

RFLPs of mtDNA or whole genomic DNA do not allow verification of species assignments within genera, or an understanding of intergeneric relationships (Kurtzman 1985) but the major strength is its capacity to identify strains (Bruns *et al.* 1991). Since mtDNA evolved faster than nuclear DNA, it is more likely to differentiate at the subspecific level (Manicom *et al.* 1990). The fast rate of change in some organisms can be up to 10-fold more rapid than that of nuclear DNA, and therefore prevent resolution sufficient to recognise the more divergent strains of a species (Kurtzman 1985). Evolutionary relationships can only be investigated with RFLP's, if a map is created

(Bruns *et al.* 1991). More distinct bands than those obtained from genomic DNA can also be generated from ribosomal RNA coding DNA (rDNA) (Bruns *et al.* 1991). With rDNA as a target, often only two to three bands are generated with any single enzyme, necessitating the use of more than one enzyme (Mills 1993). Mills (1993) had various levels of success in differentiating between species of hyphomycetes genera, when using RFLP targeting rDNA (Mills 1993).

Probes for RFLPs include random clones produced without any prior knowledge of function of structure; cloned genes from the same or a different species or repetitive sequences (Bruns *et al.* 1991). Specific probes, if available, can be hybridised to DNA on membranes (RFLPH) resulting in highly specific fingerprints (Klich & Mullaney 1992). RFLPH can be "tuned" to different relational levels such as the specific probes for vegetative compatibility groups (VCG), or isolates in *Fusarium* (Manicom *et al.* 1990). The technique is a co-dominant analysis, allowing allele flow within a population or allele segregation in a cross, to be studied (Duncan *et al.* 1998).

RFLP's have successfully been correlated with other features of fungi. Louw *et al.* (1995) correlated RFLP banding patterns to conidium length in species of *Pyrenophora* (Ascomycota) and have therefore successfully used the technique to enhance identification of morphological species. Physiological differences were confirmed in RFLP generated from forma speciales (Manicom *et al.* 1987; Manicom & Baayen 1993), pathotypes and geographical origin of strains of *Fusarium oxysporum* (Leong & Holden 1989). Ribosomal DNA RFLPs of *Cochliobolus heterostrophus* were specific for species and strains (Millar & Martin 1988). RFLPs have also been used extensively to characterize different species of *Aspergillus* (Klich *et al.* 1993; Klich & Mullaney 1992; Kozłowski & Stepien 1982; Varga *et al.* 1997), *Verticillium* (Carder & Barbara 1991) and *Trichoderma* (Meyer *et al.* 1992).

Some investigations of the systematics of the helminthosporic fungi used RFLP's as tool. RFLPs of total DNA of *Bipolaris* and *Curvularia* species were analysed using arbitrarily chosen genomic clones of DNA from *Bipolaris maydis* and *Curvularia lunata* (Nakada *et al.* 1994). Clear differences among species in both genera were observed. Infraspecific polymorphism in banding positions with these probe-enzyme combinations was slight. Based on their results, Nakada *et al.* (1994) elevated *Curvularia lunata* var. *aeria*, to a distinct species *Curvularia aeria.* Goh and co-workers (1998) studied the relationships between the helminthosporic genera *Curvularia, Drechslera, Exserohilum and Helminthosporium.* They targeted the nuclear rRNA gene using various primers and restriction enzymes. *Rsa*I digestion of ITS/28S region enabled the differentiation of genera. Their results proved *Drechslera* and *Exserohilum* to be distinct from the other genera tested but that representatives of *Curvularia* and *Bipolaris* form a heterogenous group.

### 2.5.3 Chromosomal polymorphisms

Chromosome length polymorphisms have been detected by pulse-field electrophoresis in plant pathogenic fungi such as *Septoria nodorum* (Berk.) Berk., *Erysiphe graminis D.C. f.sp. hordei* È. M. Marchal and *Ophiostoma ulmi* Buisman) Nannfeldt (Duncan *et al.* 1998). It can be used in epidemiological studies or for typing of strains, but is particularly useful for genetic mapping of fungi. It was however discovered with studies on *Phytophthora infestans* that chromosomal heteromorphisms are linked to mating-type loci, a region that displays distorted and non-Mendelian segregation (Duncan *et al.* 1998).

### 2.5.4 Polymerase Chain Reaction (PCR) based techniques

In the PCR reaction, DNA is denatured into single strands by heating before primers are allowed to anneal to the regions of sequence homology (Mitchell *et al.* 1995). DNA polymerase mediates extension of the primer when the temperature is lowered. After each cycle, the number of amplified copies increases exponentially (Mitchell *et al.* 1995). With PCR technology large amounts of amplified DNA can be produced *in vitro* from only a small initial amount. The technique is ideal in situations where material is limited i.e. certain fungi which are difficult to grow in artificial culture (Mitchell *et al.* 1995) or small pieces of herbarium material (Wingfield & Wingfield 1993). PCR-based methods are very popular mainly due to the speed with which large numbers of samples can be processed (Weising *et al.* 1995). The PCR technique is also one of the most efficient and reliable of molecular methods for phylogenetic studies: selected primers may reveal levels of genetic variability equivalent to those commonly observed following hybridisation-based DNA fingerprinting (Weising *et al.* 1995). The biggest risk with this method is contamination with foreign DNA (Van Brummelen 1993).

Research and application of PCR are often conducted with arbitrarily chosen primers (Weising *et al.* 1995). When sequence information is available, specific DNA primers may be tailored to amplify areas of interest, sometimes resulting in high levels of polymorphism (Mitchell *et al.* 1995). Since parts of the rRNA genes are heavily conserved, universal primers as designed by John Taylor for the fungi, can be used (Mitchell *et al.* 1995). These universal primers eliminated the need to know a nucleotide sequence before PCR technology can be used (Mitchell *et al.* 1995).

With high quality and sufficient quantity of prepared DNA, several different DNA-based markers can be prepared which vary in their sensitivity and specificity. Depending on the type of analysis used, markers are often neutral, i.e. without apparent selective advantage.

**a) Random amplified polymorphic DNA (RAPD)**

The RAPD technique is a modification of the polymerase chain reaction (PCR) where short arbitrarily generated single primers (usually 10-mer) are used to amplify genomic DNA (Mitchell *et al.* 1995; Duncan *et al.* 1998). Many different 10 nucleotides primers are commercially available for this purpose (Mitchell *et al.* 1995). This technique

permits polymorphisms to be identified without using cloned DNA as probes (as is necessary with RFLP's) and scans the whole genome simultaneously, binding to complementary sites (Mitchell *et al.* 1995). Any base substitutions will yield differently sized fragments that are observed after separation by electrophoresis. The types of polymorphism that are detected by RAPD's include single or multiple base substitutions, deletions of a primer binding site, and insertions that move the primers so far apart that PCR amplification fails (Erasmus 1997a). The specific banding pattern generated by this procedure is the so-called "fingerprint" that is specific to individual strains (Mitchell *et al.* 1995; Schlick *et al.* 1994). Speciation can be studied without time-consuming mating experiments and generates data to further sub-divide species (Mitchell *et al.* 1995). It is therefore possible to characterise large unknown genomes at the molecular level without the need for sequencing information.

The technique does have disadvantages in being much more dependent on variables during the reaction, than conventional PCR (Mitchell *et al.* 1995). Results are difficult to repeat consistently and are influenced by changes in template concentration, template purity, ionic strength, Mg2+ concentration and the type of thermostable polymerase. The temperature profile, especially the annealing temperature, is highly critical. RAPDs also tend to target abundant (repetitive) sequences (Weising *et al.* 1995). With the development of PCR-based restriction fragment polymorphisms (RFLP) these disadvantages were overcome (Bruns *et al.* 1991; Mills 1993).

The use of RAPD in the study of relationships among plant-pathogenic fungi is well documented (Louw *et al.* 1995). RAPD analysis of the filamentous fungi *Colletotrichum gloeosporioides* (Penz.) Sacc. and species of *Trichoderma* investigated genetic variation at sub-species level (Mills 1993). Fungi such as *Agaricus bisporus* (J. Lange) Singer, *Aspergillus fumigatus* Fr., *Fusarium solani* (Mart.) Appel en Wollenw. Emend. W. C. Snyder and H.N. Hansen (Towner & Cockayne 1993), *F. oxysporum* Schtdl. emend. W.C. Snyder and H.N. Hansen (Manicom *et al.* 1987), *Pyrenophora* spp. and *Leptosphaeria maculans* (Desm.) Ces. & De Not. (Louw *et al.* 1995) have been fingerprinted using RAPD analysis. PCR-RAPD enabled the typing of both *Beauveria bassiana* (Bals.) Vuill. and *Be. brongniartii* (Sacc.) Petch according to host specificity (Neuvéglise *et al.* 1998). Host ranges of the ovaricolous *Bipolaris* spp., based on herbarium records, were confirmed with a RAPD study on the pathological and molecular variation in the interaction between *Sporobolus* grasses and the fungi (Hetherington & Irwin 1999). RAPD analysis produces dominant markers (Duncan *et al.* 1998) and is inappropriate to distinguish between species (Weising *et al.* 1995).

**b) Amplified fragment polymorphism (AFLP)**

Genomic DNA is digested both with a regular- (usually Mse*I*) and a rare-cutting (usually Eco*RI* or Pst*I*) restriction enzyme and adaptors are ligated to the resultant

fragments. Long primers, which anneal to the adaptor sequence, are then used to amplify the entire population or restriction fragments, using PCR. Sub-populations of the DNA fragments are then amplified from this pre-amplified material using primer pairs, which overhang the adaptor sequence by one, two or three bases. The primers used in this analysis allow a high annealing temperature to be used: the procedure is thus more reproducible than RAPDs and produces a larger number of bands for study as it does not favour high copy number sequences in the genome. The high sensitivity of AFLPs provides more information on variation than any other molecular technique and is therefore used for high-resolution mapping by bulked segregant analysis (Duncan *et al.* 1998). The Peruvian population of *Phytophthora infestans* (Mont.) de Bary was studied and characterized with RFLPs and AFLPs by Péres *et al.* (1998). The authors expected AFLP fingerprinting to revealed greater differentiation and diversity.

**c) Simple sequence repeats (SSR) and inter-repeat sequences (ISSR)**

Nucleotides arranged repetitively in tandem are known as minisatellite (long sequence repeats as large as 200 base pairs) or microsatellite sequences (short repeated motifs of 2 to 6 base pairs) (Field & Wills 1996; Shimizu *et al.* 1998; Weising *et al.* 1995). All SSR motifs investigated to date i.e. CA-, CT-, GATA-, GACA-, GAA-, GTG-, GGAT-, and TCC-multimers, are present (Weising *et al.* 1995) and repeated to various extents throughout the fungi (Osiewacz, Hamann & Werner 1996; Schlick *et al.* 1994; Weising *et al.* 1995). According to Griffon (1992) they form up to 23% of the fungal genome. The AT rich repeats are more commonly found in simple organisms with GC pairs least frequent (Field & Wills 1996; Groppe *et al.* 1995). Both meiosis (sexual fungi) and mitosis (asexual fungi) appears to generate equivalent number of microsatellites (Field & Wills 1996). Little is know about the biological significance of the majority of these genetic elements but Osiewacz *et al.* (1996), as well as Field and Willis (1996), indicated that satellites control gene expression and play a role in gene transcription. They appear to be stable during ontogenesis and evolution (Osiewacz *et al.* 1996).

Variability measured with tandem repeats is most often due to particular arrays on a given chromosome having different repeat numbers in different individuals and are termed variable number of tandem repeat polymorphisms (VNTR) (Weising *et al.* 1991). These polymorphisms are generated by mitotic and meiotic unequal exchanges, or slipped strand mispairings during DNA replication (Weising *et al.* 1991). In addition to allelic variations in repeat number, polymorphisms at mini- and microsatellite loci can also be caused by sequence changes near these repeats (Erasmus 1997a).

Simple sequence analysis has at least two advantages over minisatellites. First, they are short (typically 20 to 40 bp and rarely as large as 200 bp) and easy to amplify (Weising *et al.* 1995). Minisatellites arrays are often too long (i.e. 0.5 to 30 kb) for efficient amplification. Secondly, stretches of simple sequences are more evenly distributed over

the genome than minisatellites (Weising *et al.* 1995). The length of the microsatellite used is also of importance: informativeness of the microsatellite $(CA)_N$ depends on the number of "pure" (i.e. uninterrupted), tandem repeated motifs present (Weising *et al.* 1995). Below a certain threshold (i.e. 12 CA-repeats in the study referred to by Weising *et al.* 195), the microsatellites behaved mainly in a monomorphic manner. Above this threshold, however, the probability of polymorphism to occur increased with length (Weising *et al.* 1995).

Though the advantages of PCR-amplified microsatellites over other types of markers prevail, there are also some limitations to consider. The optimum primer/species-combinations that give distinct banding patterns have to be developed: the complexity and variability obtained, strongly depend on the repeated sequence motif used (Weising *et al.* 1991). A particular repeat motif requires preliminary testing to first establish its polymorphic behaviour (Weising *et al.* 1995). Often, the identification of informative microsatellite loci, and consequently of suitable primer sequences, is more cumbersome and expensive than the generation of locus-specific polymorphic hybridisation probes (Weising *et al.* 1995). There is also a risk that random short sequences yield bands too numerous and too confused to be analysed (Longato & Bonfante 1997).

A more recent PCR strategy was introduced that combines elements of PCR microsatellite and RAPD technology (Meyer *et al.* 1991; Meyer *et al.* 1993). Microsatellite-primed PCR presents several advantages over RAPD analysis (e.g. no need for sequence information) and of microsatellite analysis (e.g. use of high-stringency annealing conditions leading to reproducible DNA patterns), although this latter contention has been challenged (Weising *et al.* 1995). With this approach, oligonucleotides complementary to microsatellites, serve as single primers. If inversely repeated microsatellites are located within an amplifiable distance of one another, the inter-repeat sequences are amplified (ISSR). Radioactive primers can be used, and the amplification products separated on agarose or highly resolving polyacrylamide gels and visualised by autoradiography (Weising *et al.* 1995). This technique, so-called microsatellite-primed PCR or inter-repeat PCR, has been used successfully to amplify hyper variable repetitive DNA sequences in a wide range of animal, plant, and some fungal species (Meyer *et al.* 1993; Weising *et al.* 1995). ISSRs allowed distinction at interspecific and intraspecific levels in ectomycorrhizal fungi (Longato & Bonfante 1997).

**d) PCR-Ribotyping (PCR-RFLP)**

The technique comprises PCR amplification with a pair of region-specific primers and subsequent RFLP analysis (Poole 2001; Wang & Szmidt 1998). The regions most commonly examined by PCR-RFLP are the rDNA sequences (Poole 2001; Wang & Szmidt 1998). This technique has some advantages to hybridisation-based RFLP

analysis in terms of its speed, sensitivity and specificity. Small initial amounts of material are needed and typing can be performed on crude DNA.

Genetic characterization of various fungi using PCR-RFLP can be found. Isolates of *Colletotrichum nupharicola* D.J. Johnson, Carris & J.D. Rogers and *Col. nymphaeae* (Pass.) Aa, proved identical with RFLP-ITS (Johnson *et al.* 1997). This type of marker was also used to assess the genetic variability of *Col. lindemuthianum* (Sacc. & Magnus) Briosi & Cavara, a fungus lacking a sexual stage. Pathogenicity could be correlated to one of the two groups defined by PCR-RFLPs (Fabré *et al.* 1996). ITS-RFLPs enabled Okabe *et al.* (1999) to place isolates of *Sclerotium* into different groups that corresponded with geographical origin and growth responses of isolates. Even though the groupings correlated with species concepts of *Sc. rolfsii* Sacc. and *Sc. delphinii* Welch, mating studies suggests that molecular groupings defined sub-specific groups. Low levels of discrimination between different species of morels, obtained with both the ITS/RFLPs and microsatellite PCRs, indicated that the high morphological diversity of morels does not correspond to a particularly high DNA polymorphism and reinforced the idea that only a few good species exists (Buscot *et al.* 1996).

**e) Sequencing**

Nucleic acid sequences of DNA were previously preferably determined by the chain termination method since this is faster and simpler than the chemical method (Towner & Cockayne 1993). The automated process surpassed both of these laborious methods and relies on discriminating fluorescent dyes attached to the nucleotides in the DNA (Towner & Cockayne 1993). The sequence is established by passing the nucleotides under a fluorescence detector (Towner & Cockayne 1993).

The real strength of molecular sequence data seems to be the recognition of monophyletic groups and not necessarily determination of taxonomic ranks (Hawksworth & Mouchacca 1994). Sequence data are widely regarded as the data of choice for phylogenetic reconstruction because they contain the maximum information possible about the primary structure of a gene, they can be analysed by a variety of methods, and they form an expanding, accessible database (e.g. Genebank; Hibbett 1992).

Analysis of sequences from different parts of the genome of the genus *Pneumocystis* (Ascomycota) suggested different affinities (Hawksworth & Mouchacca 1994). The goal of a particular study should therefore predict which part of the genome to target. Resolution levels of specific regions are:

5S rRNA gene for larger groupings on approximately family level (Hawksworth & Mouchacca 1994)

18S rDNA gene to define higher rankings within the kingdom fungi but not below the level of order (Kurtzman 1985; Mitchell *et al.* 1995; Taylor *et al.* 1993)

25S rRNA gene seem to be the most informative to separate species (1-5% difference) Mitchell *et al.* (1995), but can also resolve other taxonomic ranks i.e. 1% within a species and 3 to 20% between genera (Kurtzman & Robnett 1993; Mitchell *et al.* 1995). Conflictingly Berbee and Taylor (1993) indicated that 25S rRNA gene comparisons could not resolve the phylogeny of closely related species because of the highly conserved nature of the sequences.

ITS and IGS regions can resolve levels from species to class (Mitchell *et al.* 1995)

ITS1 region (between 18S and 5S) and ITS2 (between 5.8S and 25S) can resolve variation at generic levels (Berbee & Taylor 1993).

Alignment of sequences is a critical part of this type of analysis (Hibbett 1992). Usually sequence alignment is performed with the aid of computer programs that optimise similarity between sequences (Hibbett 1992). These methods assign penalties for mismatches and gaps and construct an alignment that minimizes the overall penalty. Sequence editors allow for interactive alignment of sequences by eye (Hibbett 1992; Holst-Jensen, Kohn & Schumacher 1997; Liou & Tzean 1997). Even though this seems to be too subjective an approach, sequences can be aligned unambiguously: some authors suggest that sequences that cannot be aligned by eye should not be used in phylogenic analysis (Hibbett 1992).

Correlation of sequencing data and specific morphological features are possible. A reliable feature to separate the Homobasidiomycetes from the Heterobasidiomycetes, the presence or absence of dolipore septa, was correlated with sequencing of 5S rDNA (Klich & Mullaney 1992). The mitosporic *Fusarium nivale* (Fr.) Ces. was transferred to the genus *Microdochium* after sequencing the 28S rRNA region of 52 strains of the hyphomycetous but no distinction could be found between forma speciales or races (Manicom *et al.* 1990). In the Pezizales (Ascomycota) the 5.8S coding region was too conserved to resolve lower-level relationships therefore sequences from variable regions such as the inter-transcribe spacers (ITS) had to be used (Kimbourgh 1994). The ITS2 region was used because it was similar in length amongst the species tested and correlated with the septal pore ultrastructure (Kimbourgh 1994). Holst-Jensen *et al.* (1997) used sequences from the nuclear rDNA and ITS regions to group genera in the Sclerotiniaceae after correlation of this data with structure of sclerotia. In the Pyrenomycetes 18S gene sequences from various species were linked to spore discharge being either forcible or not (Mitchell *et al.* 1995). Kurtzman and Robnett (1993) found ascospore shape to be the least reliable character for separating families and genera but that differences in septal types and coenzyme Q, provided better correlation with molecular sequence data.

Some strains show 100% sequence relatedness and still display morphological differences (Kurtzman 1985). This phenomenon can be explained by the analogy to the human eye colour gene. This gene, which has somewhat different sequences in individuals, does not affect fertility (Kurtzman 1985). Such small morphological differences in fungi can be contributed to small mutations within the majority of conserved DNA bases. Kurtzman (1985) therefore feels that name changes based on evidence from molecular studies is capricious but necessary if fungal taxonomy is to reflect phylogeny. To date ribosomal RNA sequencing and analysis have not helped to clarify the phylogeny of the mitosporic fungi at species level because of the difficulty in assessing sequence variation between closely related species (Mitchell *et al.* 1995).

### 2.5.5 Allele combinations

The ability of specific markers to disclose allele combinations is of importance in the information that can be expected from such an analyses. No single marker system will distinguish all the different alleles, which may occur at a locus (Duncan *et al.* 1998). Markers are of two types, either referred to as dominant, such as RAPDs and AFLPs, or as codominant, such as RFLPs and SSRs (Duncan *et al.* 1998). In dominant markers, each band observed represent a locus as either present (allele A) or absent (allele a). A fragment representing the homozygote (AA) or heterozygote (Aa) will be revealed, but the homozygote (aa) will not be revealed. It is impossible to discern between homozygosity and heterozygosity of a dominant allele, that is AA from Aa. If a band is amplified in some but not all individuals, it is polymorphic except in diploids where it is not possible to determine whether it is homo- or heterozygotic. ISSR markers are interpreted as dominant markers similar to RAPDs (Wolfe 2000). With ISSR markers, it is assumed that the priming loci on either side of the band are, in fact, present. The absence of a band can result from a lack of the priming site, mutations on either side of the site, structural rearrangement during meiosis or insertion or deletions altering the fragment size (Wolfe 2000).

Using a codominant marker (two alleles of different size), the heterozygous state (AA') can be discerned for both homozygous states (AA and A'A'). Each allele is therefore revealed as a unique band in SSRs or a number of bands in RFLPs (Duncan *et al.* 1998). In haploids, as is the vegetative state of most fungi, codominant alleles for a single locus is mutually exclusive, while in heterozygous diploids, both are represented (Duncan *et al.* 1998). Codominant markers would be the marker of choice for population studies such as determination of isolates of the same fungal species in a common host, geographical origin or distribution of isolates in a particular region (Duncan *et al.* 1998).

### 2.5.6 Resolution levels of methods and choice of marker

The various molecular methods available offer a considerable range in discriminatory power, and different levels of genetic variation can be detected depending on the method

used (Weising *et al.* 1995). Bruns *et al.* (1991) and Kohn (1992) provided particularly useful diagrams, summarizing the expected resolution of most molecular markers. Since equivalent taxonomic categories of different lineages often vary in terms of the level of molecular divergence, a given study will almost always require a preliminary assessment (Bruns *et al.* 1991). Kohn (1992) also suggest testing the selected approach and criteria to consider are the sample size, out-group selection and eventual appropriate analysis of the data. Sample selection should determine resolution above and below the required level. For example, resolution at species level should include infra-specific variation (i.e. pathotypes), inter-specific variation in the same genus, and inter-specific variation in a closely related genus or genera that will be the out-group. Sampling of several specimens, including type specimens, is important and an expert should confirm their identities. Out-groups should be as closely related to the study group as possible (Weising *et al.* 1995). Obvious additional considerations are cost, time and reliability of the selected technique (Weising *et al.* 1995).

In most cases, DNA fingerprinting has proved more sensitive in detecting genetic variation than other methods mentioned above, and is highly reproducible (Weising *et al.* 1995). PCR based techniques would be the most time efficient (Weising *et al.* 1995). Weising *et al.* (1995) stated that irrespective of the type of molecular marker used in a particular species, they usually appear to evolve in a correlated way. In most cases, consistent results can be obtained, or low levels of variation can be confirmed, if more than one type of marker technique is applied to investigate the same material (Weising *et al.* 1995). Results will then give a more comprehensive understanding of the biological process active within the group studied (Weising *et al.* 1995).

**a) Characteristics of optimal DNA-based markers**

To be sufficiently informative markers needs to be highly polymorphic, represent a significant part of the genome, provide the correct resolution levels and yield consistent and repeatable results. Numerous different microsatellites are currently available and offer such good markers. Only small amounts of DNA template are needed since satellites can be amplified with PCR. The methodology is simple and fast with no need for radiolabelling or mapping. In addition, satellites are widely, and to a large extent, evenly dispersed throughout the genomes of fungi. They are inherited in a co-dominant fashion, which allows discrimination between homo- and heterozygous states, and are therefore ideal for studies of population genetics (Weising *et al.* 1995).

## 2.6 Methods of taxonomic reconstruction

### 2.6.1 Morphometrics

The fungi are an extremely diverse group and it is unlikely that any one current method would be appropriate for either distinguishing all taxa or developing phylogenetic schemes for all major classes (Klich ) jective in processing relative

characters is to determine similarity or dissimilarity, expressed as "distance" between the various entities studied (Swofford & Olsen 1990). The visual presentation of distance trees or dendrograms, will have much greater resolution when more than one set of data i.e. morphological with molecular datasets or more than one type of molecular analysis, are included in the computation (Mitchell *et al.* 1995). Mueller and Gardes (1991) proved with their study on ectomycorrhizae that a multifaceted approach is excellent in resolving taxonomic problems. They included RFLP analysis of mtDNA and rDNA and could correlate this data with intersterility groups and morphology of basidioma.

Phylogenetic reconstruction (not intended in this investigation) might be beyond the scope of a particular study. When attempting to find the resolution level of a character, it would be irrelevant to do a parsimony analysis of molecular data of isolates representing one species (Kohn 1992). In such a study, a distance matrix or a phenogram (more visibly accessible than a matrix) would better represent the data (Kohn 1992).

**a) Cluster analysis**

Kaufman and Rousseeuw (1990) define cluster analysis as the art of finding groups in data. It is a multivariate analysis technique that in an objective manner organizes information about variables in relatively homogenous groups or "clusters" (Ramenofski 1999). The eventual goal is that clusters should be highly homogenous internally and highly heterogenous externally (members are not like members of other clusters) (Ramenofski 1999). Four basic steps should be followed in such an analysis. First collect the data, secondly generate a similarity or distance matrix, thirdly decided on the number of clusters and lastly interpretation of the clusters generated (Ramenofski 1999).

The eventual output of this process is a visual presentation of the data in a dendrogram or tree diagram. The distance matrix is a table in which both the rows and columns are the units of analysis and the cell entries are a measure of similarity or distance for any pair of cases (Van Rijsgergen 1979).

Any of several methods can be used to create similarity or distance in a phenogram and yields a more correct classification than the other (Kohn 1992). Two main types of clustering methods are recognized:

a) Partitioning methods
b) Hierarchical methods, with the latter mostly used by systematic biologists (Kaufman & Rousseeuw 1990).

It is permissible to try several algorithms on the same data, because cluster analysis is mostly a descriptive or exploratory tool in contrast with statistical tests, which are carried out for inferential or confirmatory purposes (Kaufman & Rousseeuw 1990). To optimise the results, the number of clusters to keep and the stability of the cluster should be considered. A [illegible] if clusters should agree with

existing or expected structures, or if clusters on subsets of data when added, still emerge consistently (Ramenofski 1999). Dabinett and Wellman (1978) conducted a study to develop and aid the identification of different anamorphic fungi and Ascomycota. They used various ordinary clustering techniques with a selection of 98 characters. These characters represented the morphology, conidium ontogeny, and physiology of the cultures investigated. However an unknown isolate could be assigned to any of 10 genera depending on which characters were considered the most diagnostic. The study did provide evidence to support a classification based on conidium ontogeny (Dabinett & Wellman 1978).

Kohn (1992) suggest three approaches: matrix plots, global alignment, and local alignments. She particularly referred to the popularity of the distance matrix clustering technique known as "un-weighted pair group method using arithmetic averages" (UPGMA), which is especially well suited for evaluating data in a preliminary study. Hillis (1987) states this algorithm is in fact inappropriate for reconstructing phylogenies and that most users can therefore only present their data as hypothetical. Mid-point rooting algorithms should be avoided and more appropriate methods such as out-group and Lundberg rooting should be used (Hillis 1987).

A table of means and variances of the clusters with respect to the original variables shows how the clusters differ on the original variables (Van Rijsgergen 1979). Discrimination analysis on membership/non-membership in a cluster will show which variables contributed the most to definition of a cluster (Van Rijsgergen 1979).

**b) Similarity coefficients**

Cluster analysis is based on similarity coefficients (Ramenofsky 1999). Similarity between organisms can be determined by computing similarity (or distance) and preparation of a distance matrix of pair wise comparisons (Kohn 1992). The various mathematical methods that can measure similarity are grouped in four broad categories – which are coefficients of

- Distance: measures dissimilarity
- Association: measures agreement or similarity
- Correlation: measures proportionality and independence between operational taxonomic units (OTU)
- Probabilistic similarity: measures homogeny by partitioning

Sneath and Sokal (1973) do not recommend any of the above four categories as more valuable to taxonomy since comparative studies did not define any particular choice. However, in studies such as this one where multistate characters are used, comparisons should focus on the degree of disagreement rather than just state a match or mismatch. Due to complications elaborated upon by Sneath and Sokal (1973), it might also be necessary to use a probab s. They do recommend that a

variety of similarity coefficients be used in combination with several clustering methods, and a judgement made on the robustness of similarity measures and on the relative frequency with which various taxonomic structures tend to recur.

Two coefficients are particularly useful for analysis of molecular data. Their popularity is due to simplicity and their specific treatment of character matches (Kohn 1992). These include Jaccard's coefficient that does not consider negative matches, and Dice's coefficient that weights matches more heavily than mismatches (Kohn 1992). The Nei and Li coefficient has often been used in measurement of similarity of RFLP patterns in which the number of shared fragments are compared to the total number of fragment present between both of the two isolates investigated (Mills 1993).

Errors that can affect resemblance or similarity coefficients are erroneous sampling of characters, sampling error of the taxonomic units and observational errors (Sneath & Sokal 1973). Low levels of resolution can be contributed to limited taxa sampling (Mitchell *et al.* 1995). In general, evolutionary trees obtained with data from several loci match those based on morphology (Mitchell *et al.* 1995). A higher resolution can be obtained by using more than one data set and including other characters such as morphology (Mitchell *et al.* 1995).

**c) Sufficient variables and measurements**

There is no exact answer to what the correct sample size for any variable should be (Parr Rud 2000). Similarly, there is no generally valid answer to the required number of characters necessary for a stable classification (Sneath & Sokal 1973). Sneath and Sokal (1973) argue that each different set of characters will yield somewhat different phenetic information and that phenetic similarity is not a single quantity, but a shifting concept depending on the method of measurement and the character. The usual postulate is that as the number of characters sampled increase, the value of the similarity coefficient becomes stable. When large numbers of characters are measured, the estimate of similarity obeys what might be described as a principle of inertia: as more and more characters are added, it takes an increasingly large number of characters with quite different phenetic information to alter appreciably a given estimate of similarity. Sneath and Sokal (1973) further state that classifications will change as more characters are added but will eventually converge toward a similar structure.

Sample size in molecular studies tends to be much smaller than in morphological studies because of expense, or the availability of specimens (Hillis 1987). Because systematics is a relatively poorly funded subdiscipline of biology, costs of molecular studies can be severely prohibitive (Hillis 1987). Hillis (1987) demonstrated that increased sample size and increase geographical representation do not necessarily translate into increased phylogenetic information. As the ratio of fixed to polymorphic homologies decreases, so does the need for increased sample size (Hillis 1987).

**d) Preparation of characters for computation**

The extensive publication of Sneath and Sokal (1973) provides in-depth information on character analysis in numerical taxonomy of organisms, including analysis of discrete or descriptive characters (i.e. morphological characters) (Sneath & Sokal 1973). Depending on their nature, characters are expressed in various ways to facilitate computing (Sneath & Sokal 1973). Two basic types are defined, namely two-state characters which are either present or absent, and multistate characters (Sneath & Sokal 1973). Multistate characters are either quantitative or qualitative. The former has states that may be ordered amongst others in magnitude, or continuous variables, rank orders or percentages and are therefore also numerical. Qualitative multistate characters cannot be ordered but may be illustrated by symbols. With these type of characters one can only state that characters are different but no deduction can be made if the difference between say a and b is greater or less than between c and d (Sneath & Sokal 1973). A symbol for missing or unknown values should also be incorporated.

It is often the case, such as with this study, that data sets contain a mixture of character types (Kaufman & Rousseeuw 1990). Separate cluster analysis for each set of variables might end up not being the same, which can result in problems when attempting to reconcile them (Kaufman & Rousseeuw 1990). Rather than reducing all to binary data, data should rather be combined in a single proximity matrix (Kaufman & Rousseeuw 1990). This approach expresses data as dissimilarities of interval scale and can be processed with Gower's formula (Kaufman & Rousseeuw 1990).

Kaufman and Rousseeuw (1990) illustrate that a change in measurement unit can profoundly affect the outcome of a cluster. To avoid dependence on the choice of measurement units, there is an option of standardizing data that converts the data to unitless variables. Kaufman & Rousseeuw (1990) suggest that this should be avoided where possible, since the clustering effect may become dampened. They suggest the use of the reasonably robust mean absolute deviation to recalculate all variables. They also warn that when variables have an absolute meaning, they should not be standardized.

Variables that do not contain any relevant information should be excluded since they can make clustering less apparent (Kaufman & Rousseeuw 1990). Such variables add a lot of random terms in distance, thereby hiding the useful information provided by other variables (Kaufman & Rousseeuw 1990). Selection of "good" variables often involves trial and error.

## 2.6.2 Phylogenetic reconstruction

Swofford and Olsen (1990) provide an overview and criticism of available techniques for phylogenetic reconstruction, as well as basic approaches to the analyses of most types of molecular data for systematics (Kohn 1992). Kohn (1992) mentions four categories of

techniques to estimate evolutionary trees. They are distance-matrix, parsimony, invariants, and maximum likelihood methods. It is essential to statistically test phylogenetic trees (Bruns *et al.* 1991). This can be done with re-sampling methods such as "jackknifing" and "bootstrapping" (Hibbett 1992; Kohn 1992). Indeed, it is in cases where molecular phylogenies conflict with other available evidence that it is most important to address the question of statistical significance (Bruns *et al.* 1991).

**a) Cladistics methods**

Cladistics is the formation of groups based on nested sets of derived characters. This methodology is only gradually been used by non-molecular fungal systematists (Hawksworth & Mouchacca 1994). Cladistics, a parsimony analysis, is discussed in detail by Kitching *et al.* (1998). According to Hawksworth & Mouchacca (1994) it is essential to know the pitfalls, limitations and potential of a technique when it comes to interpretation. They suggest that it would be more honest to present all equally parsimonious trees rather than a computer consensus tree or one selected by the investigator. Patterns of relationship revealed by cladistic studies on morphological data sets produce hypotheses that can then be tested by molecular methods or any other independent additional character set. Cladistics has come to the fore in the analysis of sequence data and is used almost without exception in the generation of molecular phylogenies (Hawksworth & Mouchacca 1994). Correlation with prior phylogenetic hypotheses based on morphology, ecology, physiology, or other molecular data may provide additional support for molecular phylogenies (Bruns *et al.* 1991).

**b) Conflict resolution**

Conflicts are sometimes encountered between different sets of data but fortunately some methods are available to resolve them (Taylor *et al.* 1993). The first step in conflict resolution is to use statistical methods to confirm that a conflict truly exists. Resolution of the conflict requires careful examination of the data supporting phylogenies, be they molecular data or morphological data (Taylor *et al.* 1993). False conflicts might arise with different assumptions about the evolutionary process, differences in design or differences in methods of analysis. Conflicts may arise because of inadequate sample size (Hillis 1987). If the methods of analysis are appropriate for both but the two studies still differ, the conflict should be considered real (Hillis 1987).

Fungal systematists have different concepts of species: some only accept species when morphologically distinct, and others consider species when some minimal level of genetic divergence can be demonstrated (Berbee & Taylor 1993; Hills 1987). A criticism levelled against both points of view is that evolutionary lineages may not show any particular level of divergence at either the morphological or molecular level. Complete reproductive isolation (a condition of most species concepts) can be achieved by simple changes that may not affect morphological or a particular measure of genetic

divergence (Hillis 1987). Since polymorphic genes might still be found in derived species, and these species are still capable of exchanging genes, it is more appropriate to do a population genetic analysis involving several different loci than a phylogenetic analysis of one locus (Berbee & Taylor 1993). According to Hillis (1987) some studies proved that the use of rate-independent methods of analysis (independent of the molecular-clock hypothesis), eliminate false assumptions about the evolutionary process.

Real conflicts may also be reconciled by consensus and combination techniques such as the use of Adams consensus, strict consensus and majority consensus methods. Often, however, several different techniques are required to maximise phylogenetic resolution within the group of interest (Hillis 1987). Adams proposed the construction of trees independently for molecular and non-molecular data and then used consensus methods to form a single hypothesis (Hibbett 1992). Characters should be treated as independent but if weighting of characters should be applied to resolve conflict, individual characters should be weighted only after rigorous evaluation, rather than weighting all members of a class of characters (Hibbett 1992). Weighting of characters is possible with a variety of phylogenetic reconstruction packages such as PAUP for cladistic analysis and PHYLIP for maximum likelihood calculations, but such weighting methods are not appropriate for analysis that integrates morphology and molecular data (Hibbett 1992).

# 3 THE CURRENT TAXONOMIC STATUS OF *BIPOLARIS*-LIKE FUNGI

The significance of characters used to delimit the pathogenic helminthosporic species has received considerable attention during the early 1960's (Luttrell 1963, 1964; Shoemaker 1966). Two subsequent, particularly informative studies were those of Alcorn (1983) who investigated the differences of morphological and biological attributes of conidia in many species of *Bipolaris, Drechslera* and *Exserohilum,* and that of Sivanesan (1987) who addressed the similarities between *Bipolaris* and *Curvularia.* The taxonomic developments of the *Bipolaris*-like fungi up to the current status of genera, and the significance of delimiting characteristics, will be presented in this section.

## 3.1 Taxonomic history

The taxonomic history of *Bipolaris, Curvularia, Drechslera and Exserohilum* is complex but has been researched in detail by Sivanesan (1987). A summary of his review of taxonomic developments leading to the delimitation of the above genera is as follows:

The name "*Helminthosporium*", a typographical error by Persoon 1822, is now conserved over the original *Helmisporium* by Link 1809. Subsequently, over approximately forty years, rapid name changes were introduced to the group because of taxonomic refinement. These refinements can be summarised as a progressive change in the emphasis on three basic characters i.e. conidial morphology, conidial germination and teleomorph association.

Initially *Helminthosporium* was split into two subgenera, namely *Cylindro-Helminthosporium* Nisikado for those helminthosporia with straight, cylindrical conidia that germinate by one or more germ tubes from one cell of the conidium, and *Eu-Helminthosporium* Nisikado for those species with fusoid, often curved conidia and bipolar germination. Shortly after this first division of the helminthosporia, the genus *Curvularia* was established and separated from other helminthosporia because of strongly curved conidia of which the median cells are often swollen and darker in colour than the rest of the conidial cells. Subsequently *Cylindro-Helminthosporium* was raised to generic rank and called *Drechslera* by Ito. These anamorphs were later associated with *Pyrenophora* teleomorphs. After 29 years, the subgenus *Eu-Helminthosporium* was eventually also raised to generic level by Shoemaker (1959) who proposed the name *Bipolaris* for this group. This genus was characterized as having fusoid, straight to curved conidia that germinate by one germ tube from each end and are associated with a *Cochliobolus* teleomorph. After extensive studies it became clear that *Bipolaris* sensu Shoemaker was not as well defined as originally expected. The genus contained both species of which conidia displayed protuberant hila and those without them. Furthermore,

some species had associated teleomorphs in *Cochliobolus,* while others were tentatively associated with *Trichometasphaeria.* Even though Subramanian and Jain (1966) suggested that a new genus could be established for those *Bipolaris* species with protuberant hila and *Trichometasphaeria* teleomorphs, they elected to be conservative and rejected the name *Bipolaris* as a synonym of (again) a more heterogenous *Drechslera.* Leonard & Suggs (1974) eventually assigned the group with protuberant hila to the new genus *Exserohilum.* They described the teleomorphs as *Setosphaeria,* to accommodate *Trichometasphaeria* and *Keissleriella* formerly either associated with *Bipolaris* or *Drechslera sensu* Subramanian and Jain. The investigation of hila in many *Curvularia* species confirmed their structure to be similar to those of *Bipolaris* and not *Exserohilum.* Since the establishment of *Curvularia,* species with and without protuberant hila have been associated with the teleomorphic genus *Cochliobolus.* The separation of the helminthosporia as such, has now been established by subsequent, supportive studies.

The genus name *Helminthosporium* is now restricted to a group of saprotrophs growing on woody substrates. Since the status of the genera *Helminthosporium sensu stricto, Drechslera* and their teleomorphic genera is generally accepted (Alcorn 1983; Sivanesan 1987), they will not be subject to investigation in this study.

## 3.2 Significance of characteristics differentiating the genera

### 3.2.1 Host relationships

Based on his own data and those of Drechsler, Ellis, Leonard and Suggs, Luttrell, Nisikado and Shoemaker, Alcorn (1983) documented generic host preferences within the *Bipolaris* complex of fungi. Some species seem to prefer andropogonoid, eu-panicoid and chloridoid hosts that are common in tropical and subtropical regions species (Alcorn 1983) but these grasses are also commonly host to species of *Exserohilum. Drechslera* species strongly prefer festucoid grasses that seem to be restricted to temperate climates, especially in the northern hemisphere. Subramanian and Jain (1966) also mentioned that *Drechslera* are "somewhat more specialized in parasitism" than *Bipolaris.* Specialisation to hosts is known within *Bipolaris:* the ovaricolous *B. ravenelii* and *B. crustacea* are restricted to *Sporobolus* spp. In strong contrast some species have been recorded on so many hosts that they can be regarded as plurivorous and include the species *C. hawaiiensis* (M.B. Ellis) Uchida & Aragaki, *B. maydis, B. sorokiniana, B. zeicola* and *E. rostratum.* Host range preferences in *Drechslera* are therefore clearly different from the group comprising *Bipolaris, Curvularia* and *Exserohilum.* Apart from the ovaricolous species, there seem to be no significant differences in host preferences of the genera *Bipolaris, Curvularia* and *Exserohilum.*

### 3.2.2 Morphological characters

#### a) Shape, colour, ornamentation and size of conidia

When comparing certain morphological features of the type species of *Bipolaris*, *Exserohilum* and *Drechslera*, Alcorn (1983) found these features to be mostly true for other species in the particular genus. Sivanesan (1987) contributed to the information supplied by Alcorn and argued that many aspects of conidial morphology used as distinguishing criteria between *Bipolaris* and *Curvularia* seem to be differences of degree rather than nature.

Conidia of the type species of *Drechslera*, *D. tritici-repentis* (Died.) Drechsler, are essentially cylindrical, do not taper or only taper slightly so towards the base and apex. In the type of *Bipolaris*, *B. maydis*, the conidia are mostly fusoid, sometimes obclavate- or clavate-fusoid, sometimes curved and taper towards both ends. The apical cell is hemi-ellipsoidal, and the basal cell hemi-ellipsoidal to truncate-conic. In the type species of *Exserohilum*, *E. turcicum*, they are ellipsoid-fusiform, sometimes somewhat obclavate, straight or curved. The end cells are like those of *B. maydis* (Alcorn 1983). Alcorn's (1983) study revealed the shape of *Drechslera* conidia to be distinct but that separation could not be made between the conidial shape of *Bipolaris* and *Exserohilum* and there is no significance in conidial colour as a taxonomic criterion.

Shoemaker (1959) clearly drew a distinction between his newly proposed genus *Bipolaris* and the previously described *Curvularia*. The original difference drawn by Shoemaker (1959) is that species within *Curvularia* form acutely curved spores, mostly with a swollen darkened median cell. Subsequent designation of species to any of *Bipolaris*, *Curvularia* or *Exserohilum* regarding curvature of conidia was not applied consistently (Sivanesan 1987). They all contain some species with curved conidia, with the type species of *Bipolaris* (*B. maydis*) being a good example. Alcorn (1983) also stated that the amount of curvature of spores can be changed depending on environmental conditions and that curvature therefore has no value at the generic level. The validity of *Bipolaris* against *Curvularia* can therefore be disputed. Shoemaker (1959) made no mention of teleomorph connection or other features differentiating these two genera. However, no species with disproportionately swollen median cells have been designated to either *Bipolaris* or *Exserohilum* (Alcorn 1983). Sivanesan (1987) argues that the degree of swelling of the penultimate cell is variable within and between species and in at least one *Bipolaris* species, *B. coicis* (Y. Nisik.) Shoemaker, the penultimate cell of conidia is somewhat disproportionately swollen. Similar disproportionately swollen cells are also seen in *B. ellisii* (Danquah) Alcorn and *B. papendorfii* (Aa) Alcorn but they are not usually more pigmented than the other cells. It is accepted though that this characteristic is clearly distinctive in many *Curvularia* species and a useful taxonomic characteristic at species level.

Various reports in the literature state that conidial shape depends on environmental conditions, especially on temperature and nutrition (Alcorn 1983). In both *Drechslera* and *Curvularia* this was confirmed by Alcorn (1983). Shape varies widely between species but was comparatively constant within species (Alcorn 1983). There is also evidence that *Curvularia* conidia vary in size between natural and artificial substrate (Alcorn 1990). In the monographic treatment by Ellis (1966), different ranges and means for conidial dimensions from nature and in culture are given for 11 species. For most of these, the change in size is comparatively minor, there being a tendency for conidia in culture to be slightly smaller. In *C. cymbopogonis* (Dodge) Skolko & Groves the difference is in both a reduction in length and width (Alcorn 1990). Sivanesan (1987) seemed to have ignored this type of variation when describing *Curvularia verrucose* (Tsuda & Ueyama) Sivan. Conidial features within this species vary depending on the age of colonies, ornamentation of conidia, number of septa in the conidia, degree of symmetry or curvature and pigmentation. In a novel approach Alcorn (1990) used the proportion of cell lengths to support separation of two *Curvularia* species, namely *C. andropogonis* (Zimm.) Boedijn and *C. heteropogonis* Alcorn.

**b) Structure of septum and conidial walls**

The similarity between *Bipolaris* and *Curvularia* can again be disputed by the morphology of septa in the conidia. Ellis (1971) reported a difference in septum structure with *Curvularia* as euseptate (true septa) and those of *Bipolaris* distoseptate (pseudoseptate). At first, Alcorn (1983) also drew a distinction between the septal structures of the two genera. He observed with the light microscope that the dark accentuated septa in *Curvularia* become attached to the outer periclinal wall layer and often fracture centrally. Sivanesan (1987) did not agree with Alcorn's findings and states that in all his investigations of *Curvularia*, septa at various stages of maturity were never connected to the outer wall and are therefore, strictly speaking, distoseptate. Distoseptate conidia have cells surrounded by individual sac-like layers distinct from the outer layer of the spores (Sivanesan 1987). Alcorn (1983) further stated that the structure of septa between the two genera differ in that those in *Bipolaris* appear as overlapping circles in contrast to those of *Curvularia*, a feature also observed by Sivanesan (1987) in young conidia of *Curvularia*. Sivanesan (1987) reported that this feature disappears as spores mature. He concluded, after investigating various transmission electron-microscope studies and his own observations that the term euseptate and distoseptate cannot be substantiated and therefore cannot be applied as a distinction between the two genera. The fine structure studies revealed conidia of both genera to be double-layered with an outer thin, pigmented layer, which usually splits under pressure releasing the hyaline contents and a thicker inner hyaline layer. Both

Alcorn (1983) and Sivanesan (1987) concluded that apart from the differences mentioned, the structure of septa in *Bipolaris* and *Curvularia* are fundamentally alike.

Dark- and thickened accentuated septa, is a feature of mature spores and can be found in many species of *Bipolaris, Curvularia* and *Exserohilum* i.e. *C. eragrostidis, B. ravenelii* and *E. rostratum* (Drechsler) K. J. Leonard & Suggs (Sivanesan 1987). These accentuated septa are more prominent in *Curvularia* and *Exserohilum* than in *Bipolaris* (Sivanesan 1987). Septal pores can clearly be seen in *Curvularia* and have been observed in conidia of *Bipolaris* by electron microscopic studies (Sivanesan 1987). The number of septa in conidia can also vary depending on the age of cultures (Sivanesan 1987).

**c) Hilum structure**

The term hilum is applied to the mark or scar around a pore, which indicates the point of attachment to a conidiophore (Hawksworth *et al.* 1995). Alcorn (1983) broadened the concept already applied in a similar fashion to *Cercospora*-like genera, where "thickened" or "darkened" hila are considered significant in separating genera.

Referring to Alcorn's (1987) concept of the term, the hila in the type of *Drechslera, D. tritici-repentis* are inconspicuous, rounded and have no projections or abrupt changes in contour. The pore at the bottom is visible, narrow and might sometimes have a shallow concave indentation where the pore opens to the exterior surface and is darkened around this pore. In *B. maydis*, the basal cell in the region of the hilum is commonly truncate, with the darkened areas in the form of two roughly lenticular spots, on either side of a pore. The pigmented segments are closely oppressed to the outer contour of the wall, across the flattened base and tapering up the side for a short distance. In conidia of *E. turcicum*, the type of *Exserohilum*, the hila are distinctly protruding with an abrupt change in the contour of the wall where it projects. The protrusions are double walled and appear to be a truncate cone inserted into a short cylinder. The inner wall (the cone shaped area) appears to be a continuation of the conidial wall from above the protruding section. If darkened, it can be observed where the inner wall meets the flattened extremity of the protrusion. The outer wall of the protrusion meets with the inner wall at the point where the contour changes. Alcorn (1983) concluded that the hilum structure in the three genera is distinct.

The hilum structure seems to be predictive of the teleomorphs of helminthosporia, with hila inserted in a truncate basal section of the wall (*Bipolaris*), and for conidia, with flush hila (*Drechslera*) (Alcorn 1983). *Bipolaris* species are only known to have Cochliobolus teleomorphs and *Drechslera* species *Pyrenophora* teleomorphs. However, conidia with protuberant hila have been related to two different teleomorphs namely *Setosphaeria* and *Cochliobolus* (Alcorn 1983). Conidia in *B. micropus* (Drechsler) Shoemaker also have protuberant hila. Luttrell (1958, 1973) detected an

immature *Cochliobolus* in cultures of *B. micropus*. Many species of the genus *Curvularia* closely related to the *Drechslera,* e.g. *C. cymbopogonis* (Dodge) Groves & Skolko also have protuberant hila and are also associated with *Cochliobolus* teleomorphs (Sivanesan 1987). Subsequent studies refuted this anomality: the hilum structure of *B. micropus* and those of species of some *Curvularia* proved to differ in structure from those of *Exserohilum* (Luttrell 1977; Alcorn 1983). Alcorn states that the resolution offered by light microscopy makes it very difficult to draw accurate conclusions regarding the fine structure of hila even at highest magnification. The protuberant hilum is therefore still considered one of the major distinguishing characters for the genus *Exserohilum* (Alcorn 1983; Sivanesan 1987).

Honda and Aragaki (1978) studied several *Exserohilum* species and found the formation of protuberant hila to be temperature as well as light and dark cycle dependant in culture but that these structures are consistently formed on the host. Honda and Aragaki (1978) criticised Langdon and Gibbs (1971) stating that their report lacked sufficient experimental data.

**d) Conidiophores**

Conidiophore structure is similarly in the type species of *Drechslera, Bipolaris* and *Exserohilum*. The roughening of the conidiogenous nodes can occur in both *Bipolaris* and *Curvularia* (Sivanesan 1987). Alcorn did not consider ornamentation or branching of conidiophores as a consistent, and therefore valid, taxonomic feature at generic level.

### 3.2.3 Developmental characters

In nearly all instances, the pioneering work of Luttrell (1963) was confirmed by more extensive investigation by Alcorn's (1983). One exception is the position of the basal septum in the genera with *Cochliobolus* teleomorphs (*Curvularia* and *Bipolaris*) compared to those with *Setosphaeria* teleomorphs (*Exserohilum*).

**a) Conidiogenesis**

Conidiogenesis in *Curvularia, Bipolaris, Drechslera* and *Exserohilum* is subject to controversy: Ellis (1971), Luttrell (1977) and Shoemaker (1962) believe that conidia are formed enteroblastically and can therefore be referred to as porospores. In a separate study of conidiogenesis of *B. maydis* however the process was interpreted as holoblastic, since the outer wall layers were also involved in conidium formation (Sivanesan 1987). Alcorn (1983) drew a distinction between *Bipolaris* and *Exserohilum*, both of which have protrusions on conidia indicating holoblastic conidiogenesis compared to *Drechslera* with the smooth rounded basal contour in conidia indicating an enteroblastic ontogeny. Sivanesan's (1984a) interpretation of conidiogenesis can only be confirmed by ultrastructural investigations.

**b) Septum development**

Alcorn (1983) studied the difference in sequence of septa formed during maturation in *Drechslera, Bipolaris* and *Exserohilum*. He found this process to differ between the genera, but to be significantly consistent within genera. The position of the first-formed septum in developing conidia is usually unambiguous: if formed near the base of the spore, it proved to be that septum which, in the mature conidium, delimited the basal cell. In *D. tritici-repentis* the first formed septum was found to delimit the basal cell that essentially retained its size; the second septum were formed approximately in the middle of the upper cell of the young spore and the third septum formed distal to the second. In *Bipolaris maydis* the first septum was found to be sub-median, with the second septum defining the basal cell, and the third formed toward the apex of the spore. In *E. turcicum*, the first septum forms approximately one third form the bottom of the conidium; the second develops at the apex at about the same distance from the apex as the first from the base. A third septum subsequently forms between these two and is approximately median. Septum development in the three type species is therefore significantly different. Alcorn (1983) also reported that all ovaricolous species occurring on *Sporobolus* hosts (*B. ravenelli, B. crustacea* and *B. cylindrica* Alcorn ) developed their first two to three septa simultaneously. The one ovaricolous species, known exclusively on *Eragrostis* hosts, also displayed synchronous septum formation. Alcorn could correlate the position of the primary septum with teleomorphs although exceptions were observed.

The order of formation of septa as conidia matures follow a similar pattern in both *Curvularia* and *Bipolaris* with only minor variation reported for some species (Sivanesan 1987). In these two genera the first-formed septum is median, the second delimits the basal cell and the third is distal.

**c) Germ tube formation**

The position of germ tubes that develops from conidia of helminthosporic fungi strongly signifies their placement in a particular genus (Alcorn 1983). Germ tubes formed by conidia of *D. tritici-repentis* develop from both intermediate and polar cells (amphigenous) (Alcorn 1983). The number and position of the germ tubes vary, but basal germ tubes consistently develop laterally at a point approximately midway between the hilum and the basal septum. This feature was found to be statistically significant (Alcorn 1983). In *B. maydis*, germination is not amphigenous but consistently polar or monopolar. The origin of the basal germ tube was found to be constant in emerging adjacent to the hilum and growing in the direction of the long axis of the conidium (semi-axially *sensu* Luttrell). Apical germ tubes in *B. maydis* also grow axially (Alcorn 1983). In *E. turcicum* it was not possible to establish a clear pattern: germination is mostly polar but often also amphigenous and germ tubes usually

develops semi-axially. However, in up to 6% of conidia growth of germ tubes were similar to those observed in *D. tritici-repentis* (Alcorn 1983). Often bipolar germinating spores produced conidiophores directly from the germ tube at the apex, notably so in *B. hawaiiensis* (M.B. Ellis) Uchida & Aragaki, *B. australiensis* (M.B. Ellis) Tsuda & Ueyama and *B. spicifera* (Bainier) Subram. In another group of polar germinating species such as *B. cynodontis* (Marignoni) Subram. and *B. oryzae* (Breda de Haan) Shoemaker, the end cells swell into more or less globose vesicles from which germ tubes developed. Examples are the displacement of the hilum during germination in this group, which also varied in magnitude. Alcorn also assigned a third group of species where there were no consistent pattern in number and germ tube position.

Alcorn's (1983) studies confirmed that polarity *per sé* has little value, as a taxonomic criterion at generic level, but that the position and the direction of the germ tube from the basal cell, appears to be a more reliable indicator. It is therefore possible to correlate lateral germ tubes to species with *Pyrenophora* teleomorphs and semi-axial to axial development to species with *Cochliobolus* teleomorphs. In many species of *Bipolaris*, germination often took place only from the basal cells and not polar as previously reported. The point of origin of the germ tube from the basal cell significantly separates *Drechslera* from *Bipolaris* and *Exserohilum*.

### 3.2.4 Metabolites as taxonomic characters

Many secondary metabolites such as toxins and pigments are formed by species of *Bipolaris*-like fungi (Domsch, Gams & Anderson 1980). Olufolagi (1986) and Sivanesan (1987) indicated that no taxonomic significance could be applied to these fungi as far as metabolites are concerned. However, Sivanesan (1987) remarked that toxin production in many species of *Curvularia* has not yet been investigated and until this is done, no conclusion can be drawn on whether they will support a generic synonymy between *Bipolaris and Curvularia.*

Some species of the *Bipolaris*-like complex release pigments in the agar (Ludwig 1957). These are mainly anthraquinones and vary in colour according to isolate, medium used and the conditions in which the cultures are maintained (Sivanesan 1987). The anthraquinones include the red pigment, cynodontin, formed by *Cochliobolus cynodontis* Nelson, *Coch. spicifer* Nelson, *B. euchlaenae* (Zimm.) Shoemaker and *Coch. victoriae,* a dark maroon pigment, caterin, formed by *Coch. cynodontis,* cochlioquinine and luteoleersin formed by *Coch. miyabeanus* (Ito & Kurib.) Drechsler: Dastur and alboleersin formed by *B. leersiae* (Atk.) Shoemaker. An unidentified pink pigment was reported from the mycelium of *Coch. sativus* (Ito & Kurib.) Drechsler: Dastur and was present in all strains of the fungus that were tested (Ludwig 1957). Ludwig (1957) however refuted this when he found that pigment production is greatly influenced by both the carbon and nitrogen source available to isolates. Media containing glucose and nitrogen as nitrate, alanine, l-asparagine, l-

argenine, glycine, or l-proline appeared most conducive to both growth and pigmentation. (Ludwig 1957) reported that helminthosporic species can synthesise more than one related pigment and concluded that similarity of the pigments in appearance and in their absorption spectra in the visible light range would make their use in species differentiation difficult.

### 3.2.5 Teleomorph connections

The teleomorph-anamorph association of genera within the helminthosporia has been established and is widely accepted: combinations prove consistently to be *Pyrenophora* with *Drechslera*, *Cochliobolus* with *Bipolaris* and *Curvularia* and *Setosphaeria* with *Exserohilum* (Sivanesan 1987; Alcorn 1983). The asexual part of the life cycle of these fungi can readily be isolated and cultured in artificial culture. The sexual stage is not commonly encountered and often has to be induced by mating strains (Sivanesan 1987; Alcorn 1978; Leonard 1976; Nelson 1964). The conidial characteristics are more than frequently necessary to verify the identity of very similar teleomorph species (Luttrell 1977). The description of *E. monoceras* (Drechsler) K. J. Leonard & Suggs and its teleomorph *S. monoceras* Alcorn by Alcorn (1978) is a good example: *S. monoceras* is very similar to a previously described species *S. pedicillata* (Nelson) K.J. Leonard & Suggs but the whole fungus can readily be distinguished by the differences in their anamorphs.

## 3.3 Factors influencing appearance of *Bipolaris*-like fungi

Extensive research, resulting in a vast number of publications, has been done on the effects of various environmental and nutritional factors on *Bipolaris*-like fungi. These studies have shown that *Bipolaris*, *Curvularia* and *Exserohilum* species differ in their reaction to environmental changes and that there is also variation between isolates of the same species. Despite the wealth of evidence pertaining to the effects of environmental conditions on sporulation, little effort has been made to develop and use standardised media for comparative studies within this group of fungi (Harding 1975). In this study some factors influencing the appearance of the *Bipolaris*-like fungi will be addressed.

**a) Aeration**

Studies regarding the influences of carbon dioxide and oxygen on the helminthosporia mostly concerned prevalence or survival in soil, or the ability to colonise substrates (Domsch *et al.* 1980).

Macauley and Griffin (1969) investigated the mycelial dry weight production of some helminthosporic fungi at carbon dioxide ($CO_2$) concentrations from 0%, 10%, 15% to 20%. $CO_2$ was passed through solutions inoculated with helminthosporic fungi. When a buffered series of aqueous solutions is subjected to a continuous supply of carbon dioxide with a constant concentration. The concentration of bicarbonate in the solution

increases tenfold with each unit increment of carbon dioxide and affects the pH of the solution. In *Coch. spicifer* a slight increase in dry weight production was recorded at pH4 with decreases of 33% at pH5 and 63% at pH6 and pH7. In contrast, *Coch. sativus* and some species of *Curvularia* produce more dry weight mycelium with a change in concentration of carbon dioxide from 0% to 10% (Macauley & Griffin 1969).

**b) Temperature**

The influence of different temperature on helminthosporia has been well documented and indicates differences between species. Optimal growth rate of *C. lunata* (Wakker) Boedijn is between 24-30°C (Ellis & Gibson 1975), in *B. sorokiniana* (Sacc.) Shoemaker between 27-28°C (Domsch *et al.* 1980), in *B. spicifera* between 30 -33°C and in *C. pallescens* at 25°C (Domsch *et al.* 1980). The lowest temperature required for growth is mostly unknown. *Bipolaris sorokiniana* can survive extreme fluctuation in temperature from -94 to 23°C for up to 35 days (Domsch *et al.* 1980). Temperature also influences various other features of these fungi such as rate of germination, number and length of germ tubes, germ-tube branches, conidium production, conidial survival and conidium size (Domsch *et al.* 1980).

**c) Humidity**

Humidity seems to have little influence on the prevalence or survival of these fungi in soil (Domsch *et al.* 1980). This independence was observed in *C. geniculata* (Tracy & Earle) Boedijn, *H. sativum* Pammel and *H. pedicellatum* Henry: these species survived equally well for a 1-year period when stored at an atmosphere of 100% or 0% relative humidity (Mathre 1969). High moisture levels, however, favour the death of conidia and were noticed in *B. sorokiniana* and *Coch. sativus* (Old 1967).

**d) Light**

Conflicting reports can be found on the influence of light on helminthosporic fungi. The production of conidia in *B. sorokiniana* has been reported to be accelerated in light and to have no influence (Domsch *et al.* 1980). Another report indicates the positive influence of light on the sporulation of *C. lunata* (Domsch *et al.* 1980). Sivanesan (1987) reported light not to be essential for the production of conidia of these fungi. He investigated sporulation after incubating different isolates at 20-26°C in the dark, in artificial light supplied by fluorescent light and in near UV light on a 12h light/dark diurnal cycle. A similar report is that of (Harding 1975) who states that light probably has no influence on sporulation in some *Bipolaris* species.

Luttrell noticed an effect of light on conidium size in *B. setariae* (Sawada) Shoemaker (Harding 1975) but not in *B. spicifera* (as *C. spicifera* (Bainier) Boedijn; Ruppel 1974). Tinline *et al.* (1962) speculated that difference in size and pigmentation of structures such as spores might account for the difference in resistance of helminthosporia to ultraviolet radiation.

**e) pH**

*Bipolaris*-like fungi can grow at wide ranges of pH. *Bipolaris spicifera* can grow between pH4 to pH9.2 (Domsch *et al.* 1980). Good growth and sporulation of *B. sorokiniana* was observed in the pH range 4 to 9 with longer conidia being formed in the more acid pH range (Harding 1975). Initial pH (and sucrose concentration) of nutrient media resulted in markedly affected sporulation and conidial characteristics in four *Bipolaris* species, *B. sorokiniana, B. zeicola, B. setariae* and *B. maydis* (Harding 1975). In *B. sorokiniana* and *B. zeicola* sporulated well at all pH levels. In the species *B. sorokiniana, B. zeicola, B. setariae* and *B. maydis*, conidium length and number of septa per conidium significantly decreased as the pH of the medium was increased, although some isolates of *B. sorokiniana* showed great variability in conidium size (Harding 1975). In spite of great variability within these species, it appears that optimum sporulation and production of "typical" conidia occur within the range pH4 to pH6, although the restricted vegetative growth at pH4 may limit the useful application of this feature (Harding 1975). Importantly conidium width was less markedly affected (Harding 1975). Dry weight production of both *Coch. spicifer* and *Coch. sativus* was most noticeably affected at high pH values when testing the influence of bicarbonate ion and carbon dioxide on the effect on growth of fungi (Macauley & Griffin 1969).

**f) Minerals**

There appear to be differences in mineral requirements of helminthosporic fungi but no comparative studies could be found. *Bipolaris sorokiniana, C. lunata* and *C. geniculata* can oxidize various magnesium $Mn^{2+}$ salts (Domsch *et al.* 1980). Growth of *B. sorokiniana* is stimulated by traces of zinc, manganese, and iron ions but survival of *B. sorokiniana* in soil is negatively influenced by the addition of manganese, boron and zinc ions while *C. lunata* and *C. pallescens* are tolerant to high salinity (Domsch *et al.* 1980).

**g) Nitrogen metabolism**

Numerous amino acids and some organic nitrogen sources supported growth of *Bipolaris*-like fungi but they differ in specific nitrogen source required for optimum growth (Domsch *et al.* 1980). Nitrates were reported most suitable for *B. spicifera*, but *B. sorokiniana* grew best on organic nitrogen sources (Domsch *et al.* 1980). Asparagine, urea proline, valine, serine and histidine all support good growth of *B. sorokiniana* (Domsch *et al.* 1980). Ammonium nitrate and ammonium sulphate is not good sources of nitrogen for *Coch. sativus* (Garrett 1975).

Various features of *Bipolaris*-like fungi are dependent on the particular nitrogen source available. Some nitrogen sources, such as ammonium salts, influence sporulation and conidium dimensions on *B. spicifera* (Harding 1975). There was little evidence of differential pathogenicity with inoculum of *Coch. sativus* grown on media containing

different amino acids (Garrett 1975). Four isolates of this species were consistent in their relative growth patterns. Inocula obtained from cultures produced on different nitrogen sources caused different types of lesions on barley and wheat, and were particularly striking when from media containing methionine and isoleucine (Garrett 1975). However, Hrushovetz (1957) observed that addition of 0.1% of any one of the amino acids alanine, arginine, histidine, isoleucine, leucine, methionine, serine, threonine or tryptophane to Cszapek's agar, decreased virulence in *Coch. sativus*.

D-Amino acids compared to their equivalent L-isomers, did not induce consistent reactions in growth (Harding 1975). Isolates of *B. sorokiniana* grew to some extent on the D- isomers of 15 amino acids supplied as the sole nitrogen source (Harding 1975). Conidia formed on D-isomer amino acids were often shorter than on the equivalent L-isomers (Harding 1975). D-Histidine did not support growth of any isolate (Harding 1975). The four isolates of *B. sorokiniana* tested were fairly consistent in their general behaviour. D-amino acids are also suspected of playing a role in the production of phytotoxic metabolites in *B. sorokiniana* (Harding 1975) and reduce the virulence of this fungus to wheat seedlings (Hrushovetz 1957; Domsch *et al.* 1980).

A similar effect of some D-amino acids on conidium morphology in *H. oryzae* Breda de Haan, was reported (Domsch *et al.* 1980). On the different nitrogen substrates there was a great range in the length in conidia, less difference in width of conidia but both dimensions varied considerably between isolates. There was no consistent relationship between lengths on conidia produced on D-and L-isomers of the same amino acid. The most marked difference in conidium length were found in cultures grown on both buffered and non-buffered phenylalanine, where conidia produced on the L-isomer were consistently longer than those produced on the D-isomer. All isolates of *H. oryzae* failed to produce conidia on D-arginine, D-valine and D-glutamin. Conidia produced on D-ornithine were longer than those produced on L-ornithine, although this response was less marked on buffered medium. Generally, the numbers of pseudosepta per conidium were higher in conidia produced on the L-isomer of individual amino acids although this increase usually paralleled the increased length of conidia.

**h) Carbon metabolites**

Helminthosporic fungi can utilize a great number of different carbon sources (Domsch *et al.* 1980). *Curvularia lunata* can grow on a large number of alcohols and their esters used as plasticisers (Domsch *et al.* 1980). A comprehensive study by Clarke (1972) on the utilisation of sugars by *Coch. sativus* agreed with previous findings of Lilly and Barnett for various groups of fungi, but the comparison was not extended to other *Bipolaris*-like species. *Cochliobolus sativus* was able to use L-arabinose, D-ribose, L-sorbose, D-xylose, cellobiose, melibiose, raffinose, inulin, lactose, maltose dextrin, soluble starch, pectin, D-glucose, D-fructose and D-galactose and yielded various

amounts of mycelium or radial growth rates. The amount of mycelium produced by *Coch. sativus* on some of the carbon sources, varied considerably even though the same concentrations were used. The response of the isolates on most carbon sources was fairly consistent.

As with growth on different nitrogen sources various species differ in their preference regarding carbon sources. Of six monosaccharides tested, D-fructose proved to be most suitable carbon source for growth of *C. lunata* (Domsch *et al.* 1980). Mannitol, cellobiose, lactose, maltose, sucrose, raffinose and starch can also be utilized by this species (Domsch *et al.* 1980). Dextrin was found to be more suitable than 18 other carbon sources tested, for growth and sporulation of *B. sorokiniana* (Clarke 1972). Utilisation of D-arabinose by this species was not good (Domsch *et al.* 1980). *Bipolaris spicifera* grow and sporulated well on sucrose, D- and L-xylose, D-galactose and maltose and to a lesser extent, glucose and mannose, while D- and L-sorbose, mannitol, raffinose, salicin, inulin and L-rhamnose gave poor results (Domsch *et al.* 1980). The absolute concentration of carbon required by *B. sorokiniana* were found to be relatively small, at 0.3-3.0% of the nutrient medium (Garrett 1971).

Helminthosporia tend to sporulate less abundantly on higher concentrations of a particular carbon source, while the conidia increase in length as the concentrations decrease. This was reported in *B. setariae, B. zeicola, B. oryzae, B. sorokiniana* and *B. maydis* on sucrose (Harding 1975). In most cases, lack of sporulation was noted on glucose and sucrose at concentrations of 30 to 40g / l. *Bipolaris setariae* showed much less tolerance with only one of six isolates producing conidia at sucrose concentrations higher than 5g / l (Harding 1975). Some studies also showed that conidium length and number of septa per conidium decreased as the glucose concentration of the medium was increased. It appears that low levels of sucrose or glucose encourage abundant production of "typical" conidia although vegetative growth is rather restricted (Harding 1975). This change in length and septation was reported by for *B. sorokiniana* (Harding 1975). In *B. zeicola* conidia of maximum length and number of septa were produced on sucrose concentrations of 5g / l. An exception is *B. oryzae* that sporulated well at sucrose and glucose concentrations of 30 and 40g / l (Harding 1975). Percentage germination of *B. sorokiniana* conidia however, increased with the addition of either sucrose, maltose, D-galactose, D-fructose, D-mannose, D-glucose or L-arabinose to the nutrient medium (Mathre 1969).

**i) Lipids**

Little information are available but it is known that respiration of spores of *H. sativum* is inhibited by fatty acids while the respiration of *H. pedecillatum* spores is stimulated to a certain extent by acetate. It is not clear whether this indicates that *H. pedicellatum* spores are better able to use lipids as an energy source than *H. sativum* (Mathre 1969).

**j) Vitamins**

Different vitamins result in differences in growth and sporulation of *C. lunata*. Thiamine hydrochloride was the best source of vitamin for the growth and sporulation of C. lunata. Inositol, nicotinic acid and riboflavin yielded less growth, but growth on folic acid was poorest. Thiamine, biotin and other vitamins improved growth and conidiation of *C. lunata* (Domsch *et al.* 1980). Four isolates of *Coch. sativus* showed no benefit from the addition of several vitamins in their pathogenicity to foliage of several cereals, nor did they change in the production mycelium (Clarke 1972). Addition of vitamins did not change mycelial production in four isolates of *Coch. sativus* (Clarke 1972).

**k) Inhibitory compounds**

Limited information is available but differences in tolerance to inhibitors have been noted in some species (Mathre 1969). *Helmintosporium pedicillatum* appears to be less sensitive to sodium azide than *H. sativum*. Fluoride was only slightly inhibitory to both fungi at high concentrations but stimulatory to growth of *H. sativum* at a concentration of 10-3M. Malonate was slightly inhibitory to both fungi, but completely inhibited *H. sativum* at 10-2 M. It took at least five times more dinitrophenol (DNP) to inhibit *H. pedicillatum* than it did to inhibit *H. sativum*. Tolerance of *Coch. sativus* to benzimidazole and other systemic fungicides was reported (Domsch *et al.* 1980).

## 3.4 Some unresolved aspects

During the investigation of existing published knowledge presented above, it became clear that certain issues pertaining to the delimitation of species in the *Bipolaris*-like complex must still be resolved.

### 3.4.1 The nature of variability within helminthosporia

Extensive variation in colony features and pathogenicity has been observed in helminthosporic fungi. Pathologists reported the similarity of symptoms caused by different species of helminthosporia on the same host plants (Henry 1924; Putterill 1954; Scott 1995). Alcorn (1990) also mentioned the "bewildering" array of variable and intergrading strains commonly encountered as the cause of non-specific lesions on millet, Bermuda grass, maize and many other cultivated and wild grasses. It is therefore essential that the causative organisms be isolated and correctly verified (Scott 1995). Difficulties in identification of these fungi sometimes necessitated publication of tentative names such as *Helminthosporium* N and *Helminthosporium* M (Henry 1924)

Studies in nuclear behaviour of particularly two species *B. sorokiniana* and *E. turcicum* shed light on the inherent variability of these fungi (Knox-Davies & Dickson 1960). Observations provided evidence of the perpetuation of heterokaryosis via conidia and evidence of genetic transfer though the parasexual cycle (Tinline 1962). Migration of

nuclei is common between cells in the vegetative hyphae, conidiophores and conidia (Hrushovetz 1956; Knox-Davies & Dickson 1960; Tinline 1962). Tinline (1962) speculated that interspecific heterokaryosis and parasexual recombination occur throughout the helminthosporia. Limited studies of H. sativum on host tissue provide evidence of more than one nucleolus in nuclei and implicate more DNA than would have been present under normal conditions (Knox-Davies & Dickson 1960).

Additional to heterokariosis, variation in the fungus *E. turcicum* could also be attributed to other factors. Knox-Davies & Dickson (1960) reported that nuclei within cells divided simultaneously. Such synchronous division possibly provides an opportunity for exchanges of chromosomes from non-sister nuclei into a single daughter nucleus. In *E. turcicum,* the nuclei may be aneuploid or polyploid following the coalescing of spindle fibers during synchronous nuclear division. Polyploidy and aneuploidy in vegetative mycelium of fungi is unexpected but could explain such phenomena as sectoring of cultures and the recovery of vigour after passage through a host (Knox-Davies & Dickson 1960)

Many helminthosporic species have been described on minor and inconsistent conidial characters even though it is known that morphological characteristics are affected by factors such as nutrients (Scott 1995). Variation in conidial morphology frequently makes it difficult, if not impossible, to determine whether a specific isolate is representative of one species or a closely related species (Nelson 1964). According to Scott (1995) attention should be given to cultural characters such as growth rates, development of synnemata-like hyphae and the production of sexual fruiting structures. Physiological criteria that can be used in cultural studies are pigment production and tolerance of isolates to inhibitory substances. Heterothallism in these fungi presents an opportunity to determine relationships through mating of closely related species (Nelson 1964).

### 3.4.2 Similar species within genera

Within the bipolar series of species, certain groups with common morphological tendencies exist. It is these species-complexes that make species delineation so difficult. Nelson (1964) felt that there is a definite morphological transitional between the *B. zeicola* and related species group (*B. setariae*, *B. bicolor* (Mitra) Shoemaker and *B. victoriae* (Meehan & Murphy) Shoemaker) and the various small-spored species, of which *H. spiciferum* (Bainier) Nicot are the most typical. Alcorn (1990) also mentioned the array of variable and intergrading strains commonly encountered. The validity of criteria used to differentiate these species complexes has not been clearly resolved. Nelson (1964) felt it apparent that at least certain of the species within these groups will be reduced to synonymy.

Within the genus *Exserohilum* a similar situation can be found between two specific species. When Leonard and Suggs (1974) transferred *H. longirostratum* (Subram.)

Sivanesan to *Exserohilum,* they suspected this species to be the same as *E. rostratum.* Sivanesan (1987) retained them as different because of the large difference in conidial length. Conidial shape and accentuated septa of conidia in the two species are the same. It is disputed if conidial length is a sufficiently stable character to make such a separation.

Luttrell (1977) referred to groups within *Curvularia.* The first group he mentions is the "lunata" group that includes species with conidia typically 3 septate. A second group the "geniculata" group typically has four septate conidia with the third cell forming the base being disproportionately enlarged and swollen. The walls of these swollen cells are thicker and darker than other cells, in particular the end cells which tend to remain hyaline. Conidia formed by this group of species are strongly curved. The third, the "maculans" group forms conidia where the two middle cells of the three septate conidia are enlarged and differentiated. Representatives from all three groups have been associated with a *Cochliobolus* state (Luttrell 1977).

### 3.4.3 Similarity between *Bipolaris* and *Curvularia*

Luttrell (1977) referred to the close relationship between *Curvularia* and *Bipolaris* species with flush hila. Sivanesan (1987) could not find any valid morphological characteristic to differentiate between the two genera. The consistency of the differences found was tested by determining the same characteristics for a range of species amongst the three genera. He investigated the following criteria: shape of conidia, origin of the basal germ tube, relative position of the germ tube to the conidial axis, hilum structure, ontogeny of septa in the conidia and the surface texture of conidiogenous nodes. He upheld both genera but consider them essentially identical in many features. Von Arx and Luttrell (1979) also consider the two congeneric because they share many features: both have *Cochliobolus* teleomorphs, both include species with or without protuberant hila, both include species that form stromata and species with verrucose conidiogenous nodes. Separation can only be made on certain features of conidia. Taxonomy of these two genera clearly needs to be resolved.

# 4 MATERIALS AND METHODS

## 4.1 Sources of South African records of *Bipolaris*, *Curvularia* and *Exserohilum*

The following major sources were consulted in obtaining records of the *Bipolaris*-like fungi found in South Africa:

- Herbarium records in the National Collection of Fungi, Pretoria, South Africa (PREM).
- Records of live cultures kept at the National Collection of Fungi (PPRI).

Various publications were consulted, of which the following are the most important: "The South African fungi and Lichens to the end of 1945" (Doidge 1950), indices of plant pathogens of cultivated plants (Gorter 1977, 1982) and those on wild growing plants (Gorter 1981). Gorter's (1979) list of publications on South African fungi for the period 1946-1977 was also consulted. The most recent publication listing plant pathogenic fungi in this country is by Crous *et al.* (2000). Apart from these publications, various other papers also provided information.

## 4.2 Selection of isolates investigated

Attempts were made to include at least four species representing each of the genera *Bipolaris*, *Curvularia* and *Exserohilum* and, where possible, at least five isolates per species. It was also decided to include, depending on the biology of the species, cultures from different hosts and locations so that as much variation possible would be available to study. Since Ex-type cultures of species were not always available isolates named by the author of the particular species were selected. In some instances no such culture was available in which case an isolate was obtained from a collector who is a known authority on the group under investigation. Authenticated cultures of each of the type species of the three genera, namely *B. maydis, C. lunata* and *E. turcicum* were included as primary reference.

A list of the cultures obtained is presented in Table 7 (Appendix B), with their respective collection data and condition upon receipt. Permits No.'s 14/2/2/1(9/96/130), 14/2/1(9/97/70) and 14/2/1(9/97/79) were granted by the Department of Health, Plant and Quality Control, Pretoria for the importation of these fungi from various locations aboard.

Some morphological features given in Table 8 (Appendix B) elucidate the rationale behind selecting the particular species listed in Table 7 (Appendix B). Species sharing features of two or all three genera have been selected. *Bipolaris indica* and *C. cymbopogonis* have been included since both form conidia with protuberant hila, an important character separating *Exserohilum* from *Bipolaris* and *Curvularia*. Pairs of species with similar spore morphology have been included. *Exserohilum inaequale* (euseptate) and *B. cynodontis*

(distoseptate) have similar conidial morphology but differ in conidial septal structure. *Exserohilum rostratum* and *E. longirostratum* differ only in the extreme lengths of conidia of the later species. *Curvularia lunata* resembles *E. inaequale* (Domsch *et al.* 1980), but the latter form conidia with distinct protuberant hila. Further species displaying characteristics of both *Bipolaris* and *Curvularia* are *B. ellisii, B. papendorfii, E. inaequale* and *C. cymbopogonis. Curvularia affinis* and *C. fallax* have essentially the same conidial morphology. In Table 7 (Appendix B), numbers in bold face refer to the cultures used for ISSR analysis (see paragraph 3.3.5) and the underlined numbers refer to cultures used for sequence analysis. Information about the three isolates of *Beauveria bassiana* selected as an out-group for nucleic acid based tests are included in Table 7 (Appendix B).

## 4.3 Preservation and maintenance of cultures

All cultures were deposited in the PPRI culture collection at 590 Vermeulen Street, Pretoria, South Africa. Stock cultures were made from all isolates before being preserved in water, under mineral oil and freeze-dried (Smith & Onions 1994). The buffer solution used during freeze-drying was 10% skim milk and 5% inositol. All preserved isolates and stock cultures were stored at 15°C.

## 4.4 Culture based test

Conditions were standardised, as far as possible, but due to a limitation of available facilities, not all environmental variables could be controlled.

### 4.4.1 Standardization of environmental conditions

Chemically defined media were used were possible since organic substrates, such as yeast extract and vegetable juice, tend to vary between batches (Paterson & Bridge 1994). Various defined media have been published in Booth (1971), Stevens (1974) and Smith & Onions (1994) and Paterson & Bridge (1994) but Sach's agar (Appendix C) seems to be generally preferred for *Bipolaris*-like fungi (Alcorn 1983; Sivanesan 1987). The three defined media used for various procedures were Czapek agar (Cz), Sach's agar (Sach) and Water agar (WA) (Appendix C). A complete list of media used is listed in Appendix C. It is acknowledged that liquid medium is generally preferred for biochemical tests of filamentous fungi (Paterson & Bridge 1994), but because of the large number of test repeats necessary, this was not practically feasible. There is a possibility that impurities in agar might influence test results. Many of the tests described by Paterson and Bridge (1994) can be modified for use in micro titre plates, but since the intent was to optimise the chance of obtaining additional taxonomic characteristics from colony appearance or morphological structures of cultures, this was not done. Tests cultures were grown in 90 mm Petri dishes containing 20ml of test medium, or in 65mm dishes containing 10ml of agar. The specific volume used will be reported for each particular test. Each exposure of an isolate to a test was always repeated three times.

All inoculated plates were incubated at 25°C (± 1°C), under ultraviolet light at 320nm wave length (36W, supplied by Philips) mixed with daylight fluorescent light at a distance of 30cm above the cultures and a 12 hours light / dark cycle. Changes to these conditions were sometimes required for certain tests, but such adjustments will be mentioned at the appropriate description of the method used.

Standardization of inocula was necessary to prevent differences in age, viability and amount of inoculum (Paterson & Bridge 1994). To prevent the transfer of any nutrients from the inoculum to the test medium, WA was used to prepare the inocula. Petri dishes (65mm) containing 10ml WA were inoculated with a culture and incubated for 5 days under conditions as described above. Agar plugs were removed from the WA cultures with the 6mm end of disposable glass Pasteur pipettes and placed on the test plates, as near to the centre as possible.

**a) Measurements of results**

In all culture-based tests (paragraph 3.2.2 to 3.2.7), colony width (growth rates in mm) was used to compare the performance of the different isolates. Where culture based tests yielded additional cultural characters such as sporulation or colour reactions, these were recorded. The number of days of incubation (and therefore the amount of days per tests, in which results were recorded), varied between the different test since the general growth rate of cultures predetermine the speed with which Petri dishes were covered. During some tests, it was impossible to take measurements on all consecutive days. In spite of such events assays were always completed.

### 4.4.2 Comparative variation of growth rates and spore morphology

To obtain an indication of the variability between cultures in terms of growth rates and spore shapes and sizes, five different media i.e. Potato-carrot agar (PCA), WA, Sach, Cz and Vegetable-juice agar (V8) (Appendix C) were inoculated with standardized inocula. Microscope slides of all these cultures were made in Lacto-fuchsin mounting medium (Booth 1971) on days 5 and 10 after incubation. The stain provided good contrast between cytoplasm and cell walls, enhancing the accuracy with which *camera lucida* drawings could be made. Drawings of spores were made with the aid of light microscopy using a 40 x objective. Drawings of an eyepiece micrometer were made simultaneously to ensure correct calculation of enlargements.

Drawings were converted to electronic TIFF formatted files by scanning them in at 150 X 150 dpi (dots per inch) with Hewlett Packard PrecisionScan Software ver.1.01. The spore images were filled to black, contrasted on a white background and saved as 8 bit grayscale files with Adobe Photodeluxe Business Edition software. Calibration of measurements was done by using the eyepiece micrometer bar image, included in each file. The freeware, NIH Image ver. 2.0 were used to calculate the following parameters of the spores:

- Area of the spores is measured in pixels but according to the software instruction manual is usually a slight over-estimate.
- The length of the outside boundary of the spores or perimeter but this measurement also tend to produce an over estimate.
- The roundness of spores calculated as (4x PI x area) / perimeter$^2$ result in a value between zero and one. The higher the value the rounder the spore is, with one being a perfect circle.
- Elongation is the ratio of the length of the major axis to the length of the minor axis.
- The ferret diameter is the size of a circle having the same area as the object and is computed as the square of four x area / PI.
- Compactness is computed as the square of (4 x area / PI) / major axis length and provide an indication of the objects "circleness". A value of between zero and one is provided with one being a circle.
- Major axis length is the longest line that can be drawn through the spore.
- Minor axis length is the longest line that can be drawn through the spore perpendicular to the major axis.

### 4.4.3 Growth and survival at different temperatures

Sach's agar plates were inoculated with some of the *Bipolaris*-like cultures (Appendix B: Table 7), using the standard procedure and incubated under standard conditions at 5°C, 15°C, 25°C, 37°C and 45°C for 5 days. Colony sizes were recorded to estimate the temperature yielding the best average growth of cultures and will be used as general incubation temperature for all the culture based test. Accept for 5°C, 15°C and 25°C incubation temperatures that varied up to 2°C more than the actual setting, all other incubation temperatures were stable with no measurable fluctuation.

To test survival of cultures at -20°C, they were incubated for 5 days before at least twenty, 6mm agar plugs were removed and transferred to empty previously autoclaved empty glass Petri dishes. The plugs were kept in the dark, at -20°C for 10 days. On days 10, 12, 14, 18 and 20 after the plugs were placed at -20°C, three plugs from each strain were removed, plated onto Potato dextrose agar (PDA; Biolab Diagnostics: Merck), and incubated under standardised conditions. The plugs were investigated for growth each day for up to five days after removal from the cold.

### 4.4.4 Growth at high osmotic pressure

Glycerol nitrate agar (G25N; Appendix C) is a growth medium that was developed by Pitt (1979) and is currently used in the standard procedure to identify species of *Penicillium*. *Bipolaris*-like fungi were inoculated on 65mm plates containing G25N, incubated under standardised conditions, and colony width of cultures measured each day for 10 days.

### 4.4.5 Utilization of carbon sources

Paterson and Bridge (1994) successfully used media containing various sugar alcohols as a diagnostic tool. Production of pigment in various filamentous fungi has been noticed on these compounds. Two sugar-alcohols, inositol ($CHOH.(CHOH)_4.CHOH$) and sorbitol ($CH_2(OH).(CH.OH)_4.CH_2OH$) were tested. Growth rates on a third carbon source, glucose ($C_6H_{12}O_6$), were included for comparison and as control (Botha 1998). All three media were prepared to contain a comparative molecular weight of 1% in carbon (Appendix C), inoculated and growth rates measured each day for 10 days.

### 4.4.6 Growth on inhibitory compounds

Growth of isolates on inhibitory compounds was found to be a useful diagnostic tool in separating strains and populations of many mitosporic fungi, while acetic acid proved particularly useful in diagnosis of the terverticillate species of *Penicillium* (Paterson & Bridge 1994).

The growth responses of *Bipolaris*-like isolates were tested in the presence of four inhibitory compounds namely copper sulphate, crystal violet, methylene blue and acetic acid with Czapek yeast-extract agar as base medium (CYE; Appendix C). The CYE base was used since the mycelial development was poor on WA, Cz and Sach; as a result assessment of results on the deeply stained media was impossible. A preliminary assay on authentic isolates against the four compounds in increasingly stronger concentrations enabled a selection of concentrations that yielded differences in growth rates (Table 1). The concentrations suggested by Paterson and Bridge (1994) i.e. 0.1% copper sulphate, 0.001% to 0.005% crystal violet, 0.001% methylene blue and 0.5% acetic acid, were used as starting point.

**Table 1: Preliminary tests to establish a working concentration of inhibitory compounds for culture based tests.**

| Inhibitory compound | Preliminary concentrations tested (in %) | | | | Concentration Selected (in %) |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| Acetic acid | 0.01 | 0.05 | 0.01 | 0.3 | 0.35 |
| Copper sulphate | 0.25 | 0.5 | 1.0 | 1.5 | Test abandoned due to complete inhibition |
| Crystal violet | 0.01 | 0.02 | 0.03 | 0.04 | 0.04 |
| Methylene blue | 0.05 | 0.1 | 0.2 | 0.5 | 0.3 |

Solutions of the four compounds were prepared in distilled water and filter sterilised before being added to cool but still molten CYE, and being dispensed into 9mm Petri

dishes. Inoculations on CYE base without any inhibitory compound added, served as a control. One measurement of colony diameter was made on the seventh day after incubation on crystal violet and methylene blue. Since acetic acid seemed to be least inhibitory to growth colony diameter was measured each day for ten days.

### 4.4.7 Presumptive enzymatic activity (Tween)

A variety of fungal enzymatic activities were tested to determine the ability of cultures to grow on specific substrates. All tests performed were presumptive enzymatic activity tests as described and successfully used by Paterson & Bridge (1994) on hyaline phialidic fungi.

**a) Fatty acid esterase activity**

Tween 80 agar is a mixture of fatty acids, predominantly elaidic, linoleic and palmitic acids. As fungal growth develops on Tween 80 agar (Appendix C) due to the breakdown of the fatty acids, the pH will rise. The test is positive when the originally yellow coloured medium changes to purple due to the action of the pH indicator bromocresol purple. Colony widths were measured each day for five days after inoculation. Metabolism of fatty acids can also result in the formation of insoluble calcium salts, which were visible as a white precipitate at the bottom of the medium.

However, some cultures developed only a small purple discoloration around colonies. Consequently, the assay was repeated in liquid medium and the pH of the liquid medium was measured once after 14 days of incubations. This procedure seemed more accurate and less difficult to interpret than recording the change in colour.

**b) Protease activity (Gelatine)**

Gelatine medium (Appendix C) is a presumptive test for protease activity. The medium is solidified with gelatine instead of agar. Utilization of gelatine results in liquefaction of the medium (Paterson & Bridge 1994). Ten millilitres of medium, prepared in 20ml McCartney bottles were point inoculated and incubated for 21 days. Since gelatine would be a liquid at 25°C the medium was chilled at 4°C for 30 minutes after incubation, before results were recorded.

**c) Pectinase activity (Pectin)**

Since many *Bipolaris*-like fungi are plurivorous, there is a possibility that some might be able to break down pectin. Citrus pectin, added to a synthetic medium (Appendix C) provided the only carbon source for the isolates to grow on. Ruthenium red was added to medium, since it turns pink in the presence of pectinase (Paterson & Bridge 1994). Colony widths were measured once on day five.

**d) Presumptive β-glucosidase activity (Aesculin)**

Enzymatic breakdown of aesculin will generate a small amount of sucrose and the more complex carbon source aesculin (6,7-dihydroxycoumarin 6 glucoside). The

method, widely used in bacteriology, enables limited initial growth of isolates on the molecularly less complex sucrose (Appendix C). Additional growth will only occur if the fungus forms β-glucosidase. This enzyme can split aesculin into glucose and aesculetin (6,7-dihydroxycoumarin) with the latter substance reacting to iron citrate, which turns the medium black.

Since aesculin is light sensitive, the cultures tests and controls (without aesculin) were incubated in the dark. Petri dishes from the same batch of aesculin agar were incubated in light to test the integrity of the compound since it has a short shelf life (Paterson & Bridge 1994).

### 4.4.8 Analysis of data from culture based tests

A recent data-mining technique, "First Order Inductive Logic Programming" (FOIL) and a more established technique "Decision Tree Programming" (DT), were used to estimate the value of each culture-based test in defining groups within the fungi investigated (Riddle 1999; Quinlan 1996). Castaneda DMS Software, which provides integration with MS Office, was used to perform the FOIL and DT calculations.

The strength of FOIL is its capacity to process continuous variables, process "noisy" data (such as missing or outlier data) easily process small data sets and provide "pruning" of data (Riddle 1999). During "pruning" any irrelevant determinate literals are removed without significantly compromising the accuracy of the separation rule. Using DT, relationships within data are presented in a tree structure where each split represents an attribute. This algorithm also accommodates for errors in data, such as missing values (Riddle 1999).

## 4.5 Nucleic acid based tests

### 4.5.1 Preparation of cultures

To obtain an accurate assessment of the characteristics of a given species, as many isolates as possible must be studied (Pitt 1979). However, due to financial constraints, not all isolates listed in Table 7 (Appendix B) could be analysed for genomic relatedness. The PPRI numbers of those isolates used for sequencing are underlined, and those used for ISSR analysis are in bold face.

Single spore cultures where prepared to ensure purity of the isolates under investigation. In those cultures where sporulation could not be induced, hyphal tips were isolated. Hyphal tips and single spores were transferred to WA and incubated to generate inocula. Previously sterilised Erlenmeyer flasks with 100ml Potato-dextrose broth (PDA; Merck) were inoculated as described previously (see 3.2.1). PDA, a medium rich in nutrients, was selected to generate sufficient biomass for DNA extraction. Cultures were incubated for seven days under the same conditions as described for culture-based tests.

After incubation mycelial mats were removed from the Erlenmeyer flasks and placed onto Whatman No. 1 filter paper in a Buchner funnel connected to a vacuum pump. To limit damage of mycelial mats and subsequent cell breakage, they were carefully rinsed on both sides with small amounts of distilled water to remove PDA broth medium. The water was subsequently removed by vacuum suction. The filter paper with mycelium was carefully removed from the funnel and gently squeezed between layers of tissue paper to remove excess water. The mycelium was kept in 20ml McCartney bottles at approximately -5°C and then frozen at -70°C before freeze-drying. After freeze-drying, McCartney bottles were sealed and kept at -70°C until DNA extraction could be performed.

### 4.5.2 DNA extraction and purification

Freeze-dried material was crushed to a fine power with a mortar and pestle. Freeze-dried mycelium of the *Be. bassiana* cultures had a soapy quality, making them difficult to pulverise. Liquid nitrogen was added to the mycelium and allowed to evaporate before the mycelium were ground to a fine powder.

The method of Rodrigues and Yoder (1991) was used to extract genomic DNA from freeze-dried mycelium. A small quantity (approximately 2ml) of mycelial powder was added to an Eppendorf tube and gently mixed with 1 ml lyses buffer (Appendix D). The suspension was then incubated for 30 minutes at 65°C before 1 ml of chloroform: iso-amyl alcohol (24:1) was added. This mixture was gently mixed for 5 minutes before centrifugation at 13 000 rpm for 15 minutes. The aqueous supernatant was transferred to a clean Eppendorf tube and 0.5 Vol. of 5*M* ammonium acetate added to precipitate proteins. This suspension was incubated on ice for 30 minutes and then centrifuged at 10 000 rpm for 5-10 minutes. DNA was precipitated by adding 0.6 Vol. of iso-propanol, before the tubes were placed in a freezer (approximately -5°C) for 30 minutes. The supernatant was decanted and the DNA pellet washed with 70% ethanol before centrifugation at 10 000 rpm for 5-10 minutes. After centrifugation, the 70% ethanol was decanted and the pellet redissolved in Tris-EDTA buffer (Appendix D), before storage at -5°C, in preparation for DNA amplification.

### 4.5.3 Amplification of DNA and sequencing

The forward primer with sequence LR-7 (5'-TAC TAC CAC CAA GAT CT-3') and the reverse primer LR-7R (5'-GCA GAT CTT GGT GGT AG-3'), synthesised by MWG-Biotech AG, Ebersberg, Germany were used to amplify ribosomal DNA homologous to position 17 onward within the 28S rRNA encoding gene. This primer was previously used by Meyer (1996), who successfully characterised strains of *Rhizoctonia.* A preliminary screen of *B. australiensis, B. papendorfii, C. verruculosa, C. eragrostidis, E. pedicellatum and E. rostratum* indicated differentiation between these strains (Erasmus 1997b).

The Biotechnology Unit of the Vegetable and Ornamental Plants Institute of the Agricultural Research Council [illegible] performed the sequencing-PCR

protocols using TaKaRa *Taq*™ PCR kit (R001 AM). Fungal DNA, primed with the LR-7 forward primer and the LR-7R reverse primer, was amplified during PCR with an annealing temperature of 53.5°C (Appendix D). The PCR product was visualised on a 1.5% agarose gel to confirm the successful amplification of the targeted DNA. After successful amplification the PCR reaction cycles were increased until approximately 200ng DNA was obtained. The reaction product was purified through a Qiagen column with the QIAQuick PCR purification kit (GmbH, Hilden, Germany). The purified product was send to the Core DNA Sequencing Facility, Department of Genetics, University of Stellenbosch where the nucleotide sequence was determined with an automated sequencer and the results captured electronically.

### 4.5.4 Analysis of sequence data

Electronic files containing sequence data were aligned with Dnaman for Windows version 2.71 (Lynnon BioSoft). Where automatic-sequencing readings presented ambiguities, the particular nucleotide present at that position in the sequence was identified by visual inspection of graphs generated during automatic sequencing. Statistical confidence was assigned to the indicated relationships, by 500 cycles of bootstrapping.

### 4.5.5 Inter sequence repeats (ISSR)

DNA for ISSR's was extracted from the fungal mycelium as described under paragraph 4.5.2. ARC-VOPI conducted the PCR reaction with the four anchored ISSR primers DBD-$(AC)_7$, BDB-$(CAC)_5$, DHB-$(CGA)_5$ and VHV-$(GT)_7G$ (Appendix D). These primers were chosen arbitrarily. The annealing temperature during amplification of DNA was 60°C. ISSR fragments and Molecular Weight Marker VI (M; Roche Molecular Biochemicals, Mannheim, Germany) were electrophoretically separated on 2% agarose gel, stained with ethidium bromide 10 mg /ml and photographed.

### 4.5.6 Analysis of ISSR data

**a) Cluster analysis**

Images of gels were captured in an 8-bit (256 gray-scale) TIFF graphics file format. The TIFF files were processed with GelCompar II ver. 2.5 (© Applied Maths). Different gels were normalised by including a molecular weight marker no VI (Roche Molecular Biochemicals, Mannheim, Germany) in every gel. The program calculated a similarity matrix from the densitometric curves of the fingerprint patterns. All subsequent dendrograms were based on single or combined similarity matrices. ISSR bands were scored as dominant markers and average similarity indices were calculated using algorithms that consider only band matches. Two different similarity coefficients were used namely that of Dice that are band based, and the Pearson coefficient that is curve based. The clustering method of Ward, based on Eucledian distances, was also applied. Dendrograms based on the four different primers (fingerprint types) were

assessed separately or as a combined data set of all fingerprint types in a single dendrogram.

**b) Group separation tables**

Different species were defined as groups using the Jackknife method to determine the significance of the defined groups. For the Jackknife method, average and maximal similarities were calculated derived from dendrogram similarity matrices. This method compares one species in the table with all other species in the different groups and calculates average and maximal similarities for each group. Similarities calculated for the group separation tables are not equivalent to similarity values calculated for dendrogram clusters. The values in the tables indicate the percentage of cases each species is correctly identified to the specific group originally assigned to, and between groups the percentage of cases species are identified to other groups (Gelcompar II ver. 2.5 Instruction manual).

# 5 RESULTS

## 5.1 List of South African species

The list of South African *Bipolaris*-like fungi is presented in Appendix E. Synonym - and teleomorph names are according to Sivanesan (1987), except for those species not included in his monograph or where subsequent studies incorporated nomenclatural changes. Abbreviations of province names follow the locality of each particular record and are Eastern Cape Province (EC), Gauteng (GP), KwaZulu-Natal (KZN), Mpumalanga (MP), Northern Cape Province (NC), Northern Province (NP), North-West Province (NW), Free State (FS) and Western Cape Province (WC). Records marked as "South Africa" were so reported in the original record, and where many locations for a specific host/fungus combination are known, the term "widespread" was used. Where known, the current valid teleomorph name is provided before the correct anamorph and where applicable, its synonyms. New species records are marked with an asterisk. Identifications of isolates marked with a hash (#) were confirmed by Dr J Alcorn, Department of Primary Industries, Queensland, Australia.

## 5.2 Culture based tests

### 5.2.1 Comparative variation in spore morphology between species

Nine different dimensional variables of spores were calculated namely area, perimeter, roundness, elongation, ferret diameter, compactness, major axis length and minor axis lengths as well as environmental variables including temperature (25°C and 30°C), colony age (days 5, 7 and 10) and media (Glucose, Sorbitol, Wa, Sach, PCA and Ceres). Most sets of variables included measurements of at least 20 spores. This data amounted to approximately 12 000 sets of variables, therefore only the mean values of each set were included in the final analyses. A selection of line drawings, representing each species investigated and the elongation value are provided in Appendix F (Figures 3 to 15).

An important consideration in the interpretation of the results is that the minor axis length is not necessarily equal to the width, or the shortest axis (breadth), of the spore. The minor axis length was calculated as the longest line that can be drawn through the object, perpendicular to the major axis. Since this measurement is not equivalent to spore-width commonly provided in morphological descriptions, it cannot be compared to measurements of other studies.

The effectiveness of the various dimensions to differentiate species was calculated with the DT and FOIL analyses previously described (see section 4.4.8). Ideally an optimum analysis would necessitate calculations of all possible permutations of variables but only a selected combination of calculations were performed and is presented in (Table 2).

With all variables available to the FOIL algorithm clustered 246 of 447 sets of variables (55.0%) correctly and DT 222 of 447 sets (49.7%; Table 2). With the first selection of variables FOIL (46.5) were 10% less effective in clustering than DT (56.6). The weak performance of FOIL, in contrast to DT, was ascribed to the nature of the algorithms. FOIL is considered "greedy" in finding differences and would therefore find many differences in greatly variable data sets (Castaneda help file). In contrast the DT algorithm provides a tool with which groupings are sought and defined using the minimum possible features. In further calculations only the DT results were considered as presented in Table 2.

**Table 2: Spore dimension variables used for DT analyses.**

| | **Selected variables included** | | | | | |
|---|---|---|---|---|---|---|
| All | 1 | 2 | 3 | 4 | 5 | 6 |
| Medium | -------▶ | -------▶ | -------▶ | -------▶ | | -------▶ |
| Day | -------▶ # | -------▶ # | -------▶ # | | | -------▶ |
| Temperature | -------▶ | -------▶ | | | | -------▶ |
| Area # | | | | | | -------▶ # |
| Perimeter | | | | | | -------▶ |
| Major axis length # | | | | | | -------▶ # |
| Minor axis length | | | | | | -------▶ # |
| Elongation | -------▶ # | | | | | -------▶ # |
| Roundness # | -------▶ # | -------▶ # | -------▶ # | -------▶ # | -------▶ # | |
| Ferret diameter # | -------▶ # | -------▶ # | -------▶ # | -------▶ # | -------▶ # | |
| Compactness # | -------▶ # | -------▶ # | -------▶ # | -------▶ # | -------▶ # | |
| **Percentage datasets clustered with the selected variables within the correct species** | | | | | | |
| 49.7 | 56.6 | 55.5 | 55.5 | 51.9 | 53.9 | 52.1 |

# The best differentiating variables used by the algorithms to define the various clusters.

With all nine measurements included in the calculation (first column Table 2), all variables selected as differentiating rules were those defining shape, in contrast to the use of only one variable defining size (elongation). Not one of the environmental variables (day, temperature and medium) was used. After removing all un-used variables and elongation, DT added the variable "day" to define clusters but was markedly less effective (compare selected sets 1 and 2, Table 2). When examining the DT clustering rules, "day" was used to differentiate between *B. setariae, B. maydis, E. longirostratum* and *E. rostratum* data sets, which are all the long spored species (elongation larger or equal to 5) included in the

investigation. Major axis lengths on days 5, 7 and 10 were used to separate these species. The algorithm therefore "compensated" for the loss of "elongation" in using a similar measurement. The differences in spore lengths between these species on days 5, 7 and 10 indicate a difference in tempo of development, rather than an actual difference in size. When forcing DT to use only environmental and size defining variables (compare set 1 with 6, Table 2), a marked difference in the percentage clustering was obtained. This further confirmed the preference of shape defining variables to perform clustering, rather than spore size, as indicated in the first calculation using all variables.

**Table 3: Differenciation of species by FOIL and DT using day, temperature, medium, roundness, ferret diameter and compactness.**

| Organism | FOIL | | DT | |
|---|---|---|---|---|
| | (number / total) | % | (number / total) | % |
| *B. cynodontis* | 30 / 40 | 75 | 35 / 40 | 88 |
| *B. ellisii* | 3 / 16 | 19 | 8 / 16 | 50 |
| *B. indica* | 28 / 36 | 78 | 29 / 36 | 81 |
| *B. maydis* | 11 / 23 | 48 | 14 / 23 | 61 |
| *B. papendorfii* | 14 / 27 | 52 | 26 / 27 | 96 |
| *B. setariae* | 2 / 14 | 14 | 2 / 14 | 14 |
| *C. affinis* | 16 / 29 | 55 | 11 / 29 | 38 |
| *C. cymbopogonis* | 10 / 37 | 27 | 15 / 37 | 41 |
| *C. fallax* | 21 / 43 | 49 | 16 / 43 | 37 |
| *C. lunata* | 11 / 45 | 24 | 40 / 45 | 89 |
| *E. inaequale* | 6 / 38 | 16 | 9 / 38 | 24 |
| *E. longirostratum* | 23 / 43 | 53 | 29 / 43 | 67 |
| *E. rostratum* | 13 / 29 | 45 | 18 / 29 | 62 |
| *E. turcicum* | 8 / 22 | 36 | 14 / 22 | 64 |

When using shape-defining variables with the DT technique to consider variability within the various species, *B. cynodontis*, *B. indica*, *B. papendorfii* and *C. lunata* formed highly reliable groups (80% of variables clustered together; Table 3). Inadequately differentiated species were *B. setariae*, *B. cymbopogonis*, *E. inaequale*, *C. fallax* and *C. affinis* (Table 3). When considering both FOIL (Appendix F: Table 10) and DT results (Appendix F: Table 11), *B. setariae* were often also grouped with *B. maydis*, *B. longirostratum* and *E. turcicum*; *B. cymbopogonis* were often grouped with *B. maydis* and *E. inaequale* (and with

*B. cynodontis* by DT only); *E. inaequale* were often grouped with *B. cynodontis, B. papendorfii* and *C. lunata; C. fallax* were often grouped with *C. lunata; C affinis* were often grouped with C. fallax and *C. lunata.*

### 5.2.2 Physiological characteristics

In most assays where colony diameters were recorded, the first two days of growth yielded very little growth, if any at all. To facilitate computation, a score of 0.25mm was assigned to cultures if hyphae extended from the inocula but did not reach the agar surface. A score of 0.5mm was recorded when hyphae reached the agar during this period but did not extend into the agar to a measurable distance. Any hyphal development further than 1mm from the inoculum on the agar was measured. Measurements included the 6mm diameter of the inoculum.

**a) Growth and survival at different temperatures**

None of the cultures on Sach agar survived five days of incubation at 37°C or higher. The optimum growth rate of the selected cultures seemed to be around 25°C (Table 4). In all cultures incubated at 5°C, hyphae developed on the inocula, but were never sufficiently extensive to be measured.

**Table 4: The average range of colony diameters of selected cultures incubated on Sach agar at 15°C and 25°C.**

| Name and PPRI No's | 25 °C | 15 °C |
|---|---|---|
| *B. indica* 6567, 6541 & 6538 | 44 (36-51) | 23 (21-25) |
| *C. affinis* 6428, 6652 & 6676 | 54 (53-56) | 30 (29-33) |
| *C. cymbopogonis* 6675, 6661 & 6532 | 54 (46-60) | 27.5 (26-29) |
| *E. inaequale* 6666, 6573 & 6570 | 51 (46-65) | 25 (23-27) |
| *E. rostratum*, 6658,4130 & 6530 | 65 (65) | 30 (24-38) |
| *E turcicum* 6562,6575 & 6708 | 52 (44-65) | 25 (20-34) |

Survival at -20°C on Sach, was recorded and included in the FOIL and DT analyses (presented in section 5.2.5).

**b) Growth at high osmotic pressure**

Growth on G25N was recorded and included in the FOIL and DT analyses presented in section 5.2.5.

**c) Utilization of carbon sources**

All cultures had a similar growth pattern on Sorbitol medium. The mycelium was hyaline with thin and multiple branched hyphae. Measurements had to be done with the aid of a dissecting microscope with light from below, since hyphae were often deeply imbedded into the agar. On day five after inoculation, most cultures displayed woolly

growth on top of inocula. Only five cultures released a pigment into the agar but this feature was not consistent within species. Pigment production was noted in cultures of *C. affinis* (6676), *C. cymbopogonis* (6532 & 6661) and *E. inaequale* (6527, 6666 & 6668). All isolates produced a light ocraceous buff to buffy citrine (Plate XVI 19' m) coloured metabolite (Ridgeway 1912). Representative cultures of all species, except strain 6668, developed spores on the sides of inocula after one day of incubation. Alcorn (1987) also noted this feature of the *Bipolaris*-like fungi in various species on Sach and WA media.

Three different colony textures could be recognised amongst cultures grown on Inositol medium (Table 5). Some cultures showed severely restricted radial development often with contorted hyphae. A second group formed cultures with restricted radial growth but with a dense cottony appearance. A third group of cultures were seemingly unaffected by the high concentration of inositol in the medium. The colony texture of the latter cultures was as woolly as those grown on PCA and CYE. Only one culture, *E. inaequale* (6668), developed coloured mycelium with the inoculum a sea shell pink (Plate XIV f11' f), the top of cultures salmon (Plate XIV 9' d) and reverse side of cultures carnelian red (Plate XIV 7" R-O). Two other cultures of *E. inaequale* (6668 & 6666) discharged a yellow pigment (Plate XV 15' d) into the medium, similar to the pigment they formed on Sorbitol medium. A yellow exudate was also noted in cultures of *C. affinis* (5861 & 6545).

**Table 5: Textures of five-day old colonies of *Bipolaris*-like fungi on Inositol medium.**

| Species | Colony textures | | |
|---|---|---|---|
| | Normal growth (woolly) | Restricted growth with contorted hyphae | Restricted growth but cottony |
| *B. cynodontis* | | 6660, 6185, 6584 | 6535 |
| *B. ellisii* | | 6537, 6536 | |
| *B. indica* | 6567, 6541, 6538 | | |
| *B. maydis* | | 6662, 6607, 6677 | 6564, 6430, 6667 |
| *B. papendorfii* | 6539 | 5855, 6124 | |
| *B. setariae* | | 6665, 6663 | 6665, 6572 |
| *C. affinis* | | 6428, 6676 | 6652, 6545 |
| *C. cymbopogonis* | | | 6675, 6661, 6532, 6592, 6297 |

| Species | Colony textures | | |
|---|---|---|---|
| | Normal growth (woolly) | Restricted growth with contorted hyphae | Restricted growth but cottony |
| *C. fallax* | 6445, 6574 | | 6534, 6533 |
| *C. lunata* | 6699 | 6565, 3360, 6699, 6700 | 6540, 6659, 5606 |
| *E. inaequale* | 6573, 6570, 5940 | | 6666, 6668, 6527 |
| *E. longirostratum* | 6612, 5133, 6528, 6529, 5908 | 5133 | |
| *E. rostratum* | 6658, 4130, 6531, 6530, 6119 | 6658 | |
| *E. turcicum* | 6606, 6575 | 6562, 5487, 5459 | |

### 5.2.3 Growth on inhibitory compounds

The colony diameters of *Bipolaris*-like isolates on crystal violet, methylene blue and acetic acid were recorded and included in the FOIL and DT analyses. Radial development of hyphae of all cultures tested on 0.25% copper sulphate, proved to be restricted when compared to the CYE control plates, but the intensely coloured medium made evaluation difficult and most probably inaccurate. The latter assay was therefore abandoned.

**a) Acetic acid**

Growth responses of all cultures on 0.035% and 0.01% acetic acid were recorded and included in the FOIL and DT analysis. All cultures on acetic acid developed complex assortments of coloured concentric rings. These features were difficult to include in numerical format and were not included in the FOIL or DT analyses.

**b) Crystal violet**

Crystal violet at concentrations of 0.01% and 0.025%, much higher than the 0.001 to 0.005% suggested by Paterson and Bridge (1994), had no effect on colony diameter when compared to control cultures on CYE. A preliminary test (Table 1) indicated that a concentration of 0.04% crystal violet could yield differences in colony diameter. At this concentration, growth of cultures could be rated as (0) for no growth, (1) for growth on the inoculum only, and (2) when hyphae reached the agar within 7 days of incubation. The results were included in the FOIL and DT analyses.

**c) Methylene blue**

Growth of all the isolates at concentrations of 0.05%, 0.1% and 0.2% methylene blue, one of each representing the selected species, was very similar. At a concentration of 0.5% methylene blue, 6 isolates of the 14 species tested (one isolate each), namely *B.*

*setariae* (6665), *E. turcicum* (6575), *C. cymbopogonis* (6675), *B. maydis* (6662), *B. papendorfii* (6568) and *C. lunata* (6540) did not develop any hyphae after 7 days of incubation. A weaker solution of methylene blue (0.04%) was subsequently used for the assessment of all isolates. The results were included in the FOIL and DT analyses.

### 5.2.4 Presumptive enzymatic activity

**a) Fatty acid esterase activity**

Un-inoculated Tween 80 agar has a bright yellow colour, making any positive reaction (medium turned purple due to the pH indicator bromocresol purple) easy to recognise. Colony widths were measured each day for five days after inoculation and included in the FOIL and DT analysis. Colour reaction was graded as either strongly positive when a clear colour reaction covering or nearly covering the agar plate was noted, or weakly positive when a colour change was noted close to, or underneath the colony. The test was repeated in liquid medium and the pH of cultures measured after 14 days incubation. Many cultures formed a noticeable precipitate at the bottom of the McCartney bottles. The colour of medium, pH and salt formation were included in the FOIL and DT analyses.

**b) Protease activity**

Cultures varied in their ability to utilise gelatine. Results were therefore recorded as approximately 0.25%, 0.5% and 0.75% liquefaction. Certain cultures released a yellow pigment into the medium. The presence of pigmentation and the percentage of liquefaction were recorded and included in the analysis.

**c) Pectinase activity**

Some flocculation occurred in the medium during preparation. Scanty growth of most strains on this medium made measurements of colony diameter difficult. The flocculation and the results recorded for these tests are therefore considered inaccurate, but were nevertheless included in the set of data to be FOIL and DT analysed.

**d) Presumptive β-glucosidase activity**

All cultures yielded a certain degree of black colouration of the media and therefore have the ability to break down aesculin to various degrees. Colony diameters were measured and included in the analysis. The reliability of the aesculin test was confirmed as control plates did not turn black during incubation in the dark, but only after exposure to light.

### 5.2.5 Analysis of culture-based tests

Results from all the above-mentioned culture-based tests were incorporated into an MS-Access Database before being exported to the Castaneda software for processing. Since both programs are limited in the number of variables that can be processed (256 in total), a selection of the data for inclusion in a composite analysis had to be made. DT analysis on

measurements of each individual test indicated that separation of clusters (branch or divisions) was mostly made on measurements from days 3, 4 and 5. Therefore, only the results from these three days were processed to obtain an estimate of the relative value of the different tests.

Both FOIL and DT are unbiased with no preset indication of groupings. The only precondition to FOIL and DT was that groups should include at least three samples (isolates). A group separation table was generated with DT from the physiological characters and colony diameters of all culture tests on days 3, 4 and 5. This table illustrated 67,7 % (44 of 65) of isolates correctly associated with the rest of the cultures from that particular species (Appendix G: Table 12). The FOIL analysis provided 83 % accuracy (54 of 65 isolates; Appendix G: Table 13). From these calculations, tests 5, 7, 10, 15, 18, 21 and 25 were selected for subsequent analyses since they provided the highest efficiency in grouping isolates correctly.

A complete set of measurements for each culture-based test was included in this follow up calculation. Two additional DT and FOIL calculations were run, but each time without the test that contributed least to the decision rules (Table 6). Of all culture-based tests, the colony growth rates on CYE, 1% Glucose, Sach's, Tween and WA agar seemed to have the best diagnostic capacity, when using DT as the overall accuracy of the classification did not deteriorate, but actually increased, with subsequent reductions in culture-based tests used. The FOIL technique, however, showed a marked deterioration in classification accuracy.

Other features contributing to separation of isolates cultured on the above five types of media, are the colour reactions on Tween and the capacity of cultures to produce a precipitate during the breakdown of fatty acids.

Since it is known that algorithms such as DT and FOIL can over-fit datasets, the accuracy of fitting was tested by "training" the rules used in both algorithms. For both FOIL and DT, 132 of the total 199 samples selected at random, were included in the training set. After training the generated FOIL rule set (Appendix G: Table 14) and the DT (not represented) rule set were tested on the test set (67 of 199). The DT rule set achieved 59.2 % (39 of 67) accuracy and the FOIL rule set 79.1% (53 of 67) accuracy. The high accuracy of FOIL to predict the species from the seven selected culture-based tests is therefore correct and not due to over fitting of the data.

**Table 6: Accuracy of culture-based tests to predict the species of a strain.**

| Technique | Total of 199 samples | Percentage (%) |
|---|---|---|
| **Estimate 1:tests 5, 7, 10, 15, 18, 21 and 25** | | |
| FOIL | 192 | 96.5 |
| DT | 146 | 73.4 |
| **Estimate 2: tests 5, 7, 10, 18, 21 and 25** | | |
| FOIL | 41 | 20.6 |
| DT | 151 | 75.9 |
| **Estimate 3: tests 7, 10, 18, 21 and 25** | | |
| FOIL | 45 | 22.6 |
| DT | 151 | 75.9 |

## 5.3 Nucleic acid based tests

During the interpretation of nucleic acid based test results, some constraints were encountered which should be considered. In some strains, particularly those of *E. turcicum*, DNA amplification was unsuccessful and compelled the exclusion of certain isolates from the analysis. Prior to nucleic acid analyses, confirmation of the identities of some of the strains could not be confirmed. These strains were mostly the older reference cultures growing weakly and had lost the ability to sporulate. Non-sporulating isolates included in the analyses were *B. papendorfii* PPRI 6568, *B. cynodontis* PPRI's 6535 & 6660, *E. inaequale* PPRI 6668, *E. turcicum* PPRI 5487 and *B. setariae* PPRI 6665. Some liquid cultures prepared for DNA extractions, were also contaminated and had to be discarded.

### 5.3.1 DNA sequencing

The amplified product of the LR-7 forward primer (5' end of the DNA) yielded between 600 and 900 bp in all cultures tested. Unsuccessfully sequenced regions, towards the end of DNA sequence, were excluded from the pair wise alignment. Polymorphisms amounted to approximately 2% (Figure 1). Due to the low percentage polymorphisms the amplification of DNA with the reverse primer was not done.

**Figure 1: Oligonucleotide sequence from position 17 (5' end) onward, of the 28S rRNA encoding gene of selected *Bipolaris*- like fungi.**

1 CCSKTCKACC YGSSGTCACC CCCAAGGCTT CGHCACGAGC CTCCACGCCT GCCTACTCGC

61 CGGGGCGTAA AYTYTGCCCC GGCGGAGGGG TATAGGTRAC ACGCTTGAGC GCCATCCATT

121 TTCAGGGCTA GTTCATTCGG CAGGTGAGTT GTTACACACT CCTTAGCGGA TTCCGACTTC

181 CATGGCCACC GTCCTGCTGT CTAGATGAAC CAACACCTTT TGTGGTGTCT GATGAGCGTG

241 TACTCCGGCA CCGTAACCCC TCGTTCGGTT CATCCCGCAT CGCCAGTTCT GCTTACCAAA
301 AATGGCCCAC TAATAACGTT TCATTCAAAT GCCCGCGTTC AATTAAGTAA CAAAGGGCTT
361 CTTACATATT TAAAGTTTGA GAATAGGTGA AGGKTGTTTC AACCCCCATG CCTCTAATCA
421 TTCGCTTTAC CTCATAAAAC TGAATACGTT ACTGCTATCC TGAGGGAAAC TTCGGCAGGA
481 ACCAGCTACT AGATAGTTCG ATTAGTCTTT CGCCCCTATG CCCAAATTTG ACGATCGATT
541 TGCACGTCAG AACCGNTGCG AGCCTTCACC AGAGTTTCCT NTGGCTNCAC CCTATT

A homology tree (Appendix H: Figure 16) based on these sequences presented two groupings. The first contains strains representing *Bipolaris* and *Curvularia* and the second group includes all strains of *Exserohilum*.

### 5.3.2 ISSR analysis

All four primers used produced a high diversity of bands and were sufficient to discriminate between the strains tested. The bands of amplified DNA fragments, separated according to their respective molecular weights in agarose gels, are presented as molecular fingerprints in Appendix H (Figures 18 - 2). The presence of a band of particular molecular weight in the fingerprint patterns was confirmed by densitometric curves generated by the software program. Cluster analysis produced by three of the single primers did not reveal an adequate clustering of strains when compared to the cluster analysis of all four primers combined. Only the single primer BDB-$(CAC)_5$, produced good clustering when compared to the combined data set of the four primers (Appendix H: Figures 22 & 23).

**a) Consistency of clusters indicated by cophenetic correlation values in dendrograms.**

Two similarity coefficients, Pearson's correlation coefficient (densitometric) and the Dice binary coefficient facilitated similarity measurements between the strains. Composite similarity matrixes, which included data generated by all four primers using the two coefficients, enabled illustration of ISSR results in dendrograms (Appendix H: Tables 24 & 25). Dendrograms illustrating clustering by the single primer BDB-$(CAC)_5$, is presented in Figures 22 & 23). In the following presentation, the similarity and significance values of a cluster expressed as a percentage are indicated after the relevant group (similarity value; significance value).

Referring to current morphological species Pearson's correlation coefficient (PC) revealed five major groups of strains (Appendix H: Figure 24). Each group included strains on a similarity level of approximately 40% or more. The first group included strains of *C. lunata, C. fallax, C. cymbopogonis* and two *Bipolaris* species, *B. indica* and *B. papendorfii* (40; 54). *Curvularia affinis* was exclusive to the second group (80; 64), with all *Exserohilum* species and *B. cynodontis* making up a third cluster (40; 74). The *Bipolaris* species with large spores (elongation 5 or more), *B. setariae* and *B. maydis,* made up the last group (50; 85). As expected all strains of *Be. bassiana* were significantly different

(-35; 81) to all other helminthosporic groups. *Beauveria bassiana* was intended as an out-group, and clustered itself as a clearly defined separate group in all dendrograms. Consequently it will not be discussed any further.

As indicated by PC, strains of *B. indica* (87; 100), *B. setariae* (85; 100), *B. maydis* (75; 95), *C. affinis* (80; 64), *C. cymbopogonis* (72; 72), *C. fallax* (83; 100), *E. rostratum* (78; 72) and *E. longirostratum* (86; 91) clustered with 72% or more similarity within the relevant species (Appendix H: Figure 24). Dice's coefficient (DC; Appendix H: Figure 25), confirmed the reliability of some of the above clusters. These clusters were *B. indica* (70; 100), *B. setariae* (72; 100), *B. maydis* (72; 58) and *C. fallax* (75; 100). Similarity of strains representing the species *C. affinis, C. cymbopogonis, E. rostratum* and *E. longirostratum* seems distinctly less homogenous according to Dice's coefficient. Similarity of strains using DC and shown by the single primer BDB-(CAC)5, indicated good clustering of these species except for *E. longirostratum* and *E. rostratum* (50; 75) (Appendix H: Figure 22). Similarity and significance values produced by primer BDB-(CAC)5 and PC (Appendix H: Figure 23) were *B. cynodontis* (74; 59), *C. cymbopogonis* (88; 72), *B. indica* (84; 100), *B. setariae* (91; 100), *C. affinis* (86; 69) and *C. fallax* (95; 100). *E. longirostratum* (87; 66) and *E. rostratum* (85; 55) clustered together (Appendix H: Figure 23).

The dendrogram derived from the data sets of all primers and applying DC, clustered one isolate of *E. rostratum* together and the rest with *E. longirostratum* (47:83) (Appendix H: Figure 25). Using PC, all *E. rostratum* and *E. longirostratum* isolates join at a similarity level of 73% (Appendix H: Figure 24). Both isolates of *E. inaequale* and the single isolate of *E. turcicum* were also included in the *E. rostratum* / *E. longirostratum* cluster. All four *Exserohilum* species were included in a single cluster (Appendix H: Figure 24) and joined at a similarity level of 60% and significance level of 59%. When examining the relationship of *E. turcicum* and *E. longirostratum* they seem highly similar in ISSR patterns, but this correlation is not supported by the average group separation table (Appendix H: Table 17). Only one isolate of *E. turcicum* was available for analysis.

Clustering of strains of the rest of the species investigated was less significant and the information extracted from these results, less certain. Clustering of strains of both *E. inaequale, B. papendorfii* and *C. lunata* were inconsistent with similarity values of strains of each species, being consistently below 40% (Appendix H: Figures 22 - 25).

**b) Internal stability (significance) of clusters according to group separation tables**

Maximum and average similarities were calculated for each similarity coefficient (Appendix H: Tables 15 - 18. All four tables indicate that the species clusters *B. maydis, B. setariae* and *E. longirostratum* were consistently 100% homogenous and

showed no similarity to any other species clusters. Therefore both similarity coefficients indicate a high degree of internal stability (significance) for these species clusters. However, when the internal stability values of strains using all four tables were considered, consistent 100% homogeneity could not be shown for the species *B. papendorfii, C. cynodontis, C. affinis, C. indica, C. fallax, E. inaequale* and *E. rostratum*. The least consistent group of isolates, most evidently displayed with PC (Appendix H: Table 17), were the strains representing *C. lunata*. *Curvularia lunata* showed similarities to *B. indica, C. fallax, B. papendrofii* and *C. cymbopogonis*. The main clusters containing four *C. lunata* strains supports this heterogeneity and show a similarity value of 41% at 57% significance (Appendix H: Figure 24).

Both DC (Appendix H: Tables 15 & 16) and PC (Appendix H: Tables 17 & 18) indicated some affinities between species. *Curvularia fallax* is also related to *C. cymbopogonis* and *C. affinis* while *B. cynodontis* may also be related to *C. cymbopogonis* and *C. fallax*. However, the small number of strains included in the analysis does not allow speculation on the significance of these similarities and does not justify any definite conclusions to be drawn on the probable genetic relatedness of the species groups in the *Bipolaris* complex.

# 6 DISCUSSION AND CONCLUSIONS

## 6.1 The *Bipolaris*-like species in South Africa

The firsts goal of this study was to compile a checklist of the South African species of the *Bipolaris*-like genera (Appendix E)., as described in 4.1. Up to 1995 the genus *Bipolaris* included 52 species, *Curvularia* 33 species and *Exserohilum* 20 species (Hawksworth *et al.* 1995). Approximately half of these *Bipolaris* and *Curvularia* species have been recorded from South Africa but species of *Exserohilum* are much less prevalent. The checklist compiled during this investigation includes three new records of *Bipolaris*, four of *Curvularia* and one of *Exserohilum.*

Of these newly recorded species, *B. coicis,* a strictly graminicolous species, needs specific mention because it is known to cause a serious leaf blight of *Coix* grasses, and infects leaves and inflorescences of *Zea mays* (Sivanesan 1987). *Bipolaris coicis* is here recorded from *Cenchrus ciliaris,* a fodder of high value that is also used to produce hay (Van Oudshoorn 1992). *Bipolaris nicotiae,* only known from soil (Mouchacca 1973), is reported from fodder. All newly recorded *Curvularia* species are plurivorous, occur on many different grasses and are widely distributed throughout the world (Sivanesan 1987). *Curvularia geniculata,* one of the new records, is an important causative agent of seed and seedling blights of pea, cabbage, flax, rice, sweet potato, pearl millet, wheat and some forage grasses. The record of *E. longirostratum* was from a species of *Musa* sp. and therefore in line with its previously reported plurivorous nature.

Most of *Bipolaris*-like species occurring in South Africa are species that parasitise different host genera. However, three host-specific ovaricolous parasites of *Sporobolus* are also known to occur in South Africa. They are *B. brizae*, *B. crustacea* and *B. ravenelii.* The fourth known ovaricolous species, *B. australis* Alcorn has not been isolated in South Africa (Sivanesan 1986). Of the three local species, *B. crustacea* is the most common. All specimens of *B. ravenelii* in herbarium PREM also contain *B. crustacea.* The co-existence of the two species may indicate an even stronger dependence of *B. crustacea* on specific growth conditions. This co-existence seems to be microbial succession rather than true pathogenicity of *B. crustacea* to the host. Sivanesan and Holliday (1982) reported a similar synergism of *Coch. sativus* and *C. geniculata* on seeds of *Poa pratensis* and speculated that *C. geniculata* is saprotrophic rather than parasitic.

Apart form selected publications and disease reports (Putterill 1924, 1954; Scott 1995; Van der Westhuizen 1955, 1975, 1978) there is hardly any information on the actual impact of *Bipolaris*-like fungi on agriculture in South Africa. The checklist probably represents a scant indication of the diversity of these fungi in South Africa when the large variety of natural grassveld types, different grass species and area of land inhabited by them, are considered (Acocks 1988).

## 6.2 Assessment of culture based tests and spore image-analyses.

The second goal of the investigation was to find stable characteristics to differentiate between *Bipolaris*-like species. The two criteria investigated were measurements of spore dimensions other than length and width, and growth responses of strains during culture-based tests, as described in 4.4.

### 6.2.1 Analysis of spore images

The correct approach to unresolved taxonomic issues would be, firstly, to find the characteristics that can differentiate between species and, secondly, to define the set of conditions presenting the most repeatable and consistent manifestation of such characteristics. Only features that can be obtained with consistency should be used for descriptions of species and identification keys. This concept has been successfully implemented in the identification regime proposed by (Pitt 1979) for the genus *Penicillium*.

Species concepts, as applied to fungi in contrast to other more advanced organisms, were discussed previously (see Section 2). The fungi have simple morphologies obliging taxonomists to be strongly dependent on features of spores and spore forming structures for their differentiation. However, Alcorn (1983) stated that many aspects of conidial morphology used to distinguishing between *Bipolaris* and *Curvularia* seem to be differences of degree rather than nature. Measurements of the length and width of spores are key characteristics and mostly included in descriptions of mitosporic species but the extreme variation in spore dimensions found in *Bipolaris*-like fungi limits its value within this group.

To achieve consistency, the manifestation of conidial features must be investigated and any defined hypothesis tested. Investigation of the variability of spore dimensions within species of the *Bipolaris* complex was therefore approached with the assumption that a large amount of information would enable a highly reliable, clear pattern to emerge. As more data are used to evaluate a certain feature, the statistical validity would increase providing that the data were obtained randomly and are representative of the character. At a certain point however, the addition of more data will not significantly change the validity of the information (Kohn 1992). The number and type of spore dimensions obtained form *Bipolaris*-like strains as presented in this study, exceeds that of all previous studies on the helminthosporic species. Image analysis by computer can manage a large volume of data with relative ease and provided an opportunity to obtain dimensions different to the usual length and width measurements regularly used.

When analysing values that define shape and size of spores with DT and FOIL classification techniques (explained in the 4.4.8), results (presented in 5.2.1) indicate that measurements defining shape rather than size are more useful in separating species. Of all the possible measurements used in the spore image analysis, only a few were found to be

useful: the major and minor axis measurements (defining size), ferret diameter (size), roundness (shape) and compactness (shape) were found to be most effective in differentiating between the strains studied.

FOIL and DT analysis of spore images showed that strains of the same species were not recognised as single clusters. Consequently it is not recommended that spore dimensions alone be used to differentiate between species. Important information presented in 5.2.1 illustrated the effect of environmental conditions on spore features. The shape of spores from cultures incubated at different temperatures and on the various culture media did not differ and is therefore a stable feature. In the short-spored species (elongation less than 5), cultures between five to ten days old also did not change the differential effectivety of spore shape but influenced the differential capacity of this feature in species with elongated spores (5 of more). When using DT computation, the variable "age of culture", was used only once but was essential to differentiate between the species *E. longirostratum, E. turcicum* and *B. setariae*. The DT rule used differences in roundness and ferret diameters on days five, seven and ten to separate the latter three species. This finding implies that there is a distinct and intrinsic difference in growth rate of elongated spores between species. Growth rate of spores of long-spored species would therefore facilitate their differentiation. A developmental difference was also noted during assessment of culture-based tests where strains of all species, except the strains of *B. setariae*, developed spores on the sides of inocula after one day of incubation on sorbitol agar.

Consistency in spore shape would facilitate comparison of results between laboratories. When using constant characteristics less dependant on environmental changes, it would be less critical to obtain measurements from exactly similar conditions.

### 6.2.2 Growth rate and cultural characteristics of *Bipolaris*-like fungi

Within the framework of the study, the value of physiological characteristics to differentiate between species was clearly demonstrated. Colony diameter was mostly used as the unit of measure, even though the amount of biomass produced during a test would have been a more precise measurement (Stevens 1974). This procedure is distinctly more laborious and time consuming than measuring colony diameter and was therefore not considered for this study. Other possible measurements are amongst others, colour reactions, age of colonies when sporulation started, topography or texture of cultures, nature of the colony edge, colour on the reverse side of colonies, rate of germination and the formation of torulose hyphae (Stevens 1974). Such differences were noted in this study on acetic acid medium and the sugar alcohol mediums, but were not recorded from all tests. Some of these features have been used with great success to support the differentiation of closely similar mitosporic species in other genera. Based on biochemical requirements, some species within the genus *Trichophyton* can be separated with ease

(Stevens 1974). The identification procedure for species of *Penicillium*, based on colour reaction, growth and sporulation on specific media has been developed by Pitt (1979) and is currently used as the standard for this genus.

Refinement of nearly all culture-based tests used in this study is possible and will increase their value. Some recommendations towards the refinement of these tests are proposed:

- It is important to evaluate different concentrations of the substrates tested. The resolution of tests with inhibitory compounds especially acetic acid, were highly dependent on the precise concentration of the particular compound tested. An example is the concentration of glycerol in the G25N medium that was too high since growth of all strains on this medium was severely restricted.
- Various other substrates in addition to those tested should be evaluated. Carbon sources and inhibitory compounds seem promising since inositol, sorbitol, glucose and acetic acid media were found to facilitate differentiation of species. The value of carbon utilization to differentiate species of helminthosporic fungi was also established by Garrett (1975), and in many other species of mitosporic genera (Garrett 1975; Hawksworth 1984; Patersen & Bridge 1994; Todarova *et al.* 1994). Reactions to other inhibitory compounds such as antibiotics or nutrients such as vitamins can be explored.
- The current investigation did not consider the use of specific stains or reagents as applied to identify some basidiomycetous fungi and lichens (Hawksworth *et al.* 1995). Seifert (1985) described the use of colour reactions with the reagent KOH with phloxine in his monograph of *Stilbella* and allied synnemaceous hyphomycete genera. Alcorn (1982b) used the same stain to investigate septum structure in *Curvularia* and *Bipolaris* species but did not record specific reactions between species.
- The data already accumulated from culture-based tests in this study can yield more information by using other appropriate data mining techniques, or extending calculation to more and different combinations of data sets, using FOIL and DT.

With these options in mind and from the results obtained during this investigation, the potential of physiological features to separate *Bipolaris*-like species exists. Ideally further development could result in an identification procedure similar to that of Pitt (1979) or the development of selective media. The limited use of physiological characteristics in descriptions of mitosporic fungi and the lack of its application to helminthosporic fungi therefore warrants further development and exploration.

### 6.2.3 Standardised descriptions for identification purposes

To facilitate identification of *Bipolaris*-like fungi the following features of a strain should be specified in descriptions after incubation under conditions as described in (4.4.1):

The teleomorph - attempt induction of the teleomorph as described by Sivanesan (1987). Record the spore shape, spore length, width and elongation, septum ontogeny, particular cells where germtubes emerge, the angle of germtube emergence and the hilum structure of conidia from seven-day-old cultures on Sach agar. Record colony diameter, colour reaction and texture of 5-day-old cultures on Sach, 1% Glucose, 0.01% Acetic acid and CYE agars. Record the natural host.

### 6.2.4 Nucleic acid based tests

Various publications clearly indicate that molecular markers offer reliable methods to tests the validity of a morphological taxonomic system (Bruns *et al.* 1991; Klich & Mullaney 1992; Kurtzman 1985; Mills 1993; Mitchell *et al.* 1995; Weising *et al.* 1991). The nucleic acid based facet of the investigation was therefore conducted to test the validity of the current morphological classification of the *Bipolaris*-like fungi. The techniques used were described in 4.5 and results presented in 5.3.

**a) Sequencing**

According to other molecular investigations, the 28S rRNA gene of the ribosomal DNA in the fungal genome, can be used to indicate taxonomic separation at generic level (Michell *et al.* 1995). The percentage polymorphism in the nucleic acid sequences of this region is usually between 3% and 20% and approximately 1% between species (Kurtzman & Robnett 1993; Mitchell *et al.* 1995). The level of polymorphism obtained when comparing aligned sequences of the various *Bipolaris*-like strains from this region was approximately 2%. This low percentage difference indicates high conservation of this region of the *Bipolaris*-like fungi. Nucleic acid sequences obtained with the LR-7 forward and reverse primers that target this region, only indicated a difference between *Exserohilum* species and the *Curvularia* / *Bipolaris* anamorphs of *Cochliobolus*. Sequencing data provided here support the original hypothesis of Luttrell (1977) that the anamorphic *Exserohilum* species associated with the Ascomycota teleomorph *Setosphaeria* forms a distinct group separate from the mitosporic *Bipolaris* and *Curvularia* associated with *Cochliobolus* teleomorphs. Subsequent to Luttrell's (1977) publication, many additional anamorph-teleomorph associations reported confirmed these relationships (Alcorn 1983, 1990; Sivanesan 1987). Sequencing data obtained during this investigation (see 5.3.1) also supports the observations of Sivanesan (1987), who found no evidence based on morphological data, to retain the *Cochliobolus* anamorphs, *Curvularia* and *Bipolaris* as separate genera.

**b) ISSR analysis**

Previous studies using ISSR markers were those of Longato & Bonfante (1997) who investigated inter- and intra species variation in ectomycorrhizal fungi (Basidiomycota), and that of Buscot *et al.* (1996) on morels (Basidiomycota). The current study provides t[illegible] on helminthosporic species

(Ascomycota). Since this technique has been established as an effective tool for the molecular fingerprinting of a range of plants and animals (Weising *et al.* 1991; Weising *et al.* 1995), this tool was used to test the current morphological species concepts of *Bipolaris*-like fungi, and the genetic variation within the different species.

Inter repeat PCR or ISSR analysis is one of the latest nucleic acid based techniques effectively amplifying hyper-variable repetitive DNA sequences. The combination of banding patterns obtained from four primers DBD-$(AC)_7$, BDB-$(CAC)_5$, DHB-$(CGA)_5$ and VHV-$GT_7$-G were more informative than any one of these primers separately (see 5.3). *B indica* formed a consistent group with high similarity between isolates (100%). The validity of this species based on morphology, is therefore justified. Other species which clustered separately from all others were *C. affinis, C. fallax, C. cymbopogonis, B. cynodontis, E. rostratum* and *E. longirostratum* but with somewhat greater intra-specific variation ranging between 75% and 87% similarity. Longato and Bonfante (1997) also found high similarity between strains of a species (83% to 100% similarity) in the genus *Tuber* (Ascomycota). A study of 400 strains of *Ascochyta rabiei* (Pass.) Labr. revealed approximately 70% similarity between strains (Geistlinger *et al.* 1997). The ISSR data of the long spored *Bipolaris* species, *B. setariae* and *B. maydis* indicated 20% to 75% variation between strains (Appendix H: Tables 15 - 18). Three species included in the ISSR dendrogram, *E. inaequale, B. papendorfii* and *C. lunata* were inconsistent with regard to the similarity values of strains of each species, being consistently below 40%. Based on ISSR data alone it is uncertain if these three species should be retained as separate species.

Using microsatellite markers Longato and Bonfante (1997) supported the separation of two morphologically similar species of *Tuber*, since they were only 20% to 27% similar in a fingerprint pattern. ISSR dendrograms showed a percentage similarity, for defining a species to be 70% or higher. The validity of this similarity value will be challenged in subsequent investigations.

### 6.2.5 A new approach to fungal taxonomy?

In the literature review (see 2.1, 2.2 & 2.3) the various schools of thought on fungal taxonomy were discussed. The early 1800's classification of fungi was based solely on morphology but later changed to include newly discovered developmental features of spore formation. As inadequacies of both systems became apparent, new alternatives were sought resulting in a proliferation of studies on enzymatic and molecular characterisation of fungi (Paterson & Bridge 1994). New molecular technologies resulted in uncertainty on the way forward and publications often discussed "*morphology versus molecular*" taxonomy (Bruns *et al.* 1991; Klich & Mullaney 1992; Kurtzman 1985; Mills 1993).

Molecular techniques used in this investigation did not resolve all the taxonomic questions raised at the beginning of the study (see 3.4), but certainly added reliability to observations

made. The ultimate goal of systematists is a proper phylogenic reconstruction but would be premature to attempt here, untile unresolved taxonomic issues are investigate (see 2.5.2). Molecular comparisons strongly supported the phenotypic expression of strains, specifically the behaviour of strains in culture and the nature of spore morphology. The word "*versus*" used in many publications is misleading and indicates opposites rather than the supportive role that the various different aspects true to the nature of a fungus can provide. This study of *Bipolaris*-like fungi and those of Frisvad (1994) and Pitt (1985) on phialidic mitosporic fungi, substantiate the use of various dimensions of mitosporic fungi, including morphological, physiological and nucleic-acid based characters together, to define genera and species.

## 6.3 Integrated characterisation of *Bipolaris*-like species

Before giving specific attention to the species selected for investigation, conventions in fungal classification between mycologists must be considered (Hawksworth *et al.* 1995). There is a tendency not to use intermediate ranks, or ranks below subspecies. According to convention the rank of special form is available to mycologists wishing to separate morphological identical species by host reactions but the International Code of Botanical Nomenclature does not regulate special forms or physiological races. This convention will be supported here even though the resolution level of ISSR used (discussed in 2.4.4), is on a species or below species level. According to Hawksworth *et al.* (1995) any characteristics judged to represent significant discontinuities, whether biological, morphological, ultrastructural or molecular can be used in classification. Particular emphasis is placed on reproductive structures, ultrastructure and molecular evidence at levels above family

Discussion of the taxonomic status of the various species investigated will acknowledge the conventions described in 6.2, and include information provided by the study and the relevant literature. The respective species will be discussed in groups according to the remarks on unresolved issues in the taxonomy of the *Bipolaris* complex (see 3.4) and the reasons for their selections as explained in 4.2. Each species will only be discussed once even though it might also fit into one of the other categories presented.

### 6.3.1 Species with protuberant hila

Protuberant hila have been reported in both *C. cymbopogonis* and *E. inaequale,* the most prominent morphological feature used to separate species of *Exserohilum* from other *Bipolaris*-like fungi.

**a) Spore morphology:**

Shape of conidia, in both species is described as ellipsoidal, but also as clavate in *C. cymbopogonis* and cylindrical in *E. inaequale* (Sivanesan 1987). After examining the available strains, isolates of both species were found to form two different kinds of

conidia. The first spore form is clavate (the broadest part of the conidia is almost always in the upper half of the conidia). The second spore type is ellipsoidal to cylindrical. A particularly prominent feature in the latter spore type is that spore walls of the basal cell (sometimes also the second from the base of the conidia) are parallel to one another, diverting from the parallel only from the third cell upward. This sudden change in the angle of the conidial wall gives the impression of the septum being constricted, but this is not so. Both spore types can be present, or either one of the two types can dominate, depending on the strain. Species having more than one type of spore are a common phenomenon in mitosporic fungi (i.e. *Fusarium* and *Cylindrocarpon*) and also known in *E. rostratum*.

Conidia of both species are usually four-septate. Sivanesan (1987) also noted that septa at both ends of the conidia of *C. cymbopogonis* are fairly close together with the middle cell longer and wider than the others. An enlarged middle cell has been noted in some conidia of all strains investigated. This is a distinct feature of *Curvularia* sensu Boedijn.

The conidia of both *E. inaequale* and *C. cymbopogonis* display prominent protruding hila, one of the most important features separating *Exserohilum* from the other *Bipolaris*-like fungi. No differences were observed in the structure of hila between the species. Luttrell (1977) stated that hila in *C. cymbopogonis* are distinctly different in structure than those of members of other species of *Exserohilum* an indication that *E. inaequale* does not belong in *Exserohilum*.

The range in spore size of the two species is also similar (see Appendix B: Table 8). Published length and width measurements confirm observations made during the study. Sivanesan (1987) recorded dimensions of 35-60 x 14-20µm (on host) and 28-50 x 12-20µm for *C. cymbopogonis* while Sivanesan and Holliday (1982) recorded a size range of 30-46 x 11-17 µm for conidia in culture. Conidia of *E. inaequale* are 24-32 (45) x 9-12(16) µm (Sivanesan 1987). Elongation of conidia was between 2.68 and 4.98 (average 3.6) for *C. cymbopogonis* and between 2.05 and 5.18 (average 2.98) for *E. inaequale*. The species *C. cymbopogonis* and *E. inaequale* are therefore similar in spore morphology.

**b) Physiology**

FOIL analysis of growth characteristics clearly showed differences between the two species, but the DT analysis did not. In the latter analysis *C. cymbopogonis* was grouped with *E. inaequale* and some isolates of both species grouped with *E. turcicum*. All colonies of *C. cymbopogonis* strains and some strains of *E. inaequale* were restricted on Inositol agar (Table 5). Three of six strains of *E. inaequale* however displayed normal growth on this medium. Of all strains included in the investigation only some strains of *C. affinis*, *C. cymbopogonis* and *E. inaequale* produced a pigment

on sorbitol agar. Grouping of data sets by the DT and FOIL analyses regularly included those of *E. inaequale* with those of *C. cymbopogonis* or *C. lunata* (Appendix G: Tables 11 & 12).

**c) ISSR**

*Curvularia cymbopogonis* and *E. inaequale* were often grouped together but also share these groups with other species (Appendix H: Figures 22 - 25). Similarity values between the two species were usually 20% or lower. The internal stability of the clusters containing *C. cymbopogonis* was all 4 matrixes. The internal stability of clusters containing strains of *E. inaequale* was for three out of four tables 0%, but in the fourth (Appendix H: Table 15) 50%. Not one of the two species therefore displayed intra specific homogeneity and was often clustered together even though it was in groups with only average similarity.

**d) Other important features**

*Exserohilum inaequale* was previously isolated from mango and other plant material (Sivanesan 1987). Apart from these two reports no further host data could be found, while *C. cymbopogonis* was mostly reported on species of Andropogoneae but also on other plants (Sivanesan & Holliday 1982). No teleomorph has been recorded for *E. inaequale* but for *C. cymbopogonis* a teleomorph is known in *Cochliobolus*.

**e) Taxonomic conclusion**

*Exserohilum inaequale* is a synonym of *C. cymbopogonis*. Sufficient similarity in physiology, spore morphology and to a lesser extent ISSR data exists to support the synonymy of *E. inaequale* with *C. cymbopogonis*. Although *C. cymbopogonis* prefers graminicolous hosts of the family Andropogoneae it has also been recorded on other plants. *Exserohilum inaequale* strains are scarce and host records inconclusive. It is further suggested that *C. cymbopogonis* be retained in the genus *Curvularia* since its teleomorph is known as *Cochliobolus* (Sivanesan 1987) and Luttrell (1977) found its hilum structure to be different to that of other species of *Exserohilum*.

### 6.3.2 Species with relative short spores sharing features of more than one genus

Both *B. ellisii* and *B. papendorfii* form curved conidia and sometimes a disproportionately swollen middle cell, features typical to *Curvularia*. The species *Bipolaris indica* sometimes form conidia with protuberant hila a feature typical of *Exserohilum*.

**a) Spore morphology**

The spore shape of both *B. ellisii* and *B. papendorfii* is similarly navicular but the two species are distinct in that strains of *B. ellisii* regularly produce clavate spores. No clavate spore-shapes have been observed in strains of *B. papendorfii*. The third species, *B. indica*, almost exclusively forms clavate spores. The curvature of the spores is a prominent feature of both *I* t not of *B. indica*. Boedijn, the

author of the genus *Curvularia*, relied heavily on the distinct curvature of spores to distinguish between his newly proposed genus and *Bipolaris* (Luttrell 1977). The spores of *Curvularia* species usually bend at the disproportionately enlarged and darkened middle cell of conidia. None of the three species under discussion have disproportionately enlarged and darkened middle cells in their conidia. The spore shape of *B. ellisii, B. papendorfii* and *B. indica* are however distinct in that both DT and FOIL placed the spore image data sets of all three species correctly within their respective species groups. Spore shape within each of these species stay consistent as observed under various growth conditions.

No hilum is formed on spores of *B. ellisii* and *B. papendorfii* but was irregularly observed in spores of *B. indica*. Two features of *B. indica*, the hilum structure (Sivanesan 1987) and septum ontogeny (Alcorn 1983), strongly indicate a relationship to *Exserohilum* rather than *Bipolaris*.

Clear differences in spore size between the three species were also observed (Appendix F: Figures 4, 5 & 7) and recorded by Sivanesan (1987; Appendix B: Table 8). Elongation between the tree species are highly similar: In *B. ellisii* elongation was measured between 1.99 and 2.3 (average 2.17), in *B. papendorfii* between 1.81 and 2.37 (average 2.01) and *B. indica* between 2.11 and 3.64 (average 2.66).

**b) Physiology**

Within their respective clusters, *B. indica* and *B. ellisii* were consistent in their physiological behaviour and in the textures of colonies on inositol agar (Table 5). In contrast strains of *B. papendorfii* were highly variable in reactions in a culture-based tests. The FOIL algorithm grouped strains of *B. papendorfii* in a cluster, but DT did not. DT could not differentiate a cluster. Strains representing species of all three of the genera investigated were grouped together, these were *B. maydis*, *C. lunata*, *E. inaequale* or *E. turcicum*.

**c) ISSR:**

An ISSR analysis of *B. ellisii* strains was not performed. Both the similarity coefficients used, showed *B. indica* as a reliable, consistent cluster with high internal stability (100%) (Appendix H: Tables 15 to 18). Sequencing data indicated *B. ellisii, B. indica*, and *E. inaequale* as part of a group including *Bipolaris* and *Curvularia* species. This group was separate from the other *Exserohilum* species included in the investigation. The current molecular investigation could therefore, not clarify the generic affinity of *B. indica*. ISSR analysis did not define *B. papendorfii* as a distinct group of strains. This finding is in congruence with results of culture-based tests. Alternative placement of *B. papendorfii* strains was varied, but never with any species of *Exserohilum*: *B. papendorfii* strains were grouped with *B. cynodontis, B. maydis, B. setariae, C. lunata, C. affinis* or *C. fallax* depending on the various calculations

performed. Due to the resolution level of ISSR fingerprinting no deductions about the generic affinity of this species could be made.

**d) Other important features**

*Bipolaris ellisii* is the only species with a teleomorph known in *Cochliobolus* (Sivanesan 1987; Alcorn 1983). All three species are plurivorous and have been recorded from many different hosts.

**e) Taxonomic conclusion**

All three species should be retained as distinct species. Both *B. ellisii* and *B. indica* displayed intraspecific consistency in spore morphology and physiology. This intraspecific consistency was also observed in *B. indica* in ISSR fingerprints. Due to a distinctive and consistent spore shape, *B. papendorfii* is retained as a separate species but needs further investigation because of the high molecular and metabolic variability between strains. The molecular diversity of *B. papendorfii* strains might indicate convergent evolution of a group of highly similar strains. Sequencing did not confirm similarity between *B. indica* and other species of *Exserohilum*.

### 6.3.3 *Curvularia* species separated by a single feature

The three highly similar *Curvularia* species *C. affinis, C. fallax* and *C. lunata* have been described with only subtle differences in conidium morphology.

**a) Spore morphology**

With the exception of two data sets, FOIL recognised spore shapes of *C. affinis* as those of *C. lunata*. With most data sets of the two fungi, the DT algorithm could also not establish separation of the two species. The middle cell of conidia in all three species are darkened and enlarged. Enlargement of cells is not always prominent but when spores are measured, the widest part of the spore have been across these cells (see remarks made in 6.3.2 on curvature of conidia in species of *Curvularia*).

Conidia of *C. affinis* are mostly four septate but can also be up to five septate, *C. fallax* are regularly four septate and spores of *C. lunata* are mainly three septate. Microscopic inspection of spores revealed spores of *C. lunata* to be regularly and prominently curved and in comparison those of *C. affinis* are less prominently curved. Spores of *C. fallax* are mostly straight and only rarely curved. All three species form flush hila as described by Alcorn (1983).

The spore sizes of the three species overlap in range. Sivanesan (1987) recorded 27-49 (32) x 8-9 (10) µm for *C. affinis*, 18-32 x 8-16 µm for *C. lunata* and 24 –38 (30.6) x 9-15 (12.3) µm for *C. fallax*. The spore dimensions of the three species are closely similar (Appendix F: Figures 9, 11 & 12) with elongation in *C. affinis* between 2.45 and 2.91 (average 2.64), in *C. fallax* between 1.95 and 2.79 (average 2.38) and *C. lunata* between 1.95 and 3.53 (average 2.44).

**b) Physiology**

Both *C. fallax* and *C. lunata* were presented as heterogeneous groups during culture-based tests. Clustering of some strains within the respective species were achieved with FOIL but not with the DT analysis. Similar to the growth responses in the culture-based test, strains of *C. affinis, C. fallax* and *C. lunata* were all inconsistent in colony textures.

**c) ISSR**

The *C. affinis* cluster was distinct from all other groups: strains of this species showed relatively high similarity (Appendix H: Figures 22 – 25) and 100% internal consistency for the two tables (Appendix H: Tables 17 & 18). The low degree in similarity between strains of *C. affinis* to other *Bipolaris*-like fungi investigated is in the same order as that reported by Longato and Bonfante (1997) for two species of *Tuber* (Ascomycota). Using ISSR, they reported 20% and 27% similar between the two species they investigated. *Curvularia fallax* was evident as a cluster with high degree of similarity between the two strains included in this study. Consistency within this group was indicated by group separation as 50% (Appendix H: Tables 16 & 17) and 100% (Appendix H: Table 15). *Curvularia lunata* in contrast, is a highly heterogeneous group with a similarity level between strains recorded between 0% and 49% depending on the correlation coefficient used. *Curvularia lunata* and *C. fallax* were mostly included in the same larger cluster also containing other *Curvularia* and *Bipolaris* species.

**d) Other important features**

All three species are plurivorous and can be found on many different host and substrates. *Curvularia lunata* is the only species associated with a teleomorph and belong with *Cochliobolus*.

**e) Taxonomic conclusions**

*Curvularia affinis* is a distinct species. *Curvularia fallax* and *C. lunata* cannot be separated with confidence and needs further investigation.

The only observed morphological difference between the three species is in the degree of curvature and the number of septa per conidium. Conidial septation is therefore the only morphological feature correlated with the strongly ISSR defined *C. affinis*. Even though growth responses on culture-based tests did not contribute to differentiation of this species, *C. affinis* is a distinct species.

*Curvularia fallax* and *C. lunata* cannot be separated with confidence with the only differences being in curvature and the number of septa in the spores. Both Alcorn (1983) and Sivanesan (1987) indicate curvature to be an unreliable taxonomic feature. Due to the low similarity levels between strains, *C. lunata* should be seen as a species

group. With no significant morphological or physiological distinguishing features, *C. fallax* seems to form part of this *C. lunata* group of species.

The observations made during this study confirm Luttrell's (1977) speculation that sub-generic groups exist within *Curvularia*. From the publication and illustrations of Sivanesan (1987) it would seem that the "lunata" group possibly includes species such as *C. pallescens* Boedijn, *C. clavata* Jain, *C. gudauskasii* Morgan-Jones & Karr amongst others. All these species have similar conidia: three septa, darkened middle cells and lighter end cells, spore sizes within a similar range, similarly curved at the middle cells, and in comparison to other *Bipolaris*-like fungi, similar in shape (Alcorn 1983; Sivanesan 1987). This study did not yield any information to enhance the circumscription of these closely related *Curvularia* species. The heterogeneity of the *C. lunata* group requires further investigation.

### 6.3.4 *Exserohilum* species separated on a single feature

*Exserohilum rostratum* and *E. longirostratum* have been described as separate species and are only differentiated on the length of conidia.

**a) Spore morphology**

The spore shape of both *E. rostratum* and *E. longirostratum* are rostrate to cylindrical but those of *E. turcicum* prominently fusoid. DT analyses, used on roundness, compactness and the ferret diameters clustered 65% of *E. rostratum* spore data sets and 41% of *E. longirostratum* data sets, within their respective species groups (Appendix F: Table 12). Strains of *E. rostratum* and *E. longirostratum* were often included in *B. maydis*, *C. cymbopogonis* or *E. turcicum*. However, if all the strains of *E. rostratum* and *E. longirostratum* are considered together, DT clustered 58% strains of *E. longirostratum* and 82% of *E. rostratum* within this composite group (Appendix F: Table 12). FOIL did not differentiate between spore images of *E. rostratum, E. longirostratum* and *E. turcicum*.

Conidia of all three species have prominent protuberant hila. The width of *E. turcicum* spores is distinctly and consistently greater than that of the other two species. The average length of spores of *E. longirostratum* and *E. rostratum* did not differ significantly. Elongation recorded for *E. rostratum* (4.7 - 13.73: average 8.74) and *E. longirostratum* (6.2-12.41: average 7.82) are highly similar. Darkened septa were observed in *E. rostratum* and *E. longirostratum* but not in *E. turcicum*.

**b) Physiology**

Considering all tests, the three species formed separate clusters.

**c) ISSR**

All strains of *E. rostratum* and *E. longirostratum* join at a similarity level of 50%. The consistency of this group (containing both *E. rostratum* and *E longirostratum*) ranged

between 53 and 83% (Appendix H: Figures 22 - 25). Similarity of strains within *E. rostratum* ranged from 55 - 75% and within *E. longirostratum* from 50 – 80%. The single strain of *E. turcicum* investigated was also included in this cluster. These separate species clusters occur repeatedly (specifically apparent in Appendix H: Figures 22 & 23) but with only average significance of 75% and 55% respectively (Appendix H: Figures 22 - 25). When examining the relationship of *E. turcicum* and *E. longirostratum* they seem highly similar in ISSR patterns, but average similarity between the two species does not support this correlation.

**d) Taxonomic conclusion**

*Exserohilum longirostratum* and *E. rostratum* should be retained as separate species. The current separation of *E. rostratum* from *E. longirostratum* based on spore length alone is not acceptable. The present investigation proves that ranges in spore lengths greatly overlap and the extent to which spore lengths differ within each species is similar (see elongation). However, *Exserohilum rostratum* and *E. longirostratum* were found to differ in physiological behaviour. Keeping to convention, physiological differences between morphological similar species are separated at sub-specific levels (Hawksworth *et al.* 1995). *Exserohilum longirostratum* could therefore be a physiological form of *E. rostratum*. The consistency of *E. rostratum* and *E. longirostratum* as separate clusters supports such a decision since differentiation based on sub-species variation is one of the precise strengths of this fingerprinting technique. Comparable ISSR data of more isolates are necessary to conclude the validity of this two species.

The lack of sufficiently supportive molecular data does not influence the validity of *E. turcicum* as separate species. It is distinct in spore morphology and physiological characteristics.

### 6.3.5 Species typical of the genus *Bipolaris*

*Bipolaris cynodontis, B. maydis* and *B. setariae* have been investigated as species typical of *Bipolaris* but these three species form curved conidia.

**a) Spore morphology**

DT and FOIL consistently grouped spore images of *B. cynodontis* in a cluster. Only a few data sets were grouped with *C. cymbopogonis* and *C. lunata*. In contrast, data sets of *B. maydis* were often grouped with species of both *Exserohilum* and *Bipolaris* and hardly ever formed a distinctive group. The same phenomenon is true for strains of *B. setariae*. Therefore, neither *B. setariae* nor *B. maydis* were recognized as distinct entities on spore dimensions alone. This observation can be explained by the difference between *B. maydis* and *B. setariae* for the time needed to form conidia (see 5.2.1). DT and FOIL analyses of spore images revealed a dependence on culture conditions. The synopsis is that spore shap s and *Exserohilum* species are

highly similar and are in contrast to those of *Curvularia* species included in this investigation. Spores of all three species are mostly slightly curved, fusoid (widest part in the middle) and germinate from both ends of the spores (Alcorn 1983; Sivanesan 1987).

The ranges of length and widths of *B. maydis* and *B. setariae* overlap, spores are similar in elongation (averages 4.76 & 5.29 respectively; Appendix F: Figures 3, 6 & 8). The number of conidial septa is similar (Sivanesan 1987). It is therefore difficult to distinguish between the two. The hila of conidia are flush and similar in all three species (Alcorn 1983).

**b) Physiology**

*Bipolaris cynodontis*, *B. maydis* and *B. setariae* strains form separate clusters based on culture-based tests when analysed with both DT and FOIL. The three species are similar in having the same restricted growth on inositol agar. In contrast, all species of *Exserohilum* (and some of the short-spored *Bipolaris* species *B. indica* and *B. papendorfii*), display normal growth on this media.

**c) ISSR**

Fingerprint patterns of *B. maydis* and *B. setariae* were highly distinctive. The dendrograms revealed a high measure of variability between strains of *B. cynodontis* except when Pearson's correlation coefficient was applied (compare Appendix H: Figure 23 & 24 to Figures 22 & 25). Strains of *B. cynodontis* displayed low internal stability (Appendix H: Tables 15, 16, 17 & 18) and were often included in clusters mostly containing species of *Exserohilum*.

**d) Other important features**

All three species have confirmed associations with *Cochliobolus* teleomorphs. Records of all three species indicate strong but not obligatory host preferences: *Bipolaris maydis* is commonly found on *Zea mays, B. cynodontis* on *Cynodon* spp. and *B. setariae* on *Setariae* spp.

**e) Taxonomic conclusion**

There is no doubt that *B. maydis* and *B. setariae* are distinct species as indicated by their behaviour during culture-based tests, host preferences and in their fingerprint patterns. *Bipolaris cynodontis* needs further investigation, specifically with regard to its relationship with *Curvularia*, but is retained as a separate species until subsequent investigations prove otherwise.

### 6.3.6 Genus separation

What constitutes a distinct genus in the fungi has always been a matter of debate and generic concepts vary markedly between different fungal families (Hawksworth 1974). Ideally, several distinct and unrelated features should separate genera. Genera should be

clearly recognisable groups and this is best achieved by using morphological characteristics because they are easier to recognize and apply in comparison with than characteristics requiring more sophisticated technologies (Hawksworth *et al.* 1995).

This study has shed new light on the generic status of the *Bipolaris*-like fungi. Sequencing data presented here support the genus *Exserohilum* as distinct from the genera *Bipolaris* and *Curvularia*. The current combination of features: Consistent spore morphology, darkened septa in conidia, prevalence of a hilum and germ-tube development as detailed by Alcorn (1983) should enable the assignment of a species to the genus *Exserohilum* with confidence. This combination of features is important in assigning species to *Exserohilum* since the presence of a hilum is not exclusive to this genus (see *B. indica* and *C. cymbopogonis*). As a differentiating character, the hilum should be applied much like clamp connections in Basidiomycota. Clamp connections are exclusive to Basidiomycota but not all Basidiomycota have this feature. Protuberant hila are not exclusive to *Exserohilum* but spores of all *Exserohilum* species form them. The ultimate confirmation of species placement to this genus is a *Setosphaeria* teleomorph (Sivanesan 1987).

The high level of conservation in the 28S rRNA genomic region of *Bipolaris*-like fungi present no indication of sub-groups within the *Bipolaris / Curvularia* species studied. The option of sub-groups within those defined by sequencing data should be considered, since there is no strict correlation between evolutionary time and resolution levels derived from nucleic acid based tests (Hillis *et al.* 1996). The taxonomic level of these sub-groups is not clear and can be on genus or species level. The clear separation of *Exserohilum* from the rest of the species included in this investigation, and consistent reports of the resolution level of the 28S RNA gene at generic level suggest that *Bipolaris* and *Curvularia* are synonyms (Goh *et al.* 1998, Mitchell *et al.* 1995). Investigation of nucleic acid relatedness including other genera within the Pleosporaceae and targeting a less conserved part of the genome must first confirm this.

Other characteristics investigated, highlighted aspects that define different generic groupings within the *Bipolaris / Curvularia* group. Fingerprint patterns indicated greater similarity between the species with three and four septate conidia than the multi-septate elongated (Elongation of 5 or more) spored *Bipolaris* species (see 6.3.5). ISSR patterns indicated separation between three septate and multi-septate *Curvularia* species i.e. the "lunata" and the "geniculata" type of species (see 6.3.2). Luttrell (1977) speculated on such groups but did not assign any taxonomic rank to his "lunata" or "geniculata" groups. It will also not be attempted here, but the study confirmed the necessity of further investigation of these groups.

The study did not provide sufficient justification to synonymise *Curvularia* and *Bipolaris*. A conservative approach is selected and synonymy of these two genera not proposed for two reasons. Firstly retainment of the two genera is proposed for the sake of stability

within this sometimes ostentatiously treated group. Secondly, observations made during this study indicated subgroups within the *Bipolaris* complex (see previous parts of 6).

These groups are:

- Species with short, clavate to ellipsoidal spores with elongation less than five (include *B. indica*, *B. papendorfii* and *B. ellisi*). These species are plurivorous.
- Species with ellipsoidal to cylindrical spores with elongation of five or more (include *B. maydis* and *B. setariae*). These species have graminicolous host preferences.
- The *C. lunata* group with acutely curved three-septate conidia and disproportionately swollen middle cells in conidida. These species are plurivorous.

The validity of such groups, either as separate genera or species groups, must be investigated. This is the proposed next step in clarification of the taxonomy of the *Bipolaris* complex. It is therefore proposed that, until further information becomes available, *Curvualria* should be reserved for species with spores that are ellipsoidal, clavate or navicularly shaped, have one cell in the middle of the spore that is larger and darker than the end cells, and with an average length/width ratio of less than five. The widest part of the spores should be above the median. Being plurivorous (see List of South African species in section results 5.1 and discussion of characterisation of species in this section), and having *Cochliobolus* teleomorphs will further define this group. Less important features of this genus will be curvature of spores (not a consistent feature) and developmental features such as germ-tube formation and order of septum development (laborious to find) (Alcorn 1983).

The genus *Bipolaris* should be reserved for species with cylindrical to ellipsoidal or fusoid spores, with the widest part across the median or towards the base and with a length/width ratio of five or more. Species with the spores tapering prominently towards the apex, should be placed in *Bipolaris*. Having a distinct preference for graminicolous hosts and the presence of a *Cochliobolus* teleomorph will further define this genus. Less important features of this group will be the developmental features such as germ-tube formation and order of septum development (laborious to find) (Alcorn 1983).

The proposed distinction between *Curvularia* and *Bipolaris* is strongly dependent on spore shape for two reasons. Firstly, spore shape proved to be reliable under different environmental conditions, and secondly, is an extension of the concept used to separate the genus *Drechslera* from the three genera included in this study. Conidia of the type species of *Drechslera*, *D. tritici-repentis,* are essentially cylindrical, do not taper or only taper slightly towards the base and apex. In the type of *Bipolaris, B. maydis,* the conidia are mostly fusoid (Sivanesan 1987). Alcorn (1983) concluded that no distinction can be made between the conidial shape of *Bipolaris* and *Exserohilum*. The current investigation confirmed this similarity with both DT and FOIL analysis failing to separate the long-spored species of *Bipolaris* from *Exserohilum*. There is however, sufficient other features

available to consistently differentiate between the two genera. The most prominent features separating *Exserohilum* from *Bipolaris* are the protuberant hilum of spores, accentuated septa in conidia and a teleomorph in *Setosphaeria.*

Alcorn's (1982a, b) concern about the heterogeneity of *Cochliobolus,* implicated in the differentiation of two anamorph genera, *Curvularia* and *Bipolaris,* seems unwarranted. The strong similarities between *Curvularia* and *Bipolaris* indicate unity rather than heterogeneity. Further, many reports can be found of more than one mitosporic genus being related to one teleomorph genus (Hawksworth *et al.* 1995). Both *Setosphaeria* and *Cochliobolus* (teleomorphs of *Exserohilum* and *Curvularia / Bipolaris* respectively) are classified in the family Pleosporaceae. Within this family a few examples of similar situations can be found, for example the teleomorph *Cucurbitaria* has been associated with the mitosporic fungi *Camarosporium*, *Dichomera*, *Diplodia* and *Pyrenochaeta* (Sivanesan 1984b).

In large genera, intermediate species should also be expected but are often later found to belong to one genus and not the other on the basis of features, which might not at first be apparent (Hawksworth 1974).

# 7 SUMMARY AND RECOMMENDATIONS

One of the objectives of this study was to provide a list of species of the *Bipolaris* complex in an attempt to gain a better understanding of the host relationships of this group. In this regard, the checklist did not yield any new information but only confirmed the knowledge already available in the literature. In view of the vast area of grassland biome and the importance of grain crops as an agricultural commodity, it is recommended that future investigations should focus on the impact of these fungi on the quality of grazing, crop losses and the incidence of farm animal poisoning.

A second objective of the study was to investigate the extent of variation within species of the *Bipolaris* complex. Strains representing 14 species in the three genera *Bipolaris*, *Curvularia* and *Exserohilum* were investigated. The species selected included morphologically well-defined species, some with features that could place them in more than one genus, and species that are closely similar and their separation based only on single characteristics. Investigation of variation in spore morphology and ISSR fingerprints indicated spore shape in particular, to be a reliable feature but spore sizes of lesser importance. Spore size of specifically the long-spored species is dependent on environmental conditions. When correlated with ISSR fingerprints, a protuberant hilum should not be seen as being exclusive to species of *Exserohilum*. The study also indicated that the distinct spore morphology of species do not necessarily indicate a well-defined species such as *B. papendorfii* and *C. lunata.*

The great molecular and physiological heterogeneity found in some species can easily be interpreted as being of specific value. It is, however, recommended that descriptions of new species, particularly those without a teleomorph present, be approached with caution. Careful consideration must first be given to variation in spore dimensions, host relationships and cultural behavior. The validity of such differences should be confirmed by comparing genetic similarity through nucleic-acids based techniques. Descriptions of species should include cultural and physiological features obtained for a standard set of conditions in order that identifications will be repeatable.

When combined with morphological features, physiological traits and cultural features are value to separate between species. This new approach to the identification of species within the *Bipolaris* complex should be extended. This can be achieved by refining culture-based tests already used or by exploring new tests. The classification algorithms *First order inductive logic* and *Decision tree programming*, proved decisive in analyzing the type of data presented by culture-based tests. These two classification techniques can be applied in the investigation of the differential capacity of taxonomic criteria in other groups of fungi.

Suggestions about synonymy of certain species and the emendation of the generic descriptions of both *Bipolaris* and *Curvularia* have been proposed in the discussion. Formal changes in nomenclature and descriptions require adherence to the rules of the International Code of Botanical Nomenclature. This implies the study of type material housed in various herbaria throughout the world. An integrated investigation of authentic strains of many other species will support such an emendation. In addition species groups within *Curvularia*, such as the *C. lunata* group, needs further investigation. The time and work required to formally conclude these suggestions do not fall within the scope of this study.

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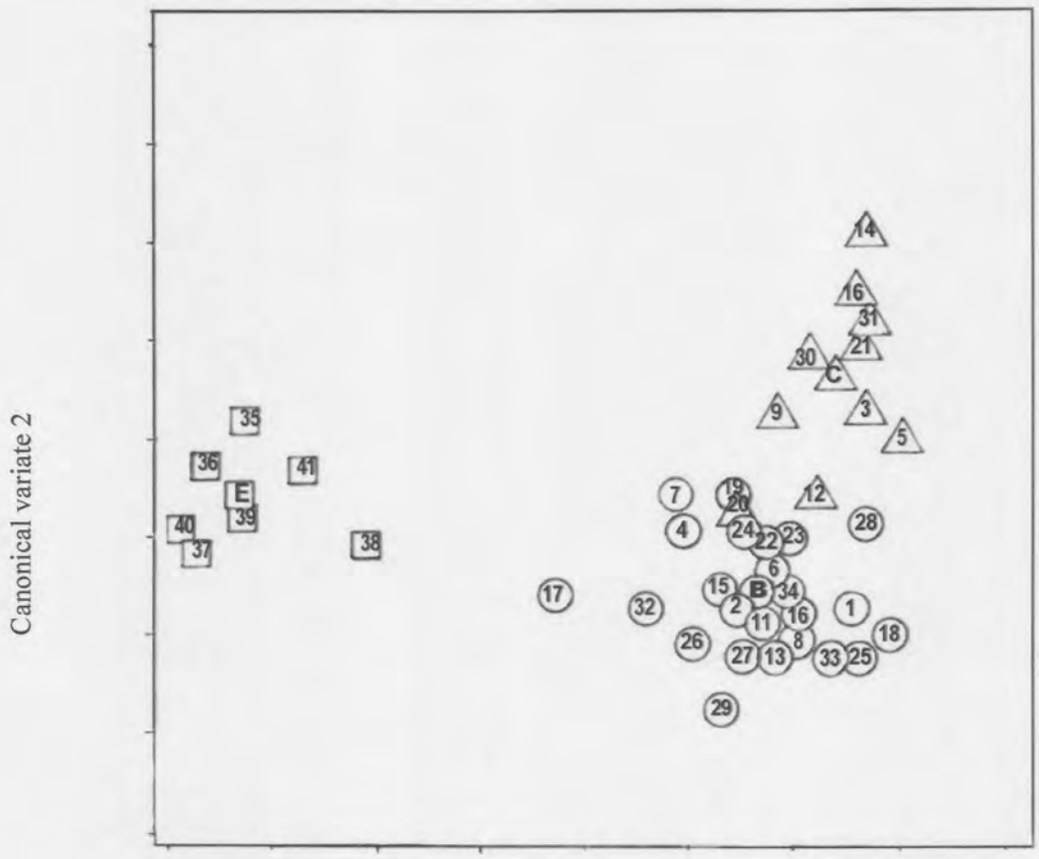
# Appendix A: Canonical variate analysis of selected species

Canonical variate analysis (CVA) or (linear) discriminant analysis is used when it is of more interest to show differences between groups than between individuals. The variability in a large number of variates is firstly reduced to a smaller set of variates, which account for most of the variability. The new set of variates, called canonical variates, is linear combinations of the original measurements, and is thus given as vectors of loadings for the original measurements. With this approach a set of directions are obtained in such a way that the ratio of between group variability to within group variability in each direction is maximised. In this study the variates were the taxonomic characteristics, which were measured on each of 41 fungal mitosporic species of the Bipolaris complex (Smith 1995).

The data used for the canonical variate analysis were obtained from the descriptions of various Bipolaris like fungi in the monograph of Sivanesan (1987). The binary variates (features) used were: stromata, conidiophore surface ornamentation, protuberant hilum, accentuated septa, conidial ornamentation, disproportionate conidial cell, end cells cut off by dark septa and curvature. Discrete variates used were spore shape, spore colour, type of germination, number of septa per conidium, length and width measurements and septum ontogeny.

**Figure 2: Differentiation of *Bipolaris*, *Curvularia* and *Exserohilum* using a canonical variate analysis and species as descriptions by Sivanesan (1987).**




Ⓑ - *Bipolaris*; Ⓒ - *Curvularia*; Ⓔ - *Exserohilum;* 1 - *B. australiens; 2 - B. bicolor;* 3 - *C. brahyspora;* 4 - *B. brizae;* 5 - *C. clavata;* 6 - *B. coicis;* 7 - *B. crustaceae;* 8 - *B. cynodontis;* 9 - *C. eragrostidis; 10 - C. geniculata; 11 - B. hawaiiensis; 12 - C. intermedia; 13 - B. leersiae; 14 - C. lunata; 15 - B. maydis; 16 - B. mediocris; 17 - B. micropus; 18 - B. miyakei; 19 - B. nodulosa; 20 - C. oryzae; 21 - C. pallescens; 22 - B. papendorfii; 23 - B. ravenelii; 24 - B. sacchari; 25 - B. setariae; 26 - B. sorghicola; 27 - B. sorokiniana, 28 - B. spicifera; 29 - B. stenospila; 30 - C. stapeliae; 31 - C. trifolii; 32 - B. urochloae; 33 - B. victoriae; 34 - B. zeicola; 35 - E. rostratum; 36 - E. holmii; 37 - E. longirostratum; 38 - E. monoceras; 39 - E. pedicellatum; 40 - E. prolatum; 41 - E. turcicum.*

# Appendix B: List of species and strains studied

**Table 7: List of strains used in this investigation according to PPRI numbers.**

| | | | |
|---|---|---|---|
| ***Beauveria bassiana*** | | | |
| **6999** | Host: Unknown<br>Locality: South Africa<br>Collector: Microbial Solutions<br>Reference: G. Limerick BB | **7000** | Host: Unknown<br>Locality: South Africa<br>Collector: H. Botha<br>Reference: Gladiator BB |
| **7001** | Host: Unknown<br>Locality: South Africa<br>Collector: Dagutat Biolab<br>Reference: Gladiator BB | | |
| ***Bipolaris cynodontis*** | | | |
| **6660** | Host: Unknown<br>Locality: Germany<br>Collector: C. Nelson, M 108, 1966<br>Reference No.: M 120, G. Arnold: (+ strain)<br>Note: This strain did not sporulate. | **AUT 6535** | Host: *Cynodon dactylon*<br>Locality: Peregian Beach, Australia<br>Collector: J L Alcorn, 27 August 1978<br>Reference No.: BRIP 12726<br>Note: No authenticated cultures could be traced. |
| **6584** | Host: *Cynodon dactylon*<br>Locality: Georgia, United States of America<br>Collector: G.W. Karr & G. Morgen-Jones<br>Reference No.: ATCC 34309 | 6185 | Host: *Eragrostis* hay<br>Locality: Humansdorp, South Africa.<br>Collector: I.H. Rong, 10 July 1996<br>Notes: J.L. Alcorn agreed this isolate to be close to *B. cynodontis* but its conidia is too short. |

| | | | |
|---|---|---|---|
| ***B. ellisii*** | | | |
| **AUT** 6537 | Host: *Dactyloctenium aegyptium*<br>Locality: Saibai Island, Australia<br>Collector: J.L. Alcorn, 1 June 1981<br>Reference No: BRIP 13598<br>Note: Culture obtained from the author of the species, and from the type locality. | 6536 | Host: Unknown<br>Locality: Karachi, Pakistan<br>Collector: S.R.H. Rizoi, 1965<br>Reference No: BRIP 13550 |
| ***B. indica*** | | | |
| **AUT 6567** | Host: *Brassica nigra*<br>Locality: Lucknow, India<br>Collector: J.N. Rai, 1967.<br>Reference No.: IMI 129.790<br>Note: Listed by Sivanesan as the Ex-type but not in the IMI catalogue. | 6541 | Host: *Amaranthus hybridus*<br>Locality: Forest Hill, Australia<br>Collector: P.E. Meyers, 30 March 1973<br>Reference No.: BRIP 11561 |
| **6538** | Host: *Trianthema portuculastrum*<br>Locality: Derby. Australia<br>Collector: R. Shivas, 28 May 1992<br>Reference No: BRIP 20259 | | |
| ***B. maydis*** | | | |
| **AUT 6662** | Host: *Zea mays*<br>Locality: Japan<br>Collector: Y. Nisikado<br>Reference No.: CBS 136.29<br>Note: The strain is from the author of the name but infertile and grew poorly. | 6564 | Host: Unknown<br>Locality: Unknown<br>Collector: C. Nelson, 1966<br>Reference No.: M 122, (+ strain) Be 5, G. Arnold |

| | | | |
|---|---|---|---|
| **AUT 6430** and 6667 | Host: *Zea mays*<br>Locality: Unknown<br>Collector: K. Böning<br>Reference No.: CBS 134.39, DSMZ 1149<br>Note: The same strain was received from two different sources. | **6607** | Host: *Zea mays*<br>Locality: Kairi, Australia<br>Collector: W. Pont, 1 May 1973<br>Reference No.: BRIP 5092<br>Note: The strain sporulated poorly. |
| **6677** | Host: *Zea mays*<br>Locality: Germany<br>Collector: Haltmann<br>Reference No.: DSMZ 62623, IMI 11635 | | |
| ***B. papendorfii*** | | | |
| **AUT 6568** | Host: Leaf litter under *Acacia karroo*<br>Locality: Potchefstroom, South Africa<br>Collector: M.C. Papendorf, 1968<br>Reference No.: IMI 136.484 Ex-type | 6709 | Host: *Dacanthium annulatum*<br>Locality: Unknown<br>Collector: B.L. Jain<br>Reference No.: DSMZ 62593, IMI 121.159 |
| **6539** | Host: *Triticum aestivum*<br>Locality: Australia<br>Collector: A.L. Alcorn, 15 October1984<br>Reference No.: BRIP 14503 | **6314** | Host: Turf grass<br>Locality: Pretoria, South Africa<br>Collector: I.H. Rong, 20 October 1996. |
| 5855 | Host: Unidentified grass<br>Locality: Onderstepoort, South Africa<br>Collector: I.H. Rong, 13 March 1996. | 6124 | Host: *Vigna unuiculata*<br>Locality: Roodeplaat, South Africa<br>Collector: I.H. Rong, 28 May 1996 |

| | | | |
|---|---|---|---|
| ***B. setariae*** | | | |
| **AUT 6665** | Host: Unknown<br>Locality: Unknown<br>Collector: S. Ito<br>Reference No.: CBS 141.31<br>Note: The strain is from the author of the teleomorph *Cochliobolus setariae* but is infertile and grew poorly. | 6572 | Host: *Setaria italica* seeds<br>Locality: Unknown<br>Collector: Schramm<br>Reference No.: DSMZ 62599<br>Note: The strain sporulated poorly. |
| **6663** | Host: *Pennisetum typhoides*<br>Locality: Hawkinsville, Georgia, USA<br>Collector: E.S. Luttrell, 3 September 1970 Reference No.: BRIP 6514<br>Note: The strain sporulated poorly. | | |
| ***C. affinis*** | | | |
| **AUT 6428** | Host: *Musa sapientum*<br>Locality: Gabon<br>Collector: J. Nicot<br>Reference No.: CBS 492.70. | **6652** | Host: *Manihot utilissima*<br>Locality: Indonesia<br>Collector: K.B. Boedijn & J. R. Reitsma<br>Reference No.: CBS 185.49, IMI 038975, QM 8064, BR 6/48<br>Note: The strain is from the author of the name. |
| **6676** | Host: *Musa sapientum*<br>Locality: Columbia<br>Collector: H. Nirenberg<br>Reference No.: DSMZ 63274 | 6545 | Host: Unknown<br>Locality: Congo<br>Collector: J. Meyer<br>Reference No.: BRIP 8521 |

| *C. cymbopogonis* | | | |
|---|---|---|---|
| **AUT 6675** | Host: *Sorghum* sp<br>Locality: Sudan<br>Collector: Fraser-Ass., East Craigs UK, 1967<br>Reference No.: IMI 130.402 Ex-type<br>Note: The strain forms immature ascomata. | **6661** | Host: *Citronella* sp.<br>Locality: Guatemala<br>Collector: J.W. Groves<br>Reference No.: CBS 185.48, ATCC 38580 |
| 6532 | Host: *Sorghum plumosum*<br>Locality: Rifle Ck, Australia<br>Collector: J.L. Alcorn, 28 October 1988<br>Reference No.: BRIP 16516 | **6592** | Host: *Rottboellia exaltata*<br>Locality: Louisiana<br>Collector: H.C. Walker<br>Reference No. ATCC 42014<br>Note: see Walker & White (1979) |
| 6297 | Host: *Hyperthelia dissoluta*<br>Locality: Rusape, Zimbabwe<br>Collector: I.H. Rong, 5 September1996 | | |
| *C. fallax* | | | |
| **AUT 6445** | Host: Air<br>Locality: Indonesia<br>Collector: K.B. Boedijn<br>Reference No.: CBS 155.34 Ex-syn-type culture | 6574 | Host: *Oryza sativa*<br>Locality: Nigeria<br>Collector: H. Nirenburg<br>Reference No.: DSMZ 631.69 |
| **6534** | Host: *Ectrosia agrostoides*<br>Locality: Horn Island, Australia<br>Collector: J.L. Alcorn, 3 June 1981<br>Reference No.: BRIP 13602 | **6533** | Host: *Spinifex longifolius*<br>Locality: Yorke Island, Australia<br>Collector: J.L. Alcorn, 2 June 1981<br>Reference No.: BRIP 13600 |

| *C. lunata* No ex type cultures available, reference culture supplied by J.L. Alcorn | | | |
|---|---|---|---|
| **6565** | Host: *Crotalaria juncia* seed<br>Locality: Thailand<br>Collector: Unknown<br>Reference No.: I 3907, DSM 63137 | **AUT 6540** | Host: *Sorghum plumosum*<br>Locality: Cape York Peninsula, Australia<br>Collector: J.L. Alcorn, 31 May 1981<br>Reference No.: BRIP 13582, IMI 266331 |
| 6659 | Host: Unknown<br>Locality: Unknown<br>Collector: Unknown, 1983<br>Reference No.: ATCC 13633, NRRL 2343, CBS 207.59; GLAXO C2100<br>Note: This strain is used for Preceptrol® but is infertile and growth very poorly. | **6699** | Host: *Zea mays*<br>Locality: North Carolina, USA<br>Collector: Unknown<br>Reference No.: NRRL 6409 |
| 6700 | Host: Ex-Japanese socks<br>Locality: New Guinea<br>Collector: Unknown<br>Reference No.: QM 2105, NRRL 2178 | **3360** | Host: *Tribulus terrestris*<br>Locality: Oudtshoorn, South Africa<br>Collector: W.F.O.M. Marasas, 6 July 1988 |
| 5606 | Host: *Persea americana* flower buds<br>Locality: Nelspruit, South Africa<br>Collector: I.H. Rong, 1 November 1994 | | |

| *E. inaequale* | | | |
|---|---|---|---|
| **AUT** 6666 and **6527** | Host: Plant material<br>Locality: Nigeria<br>Collector: D.B. Olufolagi, 31 August 1983<br>Reference No.: CBS 503.90, VTT D-79116, ATCC 60765 Ex-type<br>Note: Collection data used is from Sivanesan (1987); The same culture was received from different sources. | 6573 | Host: *Phleum pratense*<br>Locality: Iceland<br>Collector: Shramm<br>Reference No.: DSMZ 62462, IMI 12469 |
| 6570 | Host: Unknown<br>Locality: Angers, France<br>Collector: C.H.R.U, Medical Centre<br>Reference No.: CNCM 2027.92 | **6668** | Host: *Triticum aestivum*<br>Locality: Australia<br>Collector: M. Meblads, July 1984.<br>Reference No.: BRIP 14448 |
| 5940 | Host: grass<br>Locality: Rusape, Zimbabwe<br>Collector: I.H. Rong, 27 May 1995 | . | |
| ***E. longirostratum*** | | | |
| **AUT 6612** | Host: *Mentha arvensis*<br>Locality: India<br>Collector: K.K. Janardhanan<br>Reference No.: ATCC 60764, IMI 279094 | **5133** | Host: *Musa* sp<br>Locality: Nelspruit, South Africa<br>Collector: I.H. Rong, June 1993<br>Note: Pairings of this strain is fertile with 10 Australian isolates of the same species. |

| *E. longirostratum* (continue) | | | |
|---|---|---|---|
| 6528 | Host: *Cymbopogon citratus*<br>Locality: Pullenvale, Australia<br>Collector: J.L. Alcorn, 22 February 1988<br>Reference No.: BRIP 16114 | **6529** | Host: *Zea mays*<br>Locality: Atherton Tableland, Australia<br>Collector: M. Ramsey, 9 April 1985<br>Reference No.: BRIP 14916 |
| **5908** | Host: grass<br>Locality: Zimbabwe<br>Collector: I. H. Rong, 13 March 1995.<br>Note: Pairings of this strain are fertile with 10 Australian isolates of the same species. | | |
| *E. rostratum* | | | |
| **AUT 6658** | Host: *Zea mays*<br>Locality: Florida, USA<br>Collector: T.A. Kucharek, 1972<br>Reference No.: Luttrell 8868 Mating type A, IMI 197.559, ATCC 32197, CBS 466.75<br>Note: Mating of this strain (Luttrell 8868) and strain SrA3 identified by K. L. Leonard, provided the teleomorph type. | **4130** | Host: *Rhodora* sp.<br>Locality: Mariepskop, South Africa<br>Collector: C. Roux, 1990 |

| *E. rostratum* (continue) | | | |
|---|---|---|---|
| **6531** | Host: *Chrysalidocarpus lutescens*<br>Locality: Rock Hampton, Australia<br>Collector: Fitzroy Nurseries, Australia<br>Reference No.: BRIP 15403<br>Note: The strain form typical "halodes" type spores. | **6530** | Host: *Dinebra retroflexa*<br>Locality: Lawes, Australia<br>Collector: J.L. Alcorn<br>Reference No.: BRIP 12147 |
| 6119 | Host: *Parotis patens*<br>Locality: National Elephant park, Sihangwane, South Africa<br>Collector: I.H. Rong, 10 July 1996<br>Note: J.L. Alcorn remarked that "halodes" type conidia are formed on WA + straw, but that the strain might form "longirostratum" like conidia on a different medium. | | |
| *E. turcicum* | | | |
| 6562 | Host: Soil<br>Locality: Donezk, URSS<br>Collector: Unknown<br>Reference No.: I 1318, VKM F-1282 | 6708 | Host: *Zea mays*<br>Locality: Unknown<br>Collector: Heitman.<br>Reference No.: DSMZ 62618, IMI 11634<br>Note: Sivanesan (1987) did not list this isolate as one of the numerous IMI samples that he investigated. |

| *E. turcicum* (continue) | | | |
|---|---|---|---|
| **AUT** 6606 | Host: *Zea mays*<br>Locality: Illinois, USA<br>Collector: L. Hooker<br>Reference No.: ATCC 26306<br>Note: listed by ATCC as common type isolate; Culture infertile and growth poor. | 6575 | Host: *Sorghum sudanense*<br>Locality: Samford, Australia<br>Collector: R. Jones, 6 April 1981<br>Reference No.: BRIP 13326 |
| **5487** | Host: Animal feed<br>Locality: Gauteng, South Africa<br>Collector: I.H. Rong 16 November 1994. | 5459 | Host: *Zea mays*<br>Locality: Pretoria, South Africa<br>Collector: I.H. Rong, 1994. |

**Table 8: Morphologic and ontogenic features of selected species.**

| | Conidiophores | Conidial shape | Curvature | Hilum structure | Conidium size | Germination | Septum ontogeny | Number of septa | Teleomorph |
|---|---|---|---|---|---|---|---|---|---|
| ***B. cynodontis*** | Verrucose* | Fusoid* Cylindrical | Slightly curved or straight* | Flush | 30-75x10-16 | Semiaxial* Vesicles | B* | (3) 7-8 (9) | *Cochliobolus* |
| ***B. ellisii*** | Smooth | Ellipsoidal * Clavate Pyriform Navicular | Strongly curved or straight | Flush | 21-41x12-18 | Semiaxial* | B* | 3-5 | *Cochliobolus* |
| ***B. indica*** | Smooth* | Clavate Ellipsoidal | Straight | Flush* Often protruding | 40-70-17-35 | Semiaxial* | E* | (3) 5-7 | None reported |
| ***B. maydis*** | Smooth | Fusiform | Curved | Flush | 70-160x15-20 | Semiaxial* | Not known | 5-11 | *Cochliobolus* |
| ***B. papendorfii*** | Verrucose | Navicular Obpyriform | Strongly curved | Flush | 30-50x17-30 | Semiaxial* Vesicles | B* | 3 | None reported |
| ***B. setariae*** | Verrucose | Fusiform Navicular | Slightly curved | Flush | (45)50-70(100)x10-15 | Semiaxial* | B* | 5-10 | *Cochliobolus* |
| ***C. affinis*** | Smooth | Fusiform | Straight or curved | Flush | 27-32(49)x8-10(13) | Not known | Not known | 4-5; some accentuated | None reported |
| ***C. cymbopogonis*** | Not known | Clavate Ellipsoidal | Sometimes curved | Protruding | 35-60x14-20 in culture 30-46x11-17 | Not known | Not known | 4; some accentuated | *Cochliobolus* |
| ***C. fallax*** | Not known | Fusiform Ellipsoidal | Slightly curved | Flush | 24-26(30)x10-16(12) in culture 24-38(31)x9-15(12) | Not known | Not known | 4 | None reported |
| ***C. lunata*** | Not known | Clavate Ellipsoidal | Curved | Flush | 18-30x9-14 In culture 20-32x9-15 | Not known | Not known | 3; some accentuated | *Cochliobolus* |
| ***E. inaequale*** | Smooth | Fusiform Ellipsoidal | Curved | Flush or Protruding | 24-32(45)x9-12(16) | Not known | Not known | 2-4(6); some accentuated | None reported |
| ***E. longirostratum*** | Not known | Rostrate Obclavate Ellipsoidal | Sometimes curved | Protruding | 60-475x12-26 | Semi-axially | Not known | Multi-septate; end cells accentuated | None reported |
| ***E. rostratum*** | Verrucose* | Obclavate Rostrate Ellipsoidal | Straight or curved* | Protruding | 15-200x7-29 | Semi-axially* | E* | 18; End cells accentuated | *Setosphaeria* |
| ***E. turcicum*** | Smooth | Ellipsoidal Obclavate | Slightly curved | Protruding | 50-144x180-33 | Not known | Not known | 4-9 | *Setosphaeria* |

Information obtained form Alcorn (1983) and Sivanesan (1987).

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# Appendix C: Culture based tests - nutrient media and results

1. **Acetic acid agar** (Paterson & Bridge 1994)

| Components | Quantities | Method |
|---|---|---|
| Agar (Biolab) | 20.0g | Combine components and autoclave for15 minutes at 121 °C (15 p.s.i.). Let the medium cool to approximately 60 °C, before 5 ml glacial acetic acid. Using a sterile dispenser decant 10 ml medium was poured into 65 mm Petri dishes and allowed to set. |
| Distilled water | 1 litre | |
| Glucose | 20.0 g | |
| Yeast extract (Biolab) | 5.0 g | |

2. **Aesculin agar** (Paterson & Bridge 1994)

| Components | Quantities | Method |
|---|---|---|
| Czapek solution A | 50.0 ml | Combine components and autoclave for 15 minutes at 121 °C (15 p.s.i.). Using a sterile dispenser, decant 20 ml medium was poured into 90 mm plastic Petri dishes and allowed to set. |
| Czapek solution C | 50.0 ml | |
| Copper solution | 1.0 ml | |
| Zinc solution | 1.0 ml | |
| Sucrose | 5.0 g | |
| Aesculin | 3.0 g | |
| Ferric citrate | 0.2 g | |
| Agar (Biolab) | 12.0 g | |
| Distilled water | 900.0 ml | |

3. **Czapek yeast extract agar** (Pitt 1979)

| Components | Quantities | Method |
|---|---|---|
| $K_2HPO_4$ | 1.0 g | Combine components and autoclave for 15 minutes at 121 °C (15 p.s.i.). Using a sterile dispenser 20 ml medium was poured into 90 mm agar plates and allowed to set. |
| Czapek concentrate* | 10.0 ml | |
| Yeast extract (Oxoid) | 5.0 g | |
| Sucrose | 30.0 g | |
| Agar (Biolab) | 15.0 g | |

| *Czapek concentrate | | |
|---|---|---|
| $NaNO_3$ | 30.0 g | |
| KCl | 5.0 g | |
| $MgSO_4.7H_2O$ | 5.0 g | |
| $FeSO_4.7H_2O$ | 0.1 g | |
| Distilled water | 100.0 ml | |

**4. Gelatine hydrolysis medium** (Paterson & Bridge 1994)

| Components | Quantities | Method |
|---|---|---|
| Czapek solution A | 50.0 ml | Stir gelatine into a small quantity of the distilled water (room temperature). Warm the suspension at 50-60 °C until gelatine dissolves, add all other components. Dispense 10 ml medium into 20 ml McCartney bottles, close and autoclave for 15 minutes at 121 °C (15 p.s.i.). |
| Czapek solution B | 50.0 ml | |
| Czapek solution C | 1.0 ml | |
| Czapek solution E | 1.0 ml | |
| Sucrose | 10.0 g | |
| Gelatine (uniLAB) | 120.0 g | |
| Distilled water | Add up to 1 litre | |

**Czapek solution A**

| Components | Quantities |
|---|---|
| $NaNO_3$ | 4.0 g |
| KCl | 1.0 g |
| $MgSO_4.7H_2O$ | 20.0 mg |
| Distilled water | 100.0 ml |

**Czapek solution B**

| Components | Quantities |
|---|---|
| $K_2HPO_4$ | 2.0 g |
| Distilled water | 100.0 ml |

**Czapek solution C**

| Components | Quantities |
|---|---|
| $ZnSO_4.7H_2O$ | 1.0 g |
| Distilled water | 100.0 ml |

**Czapek solution D**

| Components | Quantities |
|---|---|
| $CuSO_4.5H_2O$ | 0.5 g |
| Distilled water | 100.0 ml |

**5. G25N agar** (Pitt 1979)

| Components | Quantities | Method |
|---|---|---|
| $K_2HPO_4$ | 0.75 g | Combine components and autoclave for 15 minutes at 121 °C (15 p.s.i.). Using a sterile dispenser, decant 20 ml medium into 90 mm agar plates and allowed to set. |
| Czapek concentrate[#] | 7.5 ml | |
| Yeast extract (Biolab) | 3.7 g | |
| Glycerol, analytical grade | 250 g | |
| Agar (Biolab) | 12 g | |
| Distilled water | 750 ml | |

[#] see Appendix C: 3. Czapek concentrate.

**6. Pectin agar** (Paterson & Bridge 1994)

| Components | Quantities | Method |
|---|---|---|
| $NH_4H_2PO_4$ | 0.9 g | Combine components and autoclave for 15 minutes at 121 °C (15 p.s.i.). Aseptically dispense 20 ml medium into 90 mm Petri dishes. |
| $(NH_4)_2HPO_4$ | 0.2 g | |
| $MgSO_4.7H_2O$ | 0.1 g | |
| KCl | 0.5 g | |
| Citrus pectin (uniLAB) | 1.0 g | |
| Ruthenium red | 150.0 mg | |
| Agar (Biolab) | 12.0 g | |
| Distilled water | 1 litre | |

**7. Potato carrot agar (PCA)** (Booth 1971)

| Components | Quantities | Method |
|---|---|---|
| Potato, washed and grated | 20.0 g | Boil potato and carrot in water for 1 hour and sieve to remove particles. Use the liquid and make up to 1 litre, add agar and autoclave 15 minutes at 121 °C (15 p.s.i.). |
| Carrot, washed and grated | 20.0 g | |
| Agar (Biolab) | 15.0g | |
| Distilled water | 1 litre | |

**8. Sach's agar** (Hebert 1971)

| Components | Quantities | Method |
|---|---|---|
| $Ca(No_3)_2.H_2)$ | 1.0 g | Combine components and autoclave for 15 minutes at 121 °C (15 p.s.i.). Aseptically place small pieces of previously autoclaved wheat straw and / or *Zea mays* leaves on the agar surface after it has been poured into plastic Petri dishes. Allow the plates to set. |
| $K_2HPO_4.H_2O$ | 0.25 g | |
| KCl | 0.25 g | |
| $MgSO_4.7H_2O$ | 0.25 g | |
| $CaCO_3$ | 0.85 g | |
| $FeCl_3$ | trace | |
| Agar (Biolab) | 20.0 g | |
| Distilled water | 1 litre | |

**9. Sugar alcohol and glucose medium** (Paterson & Bridge 1994)

| Components | Quantities | Method |
|---|---|---|
| $NH_4H_2PO_4$ | 1.0 g | Combine components and autoclave for 15 minutes at 121 °C (15 p.s.i.). Add, by filter sterilization, 1 % inositol, 1.01% sorbitol or 1 % glucose so that an equivalent quantity of carbon would be included. Aseptically dispense 10 ml medium into 65 mm plastic Petri dishes and allow setting. |
| KCL | 0.2 g | |
| $MgSO_4.7H_2O$ | 0.2 g | |
| Copper solution[$] | 1.0 ml | |
| Zinc solution[$] | 1.0 ml | |
| Agar (Biolab) | 12.0 g | |
| Distilled water | 1 litre | |

[$] see Appendix C: 4c and 4d.

**10. Tween 80 agar** (Paterson & Bridge 1994)

| Components | Quantities | Method |
|---|---|---|
| Mycological peptone (Biolab) | 10.0 g | Combine components, adjust the pH to 5.4 by adding HCl and dispense into 90 ml aliquots. In a separate container warm 90 ml of the water until approximately 60 - 70 °C and add 10 ml Tween 80. Autoclave the two solutions separately for 15 minutes at 121 °C (15 p.s.i.). When the media have cooled to 60 °C, add 10 ml of the Tween solution to each 90 ml basal medium and mix well. Aseptically dispense 20 ml into 90 mm plastic Petri dishes and allow to set. |
| NaCl | 5.0 g | |
| $CaCl_2.2H_2O$ | 0.1 g | |
| Bromocresol purple (Colour change at pH 5-7) | 25.0 mg | |
| Agar (Biolab) | 15.0 g | |
| Distilled water | 1 litre | |

**11. Vegetable juice agar** (Centraalbureau voor Schimmelcultures 1996)

| Components | Quantities | Method |
|---|---|---|
| Ceres vegetable and fruit concentrate | 200 ml | Combine components and adjust pH to 7.3 by adding KOH. Autoclave at 15 minutes at 121 °C (15 p.s.i.). |
| $CaCO_3$ | 3.0 g | |
| Agar (Biolab) | 15.0 g | |
| Distilled water | 1 litre | |

**12. Water agar** (Booth 1971)

| Components | Quantities | Method |
|---|---|---|
| Agar | 15.0 g | Combine, autoclave for 15 minutes at 121 °C (15 p.s.i.). Aseptically dispense 10 ml in 65 mm Petridishes or 20 ml in 90 mm Petridishes. |
| Distilled water | 1 litre | |

**Table 9: Efficacy of culture-based tests to group cultures in the correct species as predicted with Foil and DT processing.**

| | Culture based test / source of inoculum | Total number of measurements | Number of measurements placed correctly with Foil (%) | Number of measurements placed correctly with DR (%) |
|---|---|---|---|---|
| 1 | Acetic acid 0.01% / WA | 43 | 21 (54) | 91 (52) |
| 2 | Acetic acid 0.35% / WA | 175 | 51 (29) | 27 (63) |
| 3 | Aesculin / WA | 70 | 26 (37) | 11(16) |
| 4 | Ceres / PCA | 68 | 43 (63) | 39 (57) |
| 5 | Ceres / WA | 178 | 120 (67) | 0 (0) |
| 6 | Copper sulphate / WA | 22 | 11 (50) | 9 (41) |
| 7 | CYE / WA | 178 | 137 (77) | 124 (70) |
| 8 | G25N / WA | 187 | 112 (60) | 91 (49) |
| 9 | Gelatine/ WA | 176 | 86 (49) | 56 (32) |
| 10 | Glucose 1% / WA | 184 | 126 (68) | 128 (70) |
| 11 | Glucose 1.01% /WA | 184 | 98 (53) | 118 (64) |
| 12 | Inositol / PCA | 202 | 71 (35) | 99 (49) |
| 13 | Inositol / WA | 184 | 106 (58) | 100 (54) |
| 14 | PCA / PCA | 40 | 27 (68) | 20 (50) |
| 15 | PCA / WA | 190 | 160 (84) | 123 (65) |
| 16 | Pectin / WA | 199 | 14 (7) | 54 (27) |
| 17 | Sach / PCA | 71 | 45 (63) | 36 (51) |
| 18 | Sach / WA | 184 | 145 (79) | 117 (64) |
| 19 | Sorbitol / WA | 190 | 116 (61) | 21 (8) |
| 20 | Temperature >20 °C | 106 | 39 (37) | 34 (34) |
| 21 | Tween growth rates and precipitate / WA | 199 | 125 (63) | 98 (49) |
| 22 | Tween pH and precipitate / WA | 53 | 26 (49) | 25 (47) |
| 23 | V8 / PCA | 20 | 14 (70) | 11 (55) |
| 24 | WA / PCA | 56 | 33 (59) | 24 (43) |
| 25 | *WA / WA | 187 | 0 (0) | 133 (71) |

# Appendix D: Methods used in nucleic-based characterization

## 1. Lysis buffer used for DNA extraction

| Components | Quantities | Notes |
|---|---|---|
| Tris-HCl | 150 m*M* | The pH is 8.8. |
| EDTA* | 50 m*M* | |
| SLS# | 1 % | |

*Ethylenediamine tetra-acetic acid; #Sodium lauroyl sarkosine

## 2. Tris-EDTA buffer

| Components | Quantities | Notes |
|---|---|---|
| Tris-HCl | 10 m*M* | The pH is 8.8. |
| EDTA | 0.1 m*M* | |

## 3. Reaction mixture of PCR for sequencing

| | Stock | Final concentration | Amount (:l) |
|---|---|---|---|
| 10 x Buffer | 10 x | 1 x | 1.25 |
| $MgCl_2$ | 25.0 mM | 2.0 mM | 1.0 |
| dNTP* | 2.5 mM | 0.4 mM | 2.0 |
| Primer 1 LR-7 Forward | 20.0 :M | 0.16 :M | 0.1 |
| Primer 2 LR-7 Reverse | 20.0 :M | 0.16 :M | 0.1 |
| TaKaRa *Taq*# | 5U / :l | 0.75 U | 0.15 |
| $H_2O$ | | | 2.9 |
| Template DNA | 2.0 ng | 10 ng | 5.0 |
| TOTAL | | | 12.5 |

* Deoxynucleotide triphosphates namely dATP, dCTP, dGTP and dTTP; # TaKaRa Biomedicals, Japan: DNA polymerase derived from the thermo-resistant *Thermus aquaticus* (*Taq*) bacterium (Orrego 1990).

**4. Reaction mixture of PCR for ISSR's**

| | Stock | Final concentration | Amount (:l) |
|---|---|---|---|
| 10 x Buffer | 10 x | 1 x | 1.25 |
| $MgCl_2$ | 25.0 mM | 3.5 mM | 1.75 |
| dNTP | 2.5 mM | 0.4 mM | 2.0 |
| Primer * | 20.0 :M | 0.4 :M | 0.5 |
| TaKaRa *Taq* | 5U / :l | 0.5 U | 0.1 |
| PVP[$] | 25 % | 2 % | 2.5 |
| $H_2O$ | | | 2.4 |
| Template DNA | 2.0 ng / :l | 4 ng | 2.0 |
| TOTAL | | | 12.5 |

* Primers used were DBD-$(AC)_7$ ; BDB-$(CAC)_3$ ; DHB- $(CGA)_5$ ; VHV-GTG TGT GTG TGT GTG. (VHV) = (AGC)(ACT)(AGC). [$]PVP = Polyvinylpyrrolidone.

**5. Thermal profile used for both sequencing and ISSR analysis**

| Steps | Temperature °C (Initial) | Time in seconds |
|---|---|---|
| 1 | 94.5, denaturation | 90 |
| 2 | 94.5, denaturation | 15 |
| 3 | 53.5[$], anneal | 20 |
| 4 | 72, extension | 30 |
| 5 | 72, extension | 120 |
| Repeat steps 2 – 4 x 35 | | |

[$]ISSR annealing temperature 60 °C

## 6. Abbreviations for nucleic acid base ambiguities (Ting & Manos 1990)

| Ambiguity | Possible nucleic acid |
|---|---|
| M | = A or C |
| R | = A or G |
| W | = A or T |
| S | = C or G |
| Y | = C or T |
| K | = G or T |
| V | = A or C or G |
| H | = A or C or T |
| D | = A or G or T |
| B | = C or G or T |
| N | = A or C or G or T |

# Appendix E: List of South African records of *Bipolaris*-like fungi

***Bipolaris* Shoemaker 1959**

*Cochliobolus australiensis* (Tsuda & Ueyama) Alcorn

= *Pseudocochliobolus australiensis* Tsuda & Ueyama

***B. australiensis*** (M.B. Ellis) Tsuda & Ueyama

= *Drechslera australiensis* (Bugnic.) Subram. & P.C. Jain ex M.B. Ellis.

= *Helminthosporium australiensis* Bugnic. *non rite publ.*

| Host | Locality | Reference |
|---|---|---|
| Air | Nelspruit, MP | PPRI 3879 |
| *Alium cepa* | South Africa | PPRI 4234 |
| *Avena sativa* | South Africa | PPRI 5324 |
| *Casuarina* seed | Silverton, GP | PPRI 4095 |
| *Cenchrus ciliaris* | Roodeplaat, GP | Bezuidenhout (1977); PREM 45170 |
| *Citrus sinensis* leaves | Tzaneen, NP | PPRI 5122 |
| Dead plants | Vaalharts, NC | PPRI 1067 |
| Dung – Blesbok | Graaff – Reinet, EC | PPRI 4897 |
| Grass roots | Roedtan, NP | PPRI 4578 |
| *Ischaemum afrum* roots | Radium, NP | PPRI 4582 |
| *Medicago sativa* seed | Oudtshoorn, WC | Marasas & Bredell (1973); PREM 44951; PPRI 3359 |
| *Medicago* sp. | Porterville, WC | PREM 48372; PPRI 2045 |
| *Nicotiana tabacum* leaves | Rustenburg, NW | PPRI 5464, 5465; |
| *Panicum laevifolium* | Heilbron, FS | PREM 44942 |
| *Parotis patens* leaf bracts | Tembe Elephant Park, Sihangwane, KZN | PPRI 6109 |
| Pasture grass | South Africa | PPRI 6151 |
| *Salvia stenophylla* seed | Stellenbosch, WC | PPRI 7049 |
| Soil *Acacia* veld | Potchefstroom, NW | PPRI 3330 |
| Soil near *Aspalathus linearis* | Clanwilliam, WC | PPRI 5549 |
| Soil cotton field | Groblersdal, MP | PPRI 5466 |
| *Tribulus terestris* | Middelburg, EC | PREM 44795 |
| *Triticum aestivum* seeds | South Africa | Lübben 1992 |
| *Triticum* sp. debris | Heilbron, FS | PREM 45013 |

*Co. bicolor* A.R. Paul & D.G. Parbery

***B. bicolor*** (Mitra) Shoemaker.

= *H. bicolor* Mitra

= *D. bicolor* (Mitra) Subram. & P.C. Jain

= *H. bhawanii* A.P. Misra non rite publ.

= *D. bhawanii* O. Prakash & A.P. Misra

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus ciliaris* | Rustenburg, NW | Gorter (1982); PREM 44977, 45139 |
| *Cenchrus ciliaris* | Roodeplaat, GP | Bezuidenhout (1977) |
| *Cenchrus ciliaris* | GP, MP, NP and NW | Crous *et al.* (2000) |
| Grass | Hartbeeshoek | PPRI 6147# |
| Mango fruit | Pretoria, GP | PPRI 7050 |
| *Medicago sativa* seed | Oudtshoorn, WC | Marasas & Bredell (1973); PREM 44950 |
| *Panicum laevifolium* | Rusthof, FS | PREM 44926 |
| *Sorghum bicolor* subsp. *arundinaceum* (= *S. verticilliflorum*) | Widespread | Putterill 1954 |
| *Sorghum caffrorum* | Potchefstroom, NW | PREM 47227 |
| *Sorghum halepense* | Pretoria, GP | Putterill (1954); PREM 30432 |
| *Triticum aestivum* | Clarens, FS | PREM 30458 |
| *Triticum aestivum* | Bethlehem, FS | Putterill (1954); PREM 30496 |

***B. brizae*** (Y. Nisik.) Subram. & P.C. Jain

= *H. brizae* Y. Nisik.

= *D. brizae* (Y. Nisik.) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Briza maxima* | Port Elizabeth, EC | Putterill (1954); PREM 28530 |
| *Briza* spp. | South Africa | McKenzie (1992) |

*Co. nisikadoi* (Tsuda, Ueyama & Y. Nisik.) Alcorn

= *P. nisikadoi* Tsuda, Ueyama & Nisik.

***B. coicis*** (Y. Nisik.) Shoemaker

= *H. coicis* Y. Nisik.

= *Curvularia coicis* E. Castell.

= *D. coicis* (Y. Nisik.) Subram. & P.C. Jain.

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus ciliaris* | Roodeplaat, GP | PREM 45150 |

***B. crustacea*** (Henn.) Alcorn

= *H. crustaceum* Henn.

| Host | Locality | Reference |
|---|---|---|
| *Sporobolus africanis* | South Africa | SBML (2001) |
| *Sporobolus capensis* | Tweedie, KZN | Doidge (1950); PREM 9764[¥] |
| *Sporobolus capensis* | Soutpansberg, NP | PREM 1826[¥] |
| *Sporobolus fimbriatus* | Balgowan, KZN | Sivanesan (1987); PREM 23390; IMI 296455 (SBML 2001) |
| *Sporobolus pyramidalis* | Donnybrook, KZN | Doidge (1950); PREM 29835 |
| *Sporobolus pyramidalis* | Krugersdorp, GP | PREM 35543 |

*Note: Specimens also contain *B. ravenelii*

*Coch. cynodontis* R.R. Nelson

***B. cynodontis*** (Marignoni) Shoemaker

= *H. cynodontis* Marignoni

= *D. cynodontis* (Marignoni) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Azolla filliculloides* | Grahamstown, EC | PPRI 6794 |
| *Cynodon bradleyi* | Brooklyn, GP | PREM 30242; PREM 29976 |
| *Cynodon bradleyi* | Redan, EC | Putterill (1954) |
| *Cynodon dactylon* leaf spot | EC, WC, KZN and "Transvaal" | Doidge *et al.* (1953); Gorter (1981) |
| *Cynodon dactylon* | Pilgrims Rest, MP | Putterill (1954); PREM 27359 |
| *Cynodon incompletus* | Middelburg, EC | Roux and Van Warmelo (1997) |
| *Eragrostis* sp. hay | Humansdorp, EC | PPRI 6182 |
| *Eragrostis* sp. hay | Groblersdal, MP | PPRI 6158 |
| *Trachus koelroides* debris | Middelburg, EC | PPRI 4889 |
| *Triticum aestivum* | Heilbron, FS | PREM 44898, 44897 |
| *Vigna unguiculata* | Roodeplaat, GP | PPRI 6185[#] |

*Co. hawaiiensis* Alcorn

= *P. hawaiiensis* (Alcorn) Tsuda & Ueyama

***B. hawaiiensis*** (M.B. Ellis) J.Y. Uchida & Aragaki

= *D. hawaiiensis* M.B. Ellis

= *H. hawaiiensis* Bugnic.

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus ciliaris* | Dendron, NP | PREM 45540 |
| *Cenchrus ciliaris* | Roodeplaat, GP | Bezuidenhout (1977) |
| *Cynodontis incompletus* | Middelburg, EC | Roux and Van Warmelo (1997) |
| Fodder | Tshipise, NP | PPRI 5159 |
| *Lupinus* sp. | WC | Crous *et al.* (2000); PREM 45331 |
| Mango leaves | Lisbon Estates, MP | PPRI 5325 |
| *Triticum aestivum* seeds | South Africa | Lübben (1992) |

***B. indica*** J.N. Rai, Wadhwani & J.P. Tewari

= *D. indica* (J.N. Rai, Wadhwani & J.P. Tewari) Mouch.

= *D. boeremae* A.S. Patil & V.G. Rao

| Host | Locality | Reference |
|---|---|---|
| Fodder | Lichtenburg, NW | PPRI 6155 |
| *Parotis patens* roots | Tembe Elephant Park, Sihangwane, KZN | PPRI 6111 |
| Soil | Vioolsdrift, NC | PPRI 4239 |

***B. leersiae*** (G.F. Atk.) Shoemaker

= *H. leersiae* G.F. Atk.

= *D. leersiae* (G.F. Atk.) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Leersia hexandra* | Nottingham Road, KZN | Putterill (1954), PREM 33115 |
| *Setaria* sp. | Nottingham Road, KZN | Putterill (1954); PREM 33116 |

*Co. heterostrophus* (Drechsler) Drechsler

= *Ophiobolus heterostrophus* Drechsler

***B. maydis*** (Y. Nisik. & C. Miyake) Shoemaker

= *H. maydis* Y. Nisik. & C. Miyake

= *D. maydis* (Y. Nisik. & C. Miyake) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| Debris | Berg-en-Dal Rest camp, Kruger National Park, MP | PPRI 4240 |
| Grass | Jozini, KZN | PPRI 2957 |
| *Zea mays* | Hillcrest, KZN | PREM 45753; PPRI 3331 |
| *Zea mays* seed and leaves | Lydenburg, MP | Gorter (1977, 1979); PREM 44938, 44980 |
| *Zea mays* | Komatipoort, MP | PREM 45080 |
| *Zea mays* | Pietermaritzburg, KZN | PREM 45032, 45033, 45089 |

***B. mediocris*** (K.M. Putterill) Shoemaker

= *H. mediocre* K.M. Putterill

= *D. mediocris* (K.M. Putterill) Subram. & P.C. Jain as *D.* "*mediocre*"

| Host | Locality | Reference |
|---|---|---|
| *Pennisetum clandestinum* | Schagen, MP | Putterill (1954); PREM 30756 Type |

***B. micropus*** (Drechsler) Shoemaker

= *H. micropum* Drechsler

= *H. leptochloae* Y. Nisik. & C. Miyake

| Host | Locality | Reference |
|---|---|---|
| Zea mays | Klipdrift, GP | PPRI 4689 |

***B. miyakei*** (Y. Nisik.) Shoemaker

= *H. miyakei* Y. Nisik.

= *D. miyakei* (Y. Nisik.) Subram. & B.L. Jain

| Host | Locality | Reference |
|---|---|---|
| *Eragrostis curvula* | Pietermaritzburg, KZN | Gorter (1979); PREM 7761 |

***B. multiformis*** (Jooste) Alcorn

= *D. multiformis* Jooste (Alcorn 1983)

| Host | Locality | Reference |
|---|---|---|
| *Tribulus terrestris* | Middelburg, EC | Jooste (1975); PPRI 3367 |

***B. nicotiae*** (Mouch.) Alcorn (Alcorn 1983)

= *D. nicotiae* Mouch.

| Host | Locality | Reference |
|---|---|---|
| Fodder | Lichtenburg, NW | PPRI 6155[#] |
| Soil | Vioolsdrift, NC | PPRI 4239[#] |

*Co. nodulosus* Luttr.

***B. nodulosa*** (Berk. & M.A. Curtis) Shoemaker

= *H. nodulosum* Berk. & M.A. Curtis

= *H. nodosum* Berk. & M.A. Curtis

= *H. leucostylum* Drechsler

= *B. leucostyla* (Drechsler) Shoemaker

= *D. nodulosa* (Berk. & M.A. Curtis) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Aestivum sativum* hay | Porterville, WC | PPRI 6196 |
| *Cenchrus ciliaris* | Moedville, NW | PREM 45343 |
| *Eleusine coracana* subsp. *africana* (= *E. africana*) | Heilbron, FS | PREM 44745, 44899 |
| *Eleusine indica* | White River, MP; Vereeniging GP; Ladysmith, KZN | Putterill (1954); PREM 23689, 25908, 10036 |
| *Eleusine* spp. | KZN; "Transvaal" | Gorter (1981) |
| *Eriocephalus cricoides* debris | Middelburg, EC | PPRI 4886 |
| *Gossypium hirsutum* seeds | Limpopo river, NP | PPRI 6808 |
| *Sorghum vulgare* | NW | PREM 47234 |

*Co. miyabeanus* (S. Ito & Kurib.) Drechsler ex Dastur

= *O. miyabeanus* S. Ito & Kurib

***B. oryzae*** (Breda de Haan) Shoemaker.

= *H. oryzae* Breda de Haan

= *H. oryzae* Miyabe & Hori

= *D. oryzae* (Breda de Haan) Subram. & P.C. Jain

= *Luttrellia oryzae* (Breda de Haan) Gornostaì

| Host | Locality | Reference |
|---|---|---|
| *Oryza sativa* | Middelburg, MP | Gorter (1977) |
| *Oryza sativa* | GP, MP, NP and NW | Crous *et al.* (2000) |

***B. papendorfii*** (Aa) Alcorn

= *C. siddiquii* S. Ahmad & Quraishi

= *C. papendorfii* Aa

= *D. papendorfii* (Aa) M.B. Ellis

| Host | Locality | Reference |
|---|---|---|
| *Acacia karroo* leaf-litter | Potchefstroom, NW | Van der Aa (1967) |
| Air | Mariepskop, NP | PPRI 4094 |
| *Cenchrus ciliaris* | Roodeplaat, GP | Bezuidenhout (1977); PREM 45149 |
| *Citrus sinensis* leaves | Tzaneen, NP | PPRI 5342 |
| *Cynodontis incompletus* | Middelburg, EC | Roux 1997 |
| Grass | Leeudoringstad, NW | PPRI 5852 |
| Grass | Onderstepoort, GP | PPRI 5852 |
| Grass | Pretoria, GP | PPRI 6314 |
| *Oryza* sp. | Levubu, NP | PREM 45026 |
| Soil under *Acacia karroo* | Potchefstroom, NW | Papendorf (1976) |
| Soil under *Acacia karroo* | KZN | PPRI 3830 |
| *Sorghum vulgare* seed | Lichtenburg, NW | PREM 47232 |
| Teff | South Africa | PPRI 5667 |
| *Tribulus terrestris* | Middelburg, EC | PREM 45466 |
| *Triticum aestivum* seed | South Africa | Lübben 1992 |
| *Vigna unguiculata* | Roodeplaat, GP | PPRI 6124[#] |

***B. priskaensis*** W.Q. Chen & W.J. Swart (Chen, Swart & Niewoudt 2000)

| Host | Locality | Reference |
|---|---|---|
| *Pistachia vera* | Prieska, NC | Chen *et al.* (2000); PREM 56306 Holotype; PPRI 6806 ex-Type |

*Co. ravenelii* Alcorn

***B. ravenelii*** (M.A. Curtis) Shoemaker

= *Heterosporium callospermum* Speg. (Shoemaker 1959)

= *H. crustaceum* Henn. (Shoemaker 1959)

= *H. ravenelii* M.A. Curtis

= *H. hoffmanii* Berk.

= *Napicladium ravenelii* (M.A. Curtis) Speg.

= *H. tonkinense* P. Karst. & Roum.

= *D. ravenelii* (M.A. Curtis) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Sporobolus africanus* (as *S. capensis*) | Widespread | Gorter (1981); PREM 1453, 1826[$], 2201, 6692, 6921, 9769[$], 10065, 10097, 11643, 20371, 31783, 40533, 45730 |
| *S. fimbriatus* | Kentani, KZN | Doidge (1950); Putterill (1954); PREM 6667 |
| *S. pyramidalis* | Nelspruit, MP | PREM 57261 |
| *S. pyramidalis* | Schagen, MP | PREM 32909 |
| *S. pyramidalis* | Lydenburg, MP | Doidge (1950); PREM 26093 |
| *Sporobolus* sp. | Schagen, MP | PREM 47603 |

Note: [$] Specimens also contain *B. crustacea*

***B. sacchari*** (E.J. Butler) Shoem.

= *H. sacchari* E.J. Butler

= *D. sacchari* (E.J. Butler) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Aspalathus linearis* seed | Clanwilliam, WC | PPRI 5399 |
| *Cenchrus cilliarus* | Roodeplaat, GP | Bezuidenhout (1977) |
| *Saccharum officinarum* seed | Northern areas of KZN | Gorter (1977) |
| *Saccharum officinarum* seed | Mt. Edgecombe, KZN | Putterill (1954); PREM 30245, 30988 |

*Co. setariae* (S. Ito & Kurib.) Drechsler ex Dastur

= *O. setariae* S. Ito & Kurib.

***B. setariae*** (Sawada) Shoemaker

= *H. setariae* Sawada

= *D. setariae* (Sawada) Subram.

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus ciliaris* | Roodeplaat, GP | PREM 45151 |
| *Cynodon* sp. | South Africa | McKenzie (1992); SBML (2000) |
| *Persea* sp | NP | Crous *et al.* (2000) |

***B. sorghicola*** (Lefebvre & Sherwin) Alcorn

= *H. sorghicola* Lefebvre & Sherwin

= *D. sorghicola* (Lefebvre & Sherwin) M.J. Richardson & E.M. Fraser

| Host | Locality | Reference |
|---|---|---|
| *Parotis patens* | Tembe Elephant Park, Sihangwane, KZN | PPRI 6118 |
| *Sorghum vulgare* | NP | PREM 45019 |
| *Sorghum vulgare* seed | Lichtenburg, NW | PREM 4723 |
| *Sorghum caffrorum* seed | Lichtenburg, NW | PREM 47237 |
| *Zea mays* | Buffelspoort, NW | PPRI 4690 |

*Co. sativus* (S. Ito & Kurib.) Drechsler ex Dastur

= *O. sativus* S. Ito & Kurib.

***B. sorokiniana*** (Sacc.) Shoemaker

= *H. sorokinianum* Sacc.

= *H. sativum* Pammel

= *H. acrothecioides* Lindf.

= *H. californicum* Mackie & G. E. Paxton

= *D. sorokiniana* (Sacc.) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Acacia karroo* | Potchefstroom, NW | PREM 43015, 43018, 42062, 43062 |
| *Hordeum vulgare* leaves, roots and seeds | WC, FS and "Transvaal" | Gorter (1977) |
| *Hordeum* sp. | Grahamstown, EC; Louis Trichardt, NP; Lydenburg, MP | Putterill (1954); PREM 25890l, 30857, 30858, 30859, 30860 |
| *Panicum* sp. | Humansdorp, EC | PPRI 6180# |
| *Triticum aestivum* roots and seed | "Cape", FS and "Transvaal" | Gorter (1977) |
| *Triticum aestivum* seeds | South | Lübben (1992); PPRI 1026 |
| *Triticum vulgare* | Bethlehem, FS; Lydenburg, MP | Putterill (1954); PREM 30990, 30991, 30493, 30421 |

*Co. spicifer* R.R. Nelson

= *P. spicifer* (R.R. Nelson) Tsuda

***B. spicifera*** (Bainier) Subram.

= *Brachycladium spiciferum* Bainier

= *H. tetramera* McKinney

= *C. spicifera* (Bainier) Boedijn

= *H. spiciferum* (Bainier) Nicot

= *C. tetramera* (McKinney) Boedijn ex J.C. Gilman

= *B. tetramera* (McKinney) Shoemaker

= *D. tetramera* (McKinney) Subram. & P.C. Jain.

= *D. spicifera* (Bainier) Arx

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus ciliaris* | Roodeplaat, GP | PREM 45173 |
| *Citrus* sp. | Letaba Estate, Tzaneen, NP | PPRI 5107 |
| *Cynodon transvaalensis* | Johannesburg, GP | Putterill (1954); PREM 30246 |
| Debris | D'Nyala Provincial Nature Reserve, NP | PPRI 3824 |
| *Eragrostis* sp. hay | Humansdorp, EC | PPRI 6184 |
| Groundnuts | Vaalharts, NC | PPRI 3766 |
| Soil and plant debris | Upington, NC | PREM 47830 |
| Soil under *Acacia karroo* | Potchefstroom, NW | Papendorf (1976) |
| *Triticum aestivum* seeds | South Africa | Lübben (1992) |
| *Triticum* sp. | Bethlehem, FS | Putterill (1954); PREM 30494 |
| *Triticum* sp. | WC | Crous *et al.* (2000) |

*C. stenospilus* Matsumoto & W. Yamamoto, *nom. inval.* (SBML 2001)

***B. stenospila*** (Drechsler) Shoemaker.

= *H. stenospila* Drechsler

= *D. stenospila* (Drechsler) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Saccharum officinarum* stems | Mt. Edgecombe, KZN | Gorter (1977) |

*B.* ***urochloae*** (K.M. Putterill) Shoemaker

= *H. urochloae* K.M. Putterill

= *D. urochloae* (K.M. Putterill) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| Onion seed | Oudshoorn, WC | PPRI 3358 |
| Onion seed | Beaufort West, WC | |
| Onion seed | Pretoria, GP | PREM 44946, 4985, 44983 |
| Soil and debris from cotton-field | Groblersdal, MP | PPRI 5494 |
| *Urochloa panicioides* (= *U. helopus* Crous *et al.* 2000) | Barberton, MP | Putterill (1954); PREM 26148 Holotype |
| *Urochloa panicioides* (= *U. helopus* Crous *et al.* 2000) | Schagen, MP | Putterill (1954); PREM 26182 Paratype |

*Co. victoriae* R.R. Nelson

***B. victoriae*** (F. Meehan & H.C. Murphy) Shoemaker

= *H. victoriae* F. Meehan & H.C. Murphy

= *H. sativum* Pammel, J.E. King & Bakke var. *victoriae* (F. Meehan & H.C. Murphy) H.R. Rosen

= *D. victoriae* (F. Meehan & H.C. Murphy) Subram. & P.C. Jain.

| Host | Locality | Reference |
|---|---|---|
| *Triticum aestivum* seeds | South Africa | Lübben (1992) |
| Soil under *Acacia karroo* | Potchefstroom, NW | Papendorf (1976) |

*Co. carbonum* R.R. Nelson

***B. zeicola*** (G. L. Stout) Shoemaker.

= *H. zeicola* G.L. Stout

= *H. carbonum* Ullstrup

= *D. zeicola* (G. L. Stout) Subram. & P.C. Jain.

= *D. carbonum* (Ullstrup) Sivan.

| Host | Locality | Reference |
|---|---|---|
| *Pennisetum glaucum* | KZN | Crous *et al.* (2000) |
| *Cenchrus ciliarus* | Roodeplaat, GP | Bezuidenhout (1977) |
| *Cynodontis incompletus* | Middelburg, EC | Roux and Van Warmelo (1997) |
| *Zea mays* leaves | Derdepoort, GP; Carltonville, GP; Ventersdorp, NW; Potchefstroom, NW; Delmas, MP; Roodeplaat, GP; Delmas, MP; Springbok flats, NP | Gorter (1979); PREM 45079, 44683, 45045, 45171, 45572; PPRI 5461, 6201, 3362 |
| *Triticum aestivum* seeds | South Africa | Lübben (1992) |
| *Triticum aestivum* seeds | Springbok flats, NP | Gorter (1977) |
| *Triticum aestivum* seeds | KZN | Crous *et al.* (2000) |

***Curvularia*** Boedijn 1933

= *Curvisporium* Corbetta 1963

= *Acrotheciella* Koord.1907

= *Malustella* Batidys & J.A. Lima 1960.

***C. affinis*** Boedijn

| Host | Locality | Reference |
|---|---|---|
| *Ipomoea batatas* | Levubu, NP | PPRI 5861 |
| *Zea mays* | Klipdrift, GP | PPRI 7051 |

***C. borreriae*** (Viégas) M.B. Ellis

= *H. borreriae* Viégas

=*C. borreriae* (Viégas) Martin apud Viégas *nom. inval.* (Art. 33.2)

| Host | Locality | Reference |
|---|---|---|
| *Citrus cinensis* leaves | Letsitele, NP | PPRI 5109 |
| Debris | Ozwatini Plateau, Greytown, KZN | PPRI 3820 |

Note: The initials of Martin, co author of the name *C. borreriae* (Viégas) Martin apud Viégas, could not be verified since the original publication was not available and subsequent publications referred to the name as above (Ellis 1966; Sivanesan 1987).

***C. brachyspora*** Boedijn

| Host | Locality | Reference |
|---|---|---|
| Debris | Uitsoek trail, MP | PPRI 4060 |
| Moss | Mariepskop, NP | PPRI 4096 |
| *Panicum laevifolium* | Heilbron, FS | PREM 44931; PPRI 3343 |
| *Pinus patula* shoots | Rhenosterhoek, NW | Van der Westhuizen (1955) |
| Soil cotton field | Groblersdal, MP | PPRI 5463 |

***C. clavata*** P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Aspalathus linearis* | Clanwilliam, WC | PPRI 5212 |
| *Brachychloa* leaf bracts | Tembe Elephant Park, Sihangwane, KZN | PPRI 6120 |
| *Cenchrus cilliarus* | Roodeplaat, GP | Bezuidenhout (1977) |
| *Citrus cinensis* leaves | Tzaneen, NP | PPRI 5108 |
| *Panicum coloratum* phylloplane of / and litter | Delmas, MP | Eicker ( 1976) |
| *Triticum aestivum* seeds | South Africa | Lübben (1992) |
| *Triticum aestivum* seeds | Heilbron, FS | PPRI 3344 |

*Co. cymbopogonis* J.A. Hall & Sivan.

***C. cymbopogonis*** (C.W. Dodge) J.W. Groves & Skolko

= *H. cymbopogonis* C.W. Dodge

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus cilliarus* | Roodeplaat, GP | Bezuidenhout 1977 |
| *Hyparrhenia quarrei* | Royal Natal National Park, KZN | PPRI 6273[#] |
| *Zea mays* | Klipdrift, GP | PPRI 6633[#] |

*C. eragrostidis* (Tsuda & Ueyama) Sivan.

= *P. eragrostidis* Tsuda & Ueyama

***C. eragrostidis*** (Henn.) J.A. Meyer

= *Brachysporium eragrostidis* Henn.

= *Spondylocladium maculans* C.K. Bancr.

= *C. maculans* (C.K. Bancr.) Boedijn

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus cilliarus* | Roodeplaat, GP | Bezuidenhout (1977) |
| Grass | Beech Wood, KZN | PPRI 3221 |
| Grass | Severn, NW | PPRI 5666 |
| *Triticum aestivum* seeds | South Africa | Lübben (1992) |
| *Pinus patula* shoots | Louis Trichardt, NP | Van der Westhuizen (1955) |

*Co. geniculatus* R.R. Nelson

= *P. geniculatus* (R.R. Nelson) Tsuda, Ueyama & Nishihara

***C. geniculata*** (Tracy & Earle) Boedijn

= *Helminthosporium geniculatum* Tracy & Earle

= *Brachysporium sesami* Sawada

| Host | Locality | Reference |
|---|---|---|
| Avocado leaves | Tzaneen, NP | PPRI 3114 |

***C. harveyi*** Shipton

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus cilliarus* | Roodeplaat, GP | Bezuidenhout (1977) |
| Debris | Uitsoek trail, MP | PPRI 4059 |

*Co. intermedius* R.R. Nelson

***C. intermedia*** Boedijn

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus cilliarus* | Roodeplaat, GP | Bezuidenhout (1977) |
| Debris | Mariepskop, NP | Sinclair, Eicker and Morgen-Jones (1984) |

*Co. lunatus* R.R. Nelson & F.A. Haasis as *Co.* "*lunata*"

= *P. lunatus* R.R. Nelson & F.A. Haasis) Tsuda, Ueyamma & Nisihih.

***C. lunata*** (Wakker) Boedijn

= *Acrothecium lunatum* Wakker

= *H. caryopsidum* Sacc.

= *H. sudanensis* Cif. & Gonz. Frag.

= *C. caryopsidum* (Sacc.) Teng

| Host | Locality | Reference |
|---|---|---|
| Avocado flower buds | Nelspruit, MP | PPRI 5606 |
| *Cenchrus ciliaris* | Roodeplaat, GP | Bezuidenhout (1977) |
| *Gladiolus* sp. | South Africa | SBML (2000); BPI 443711 |
| L.M. lawn | Roodeplaat, GP | PPRI 6099# |
| *Lupinis* sp. | WC | Crous *et al.* (2000) |
| Mango leaves | Hazyview, MP | PPRI 5331 |
| *Tribulis terrestris* | Oudtshoorn, WC | PPRI 3360# |
| *Triticum aestivum* | Bethlehem, FS | Sivanesan (1987); PREM 30495 |
| *Triticum aestivum* seeds | South Africa | Lübben (1992) |
| Unknown plant leaves | Mariepskop, NP | PPRI 4137 |

*Co. pallescens* (Tsuda & Ueyama) Sivan.

= *P. pallescens* Tsuda & Ueyama

***C. pallescens*** Boedijn

= *C. leonensis* M.B. Ellis

| Host | Locality | Reference |
|---|---|---|
| *Aspalathus linearis* seed | Clanwilliam, WC | PPRI 5418 |
| *Cenchrus cilliarus* | Roodeplaat, GP | Bezuidenhout (1977) |
| *Citrus cinensis* leaves | Tzaneen, MP | PPRI 5121 |
| Dung – Golden Pheasant | Pretoria, GP | PPRI 3395 |
| Grass | Pretoria, GP | PPRI 6313 |
| *Pinus patula* | Sabie, MP | Van der Westhuizen (1955) |

***C. senegalensis*** (Speg.) Subram.

= *C. senegalensis* (Speg.) Muntoñola, nom. illeg. (Art. 63)

= *Brachyspora senegalense* Speg.

= *Acrothecium falcatum* Tehon

= *C. falcata* (Tehon) Boedijn (as C. "flacata")

| Host | Locality | Reference |
|---|---|---|
| Wheat straw | Heilbron, FS | PPRI 1091 |

***C. stapeliae*** (du Pless.) S.J. Hughes & du Pless.

= *Tripospórium stapeliae* du Pless. (Ellis 1966)

| Host | Locality | Reference |
|---|---|---|
| *Huernia oculata* | Stellenbosch, WC | Hughes (1951); Alcorn (1990); PREM 46707 |
| *Stapelia schinzii* | Stellenbosch, WC | PREM 46710, 46712, 46713 |
| *Stapelia* spp. | Stellenbosch, WC | PREM 46710, 46712, 46713 |
| *Tavaresia grandiflora* | Stellenbosch, WC | PREM 46709 |
| *Trichocaulon cactiforme* | Stellenbosch, WC | Hughes 1951, PREM 46708 |

*Co. tuberculatus* Sivan.

* ***C. tuberculata*** P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| Soil around *Acacia karroo* | Potchefstroom, NW | PPRI 3240 |

***C. trifolii*** (Kauffmann) Boedijn

= *Brachysporium trifolii* Kauffmann

= *Blennoria trifolii* Bonar *nom. nud.* (SBML 2000)

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus cilliarus* | Roodeplaat, GP | Bezuidenhout (1977) |
| *Gladiolus hortulanus* stem | Barberton, MP | Gorter (1977) |
| *Zea mays* | Springs, GP | Marasas *et al.* (1966) |

*Co. verruculosus* (Tsuda & Ueyama) Sivanesan

= *P. verruculosus* Tsuda & Ueyama

***C. verruculosa*** M.P. Tandon & Bilgrami ex M.B. Ellis

= *C. verruculosa* M.P. Tandon & Bilgrami *nom inval.* (Art. 37)

| Host | Locality | Reference |
|---|---|---|
| *Eragrostis* hay | Humansdorp, EC | PPRI 6183 |
| *Ipomoea batatas* | Groblersdal, MP | PPRI 6162 |
| *Portulaca* sp. | Karoo National Reserve, Graaff-Reinet, EC | PPRI 4894 |

***Exserohilum*** K.J. Leonard & Suggs 1974

*Setosphaeria holmii* (Luttr.) K.J. Leonard & Suggs

= *Trichometasphaeria holmii* Luttr.

= *Keissleriella holmi* (Luttr.) Arx

***E. holmi*** (Luttr.) K.J. Leonard & Suggs

= *H. holmii* Luttr.

= *D. holmii* (Luttr.) Subram. & P.C. Jain

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus ciliaris* | Roodeplaat, GP | Bezuidenhout (1977); PREM 45152 |

***E. longirostratum*** (Subram.) Sivan.

= *H. longirostratum* Subram.

= *B. longirostratum* (Subram.) Subram.

= *D. longirostratum* (Subram.) Subram.

| Host | Locality | Reference |
|---|---|---|
| *Musa* sp. | Nelspruit, MP | PPRI 5133# |

*S. monoceras* Alcorn

***E. monoceras*** (Drechsler) K.J. Leonard & Suggs

= *H. monoceras* Drechsler

= *B. monoceras* (Drechsler) Shoemaker

= *D. monoceras* (Drechsler) Subram. & P.C. Jain

= *L. monoceras* (Drechsler) Khokhr.

| Host | Locality | Reference |
|---|---|---|
| *Cenchrus ciliaris* | Roodeplaat, GP | Bezuidenhout (1977), PREM 45179 |

*S. pedicellata* (R.R. Nelson) K.J. Leonard & Suggs

=*T. pedicellata* R.R. Nelson

***E. pedicellatum*** (A.W. Henry) K.J. Leonard & Suggs

= *H. pedicellatum* Henry

= *B. pedicillata* (Henry) Shoemaker

= *D. pedicellata* (Henry) Subram. & P.C. Jain.

| Host | Locality | Reference |
|---|---|---|
| Soil and *Zea mays* debris in cotton field | Groblersdal, MP | PPRI 6160 |
| *Zea mays* | Settlers, NP | PPRI 1047 |
| *Zea mays* | Ventersburg, FS | PREM 45765; PPRI 3333 |
| *Zea mays* roots | Potchefstroom, NW | PPRI 5087 |
| *Zea mays* | FS and "Transvaal" | Gorter (1977) |
| *Zea mays* | Roodeplaat, GP | Marasas *et al.* (1966); PREM 42940 |

*S. prolata* K. J. Leonard & E.G. Suggs

***E. prolatum*** K.J. Leonard & E.G. Suggs

| Host | Locality | Reference |
|---|---|---|
| *Musa* sp. | Nelspruit, MP | PPRI 5134 |

*S. rostrata* K.J. Leonard

***E. rostratum*** (Drechsler) K.J. Leonards & E.G. Suggs

= *H. halodes* Drechsler

= *H. rostratum* Drechsler

= *H. halodes* Drechsler var. *tritici* Mitra

= *H. halodes* Drechsler var. *elaeidicola* Kovachich

= *B. halodes* (Drechsler) Shoemaker.

= *B. rostrata* (Drechsler) Shoemaker.

= *D. halodes* (Drechsler) Subram. & P.C. Jain.

= *H. appatternae* K.S. Deshpande & K.B. Deshpande

= *D. rostrata* (Drechsler) M.J. Richardson & E.M. Fraser

= *E. halodes* (Drechsler) K.J. Leonards & E.G. Suggs

= *L rostrata* (Drechsler) Gonorstai

| Host | Locality | Reference |
|---|---|---|
| Animal feed | Onderstepoort, GP | PPRI 3392, 5149 |
| Bananas | Nelspruit, GP | PPRI 5483 |
| *Brachychloa* leaf bracts | Tembe Elephant Park, Sihangwane, KZN | PPRI 6119 |
| *Cenchrus ciliaris* | Roodeplaat, GP | Bezuidenhout (1977); PREM 45172 |
| *Chrysalidocarpus leitescens* | Tongaat, KZN | PREM 47561 |
| *Citrus sinensis* leaves | Tzaneen, NP | PPRI 5105 |
| *Cynodontis incompletus* | Middelburg, EC | Roux and Van Warmelo (1997) |
| Debris | D`Nyala Nature Reserve, NP | PPRI 3817 |
| *Eragrostis lehmanniana* debris and soil from | Middelburg, EC | PPRI 4874 |
| Fodder | Lichtenburg, NW | PPRI 6154 |
| Kikuyu grass | Pretoria, GP | PPRI 4852 |
| L.M. lawn | Roodeplaat, GP | PPRI 6148 |
| *Medicago sativa* seed | Ladysmith, KZN | PREM 44525, 44527 |
| *Panicum* hay | Humansdorp, EC | PPRI 6178 |
| *Rhodora* fern | Mariepskop, NP | PPRI 4130 |
| *Saccharum officiarum* | KZN | PREM 32488 |
| Unknown mangrove plant seeds | Pietermaritzburg, KZN | PPRI 5904 |

| Host | Locality | Reference |
|---|---|---|
| *Setaria incressata* roots | Morolong, NP | PPRI 4588 |
| Soil Cotton field | Groblersdal, MP | PPRI 5460, 5462, 5489 |
| *Sorghum caffrorum* seed | NP | PREM 47224, 47238 |
| *Triticum aestivum* | Bethlehem, FS | Putterill (1954); PREM 30456, 30460 |
| *Triticum aestivum* seeds | South Africa | Lübben (1992) |
| *Triticum* | Brits, NW | Putterill (1954); PREM 30434 |
| *Zea mays* | Pretoria, GP | PREM 47494; PPRI 1025, 3346 |
| *Zea mays* | Bloemfontein, FS | PREM 44659 |

*S. turcica* (Luttr.) K.J. Leonard & E.M. Suggs

= *T. turcica* Luttr.

= *Keissleriella turcica* (Luttr.) Arx

***E. turcicum*** (Pass.) K.J. Leonard & E. M. Suggs

= *H. turcicum* Pass.

= *H. inconspicuum* Cooke & Ellis

= *B. turcica* (Pass.) Shoemaker

= *D. turcica* (Pass.) Subram. & P.C. Jain

= *L. turcica* (Pass.) Khokhr.

| Host | Locality | Reference |
|---|---|---|
| *Pennisetum glaucum* | KZN | Crous *et al.* (2000) |
| *P. typhoides* | KZN | Crous *et al.* (2000) |
| Pigs feed | GP | PPRI 5487 |
| *S. halepense* | Ashburton KZN; Prinshof, GP | Putterill (1954); PREM 28600, 30431, 30432, 30710, 10099 |
| *S. sudanense* leaves and seeds | Pietermaritzburg KZN; Cedara KZN; Prinshof, GP | Putterill (1954); PREM 15448, 15462, 30709 |
| *S. verticilliflorum* leaves | KZN and "Transvaal" | Doidge (1950); Gorter (1981) |

| Host | Locality | Reference |
|---|---|---|
| *S. verticilliflorum* leaves | Ficksburg, FS | PREM 42548 |
| *S. verticilliflorum* leaves | Dalton, KZN; Prinshof, GP | Putterill (1954); PREM 30708, 9729 |
| *Sorghum caffrorum* leaves & seeds | Bloemfontein, FS | Doidge (1950); PREM 31789 |
| *Zea mays* | Cedara, KZN; Naboomspruit, NP | Putterill (1954); PREM 2187, 20386 |
| *Zea mays* on leaves | Widespread | Gorter (1977) |

# Appendix F: ANALYSIS OF SPORE IMAGES

**Table 10: Group separation table of FOIL analysis using the variables medium, day, temperature, roundness, ferret diameter and compactness.**

| | Total | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 40 | 30 | | | | | | 3 | 4 | 1 | | 2 | | | |
| **B** | 16 | | 3 | | | 2 | | 3 | | 4 | 3 | 1 | | | |
| **C** | 36 | 1 | | 28 | | 1 | | | 3 | | | | 1 | 1 | 1 |
| **D** | 24 | 2 | | 1 | 11 | 2 | 1 | | | | | | 3 | 3 | 1 |
| **E** | 27 | 2 | 1 | 6 | | 14 | | | 2 | | | 2 | | | |
| **F** | 14 | | | | 1 | | 2 | | | | | | 5 | 4 | 2 |
| **G** | 29 | | | | | | | 16 | | 7 | 6 | | | | |
| **H** | 39 | 16 | | 1 | 2 | 1 | 2 | | 10 | | | 1 | 2 | 2 | 2 |
| **I** | 43 | | | 2 | | | | 13 | | 21 | 7 | | | | |
| **J** | 45 | 1 | | 1 | 1 | | | 14 | | 16 | 11 | | 1 | | |
| **K** | 40 | 9 | 2 | 2 | | 3 | | 4 | 5 | 6 | 2 | 6 | 1 | | |
| **L** | 44 | 2 | | 2 | 5 | | | | | | | | 23 | 9 | 3 |
| **M** | 29 | | | | 6 | | 1 | | | | | | 7 | 13 | 2 |
| **N** | 22 | | | | 3 | | | | | | | | 5 | 6 | 8 |

**A** = *B. cynodontis*; **B** = *B. ellisii*; **C** = *B. indica*; **D** = *B. maydis*; **E** = *B. papendorfii*; **F** = *B. setariae*; **G** = *C. affinis*; **H** = *C. cymbopogonis*; **I** = *C. fallax*; **J** = *C. lunata*; **K** = *E. inaequale*; **L** = *E. longirostratum*; **M** = *E. rostratum*; **N** = *E. turcicum*.

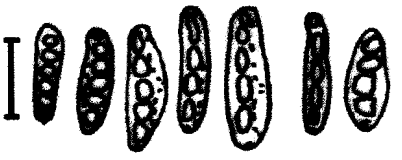
**Table 11: Group separation table of DT analysis using the variables medium, day, temperature, roundness, ferret diameter and compactness.**

| | Total | A | B | C | D | E | F | G | H | I | J | K | L | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 40 | 35 | | | | | | | 1 | 1 | 2 | 1 | | |
| B | 16 | 1 | 8 | | | 6 | | | | | 1 | | | |
| C | 36 | | | 29 | 1 | 3 | | | 1 | | | 1 | 1 | |
| D | 24 | | | 4 | 14 | 1 | 1 | | 1 | | | 2 | 1 | |
| E | 27 | | | | | 26 | | | | | | 1 | | |
| F | 14 | | | | 6 | | 2 | | | | | | 2 | 4 |
| G | 29 | 1 | | | | | | 11 | | 8 | 9 | | | |
| H | 39 | 10 | | | 7 | | | | 15 | | | 7 | | |
| I | 43 | | | | | | | 2 | | 16 | 25 | | | |
| J | 45 | | 2 | 1 | 1 | | | | | | 40 | 1 | | |
| K | 40 | 9 | | | | 6 | 1 | 2 | 2 | | 11 | 9 | | |
| L | 44 | 2 | | 1 | 7 | | | | 2 | | | | 29 | 3 |
| M | 29 | | | | 8 | | 1 | | | | | | 18 | 2 |
| N | 22 | | | | 3 | | | | | | | | 5 | 14 |

**A** = *B. cynodontis*; **B** = *B. ellisii*; **C** = *B. indica*; **D** = *B. maydis*; **E** = *B. papendorfii*; **F** = *B. setariae*; **G** = *C. affinis*; **H** = *C. cymbopogonis*; **I** = *C. fallax*; **J** = *C. lunata*; **K** = *E. inaequale*; **L** = *E. longirostratum*; **M** = *E. rostratum*; **N** = *E. turcicum*.

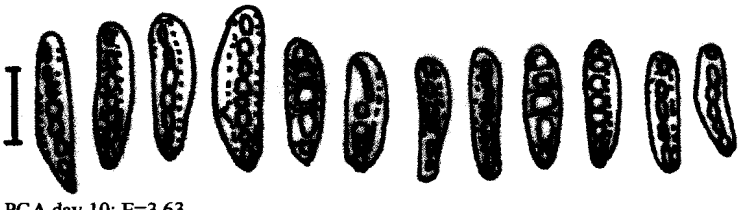
**Figure 3:** **Line drawings of *B. cynodontis* PPRI 6535 spores formed under different cultural conditions. Scale bar = 10 µm; average elongation E=3.41**

Ceres day 10; E=2.74

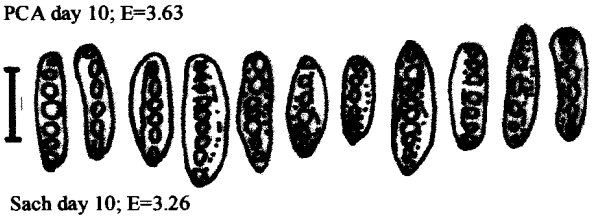


PCA day 5; E=3.58

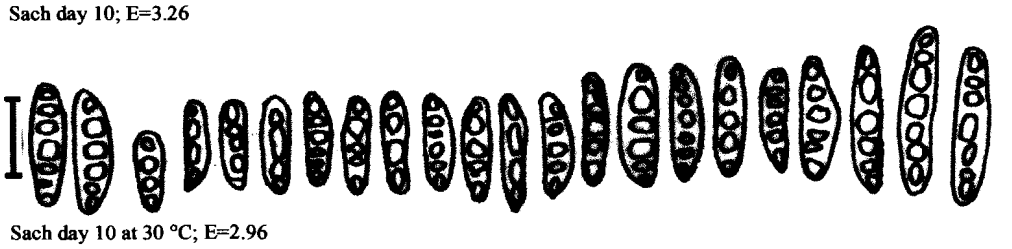
Glucose day 10; E=3.97



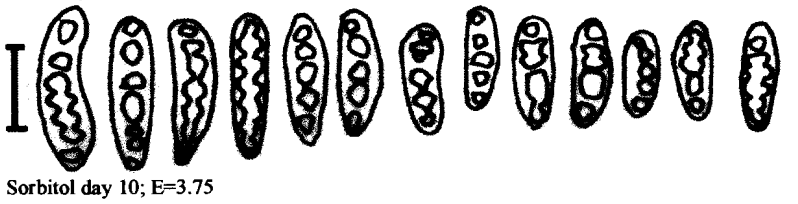
PCA day 10; E=3.63



Sach day 10; E=3.26



Sach day 10 at 30 °C; E=2.96

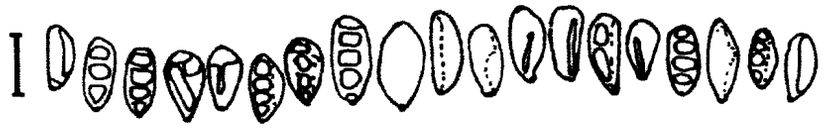


Sorbitol day 10; E=3.75

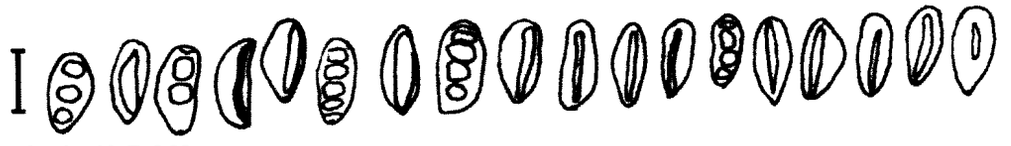
**Figure 4: Line drawings of *B. ellisii* PPRI spores formed under different cultural conditions. Scale bar = 10 µm; average E=2.17**

Ceres day 5; E=1.99

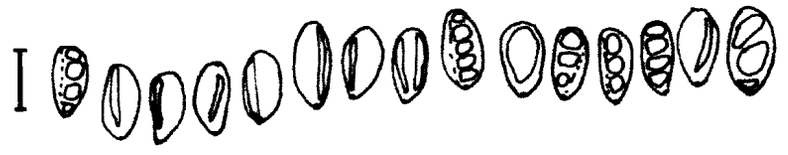
Ceres day 10; E=2.07



PCA day 5; E=2.13



PCA day 10; E=2.28

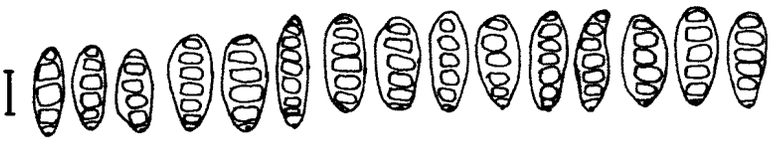


Sach day 5; E=2.10

Sach day 8 at 30°C; E=2.29

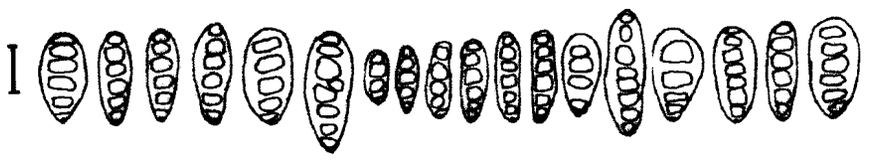
WA day 10; E=2.25

**Figure 5: Line drawings of *B. indica* PPRI 6567 spores formed under different cultural conditions. Scale bar = 10 µm; average E=2.66**

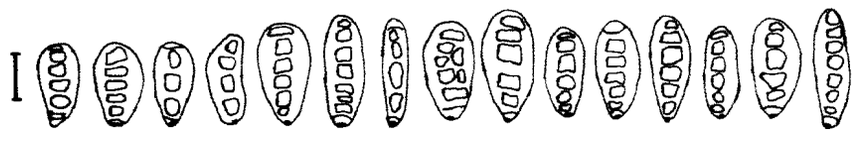


Ceres day 5; E=2.69

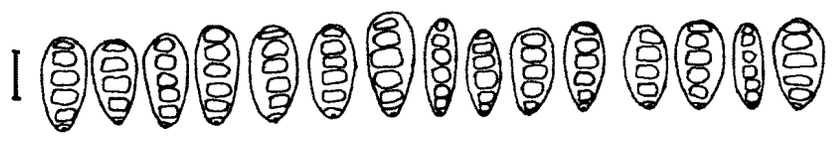
Ceres day 10; E=2.11



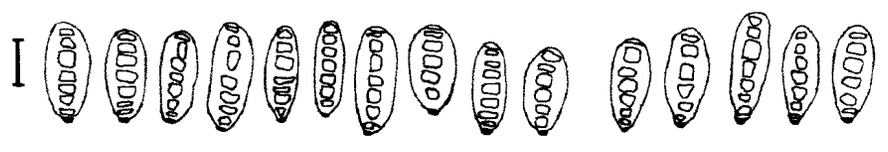
Sach day 5; E=2.43



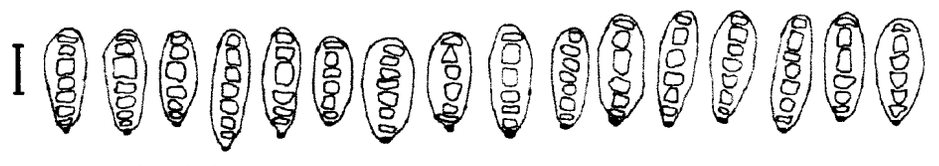
Sach day 8 at 30 °C; E=2.59



WA day 5; E=2.30

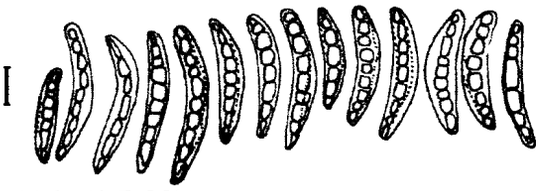


Sorbitol day 10; E=3.64

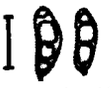


Glucose day 10; E=3.40

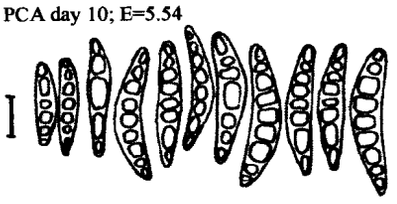
**Figure 6: Line drawings of *B. maydis* PPRI 6564 spores formed under different cultural conditions. Scale bar = 10 µm; average E=4.76**



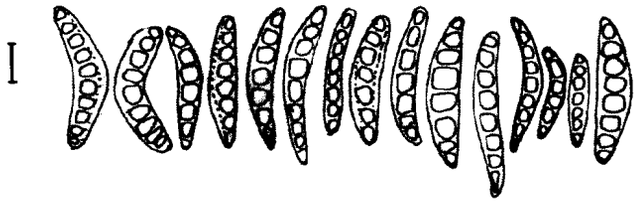
PCA day 10; E=5.54



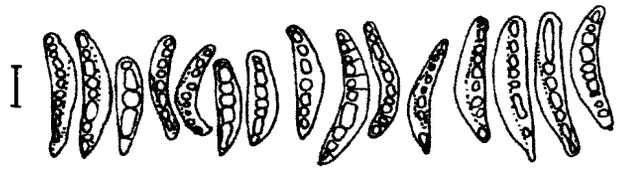
Ceres day 10; E=2.86



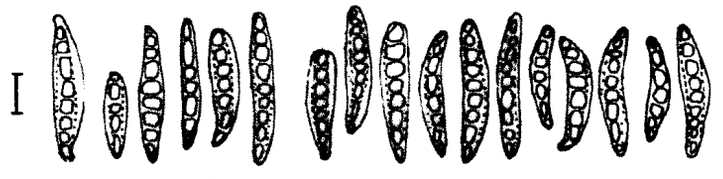
Sach day 8 at 30 °C; E=4.84



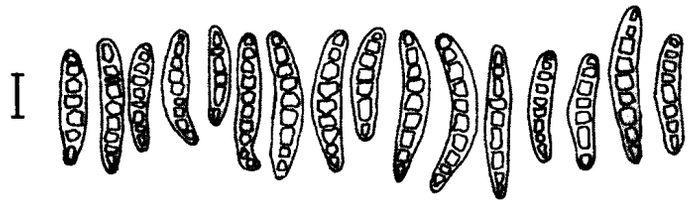
Sach day 10; E=4.69



WA day 10; E=5.11

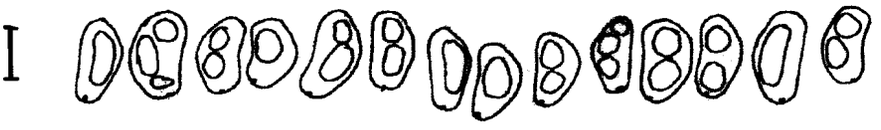


Sorbitol day 10; E=5.15



Glucose day 10; E=5.15

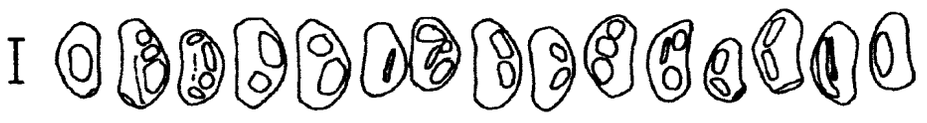
**Figure 7: Line drawings of *B. papendorfii* PPRI 5855 and PPRI 6539 spores formed under different cultural conditions. Scale bar = 10 µm; average E=2.01**



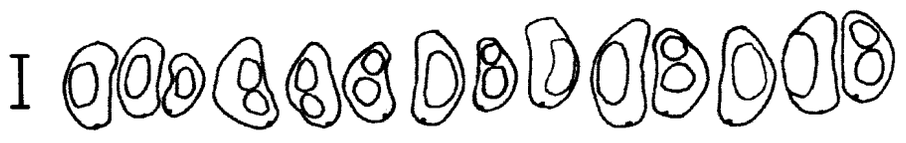
Ceres day 5; E=1.94

Ceres day 10; E=1.83

PCA day 5; E=1.96



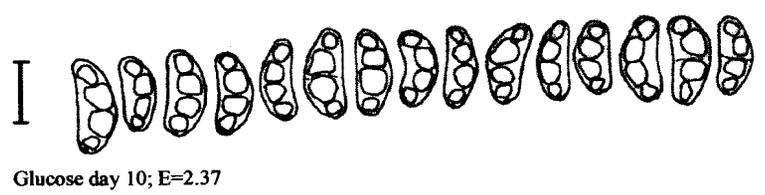
PCA day 10; E=1.85



Sach day 5; E=1.81

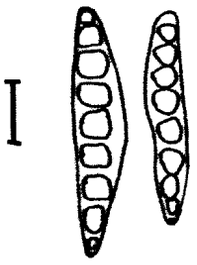
Sach day 8 at 30°C; E=2.13

Sorbitol day 10; E=2.26



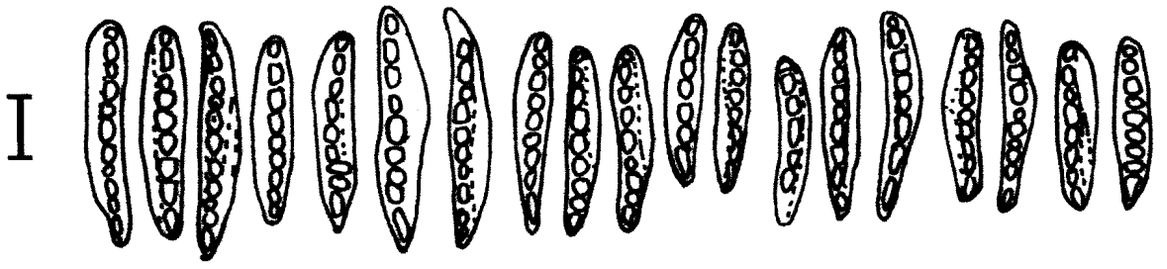
Glucose day 10; E=2.37

**Figure 8:** **Line drawings of *B. setariae* PPRI 6633 spores formed under different cultural conditions. Scale bar = 10 µm; average E=5.22**

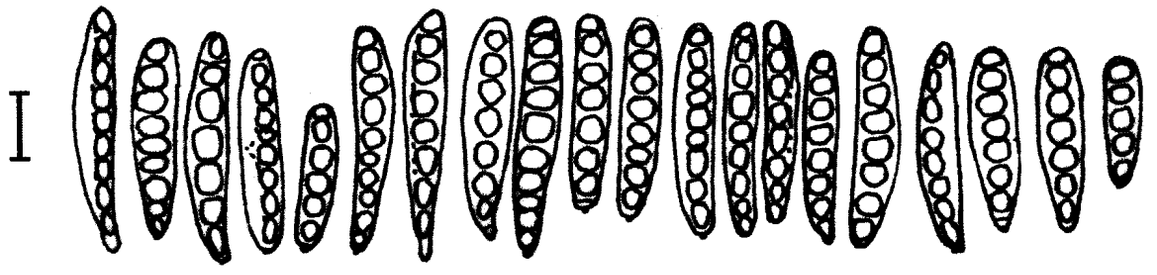


Ceres day 5; E= 5.24

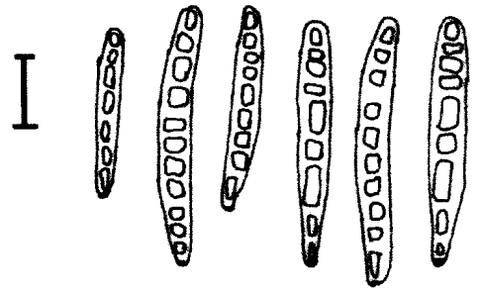
Ceres day 10; E= 5.20



PCA day 10; E= 5.19



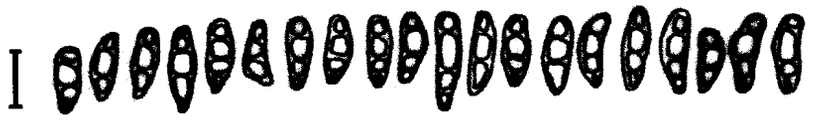
WA day 10; E= 5.12



Glucose day 10; E= 5.37

**Figure 9: Line drawings of C. *affinis* PPRI 6428 spores formed under different cultural conditions. Scale bar = 10 µm; average 2.64**

Ceres day 5; E=2.49



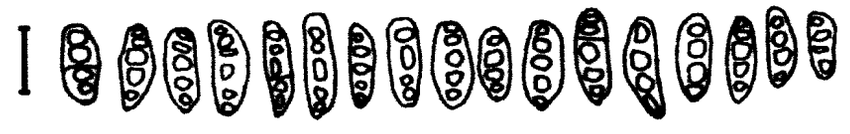
PCA day 5; E=2.91

PCA day 10; E=2.58

Sach day 5; E=2.5

Sach day 7 at 30 °C; E=2.79

WA day 10; E=2.45



Sorbitol day 10; E=2.77

Glucose day 10; E=2.70

**Figure 10: Line drawings of C. *cymbopogonis* PPRI 6633 and PPRI 6592 spores formed under different cultural conditions. Scale bar = 10 µm; average E=3.6**

Ceres day 5; E=3.04

PCA day 5; E=3.38

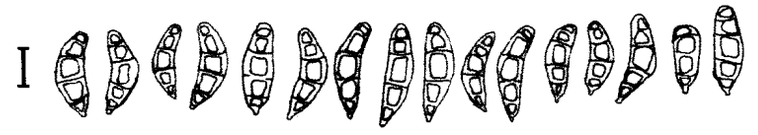
PCA day 10; E=2.87

Sach day 5; E=3.37

Sach day 10; E=3.61

WA day 5; E=2.68

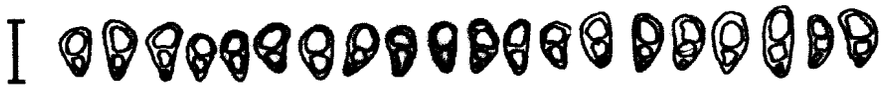
Sorbitol day 10; E=3.00



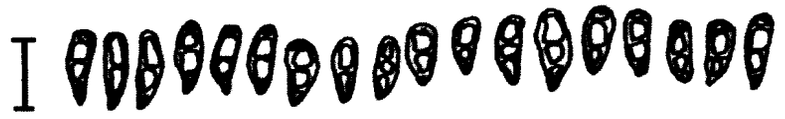
Glucose day 10; E=3.79

**Figure 11: Line drawings of C. *fallax* PPRI 6445 spores formed under different cultural conditions. Scale bar = 10 µm; average E=2.38**

Ceres day 5; E=1.95



Ceres day 10; E=2.26

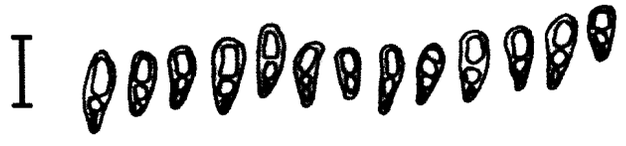


PCA day 5; E=2.65

PCA day 10; E=2.5

Sach day 5; E=2.65

Sach day 10; E=2.67



WA day 10; E=2.08

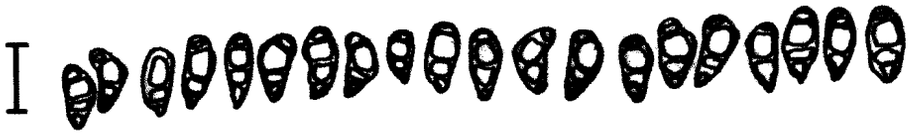
Sorbitol day 10; E=2.24

Glucose day 10; E=2.79

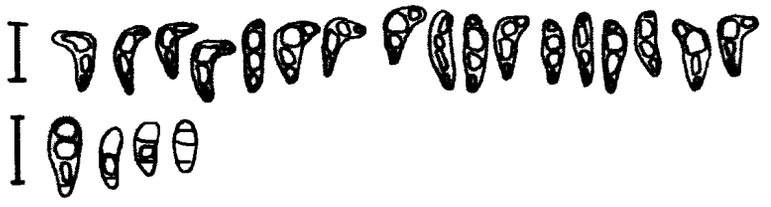
**Figure 12: Line drawings of C. *lunata* PPRI 6540 and PPRI 6699 spores formed under different cultural conditions. Scale bar = 10 µm; average E=2.44**

Ceres day 5; E=2.53

Ceres day 10; E=2.16

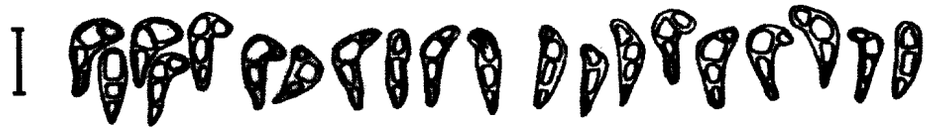


Sach day 5; E=3.53



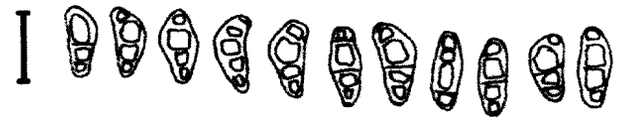
Sach day 10; E=2.76

Sach day 7 at 30°C; E=1.95



WA day 5; E=2.98

WA day 10; E=2.1

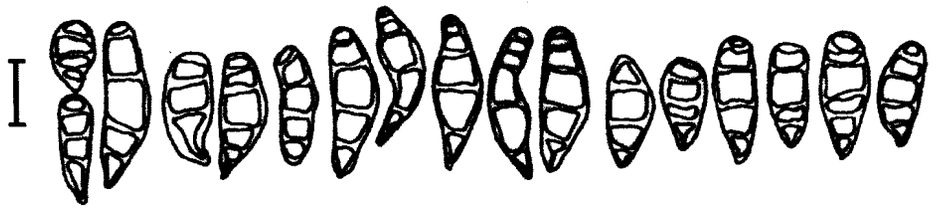


Sorbitol day 10; E=2.05

Glucose day 10; E=2.11

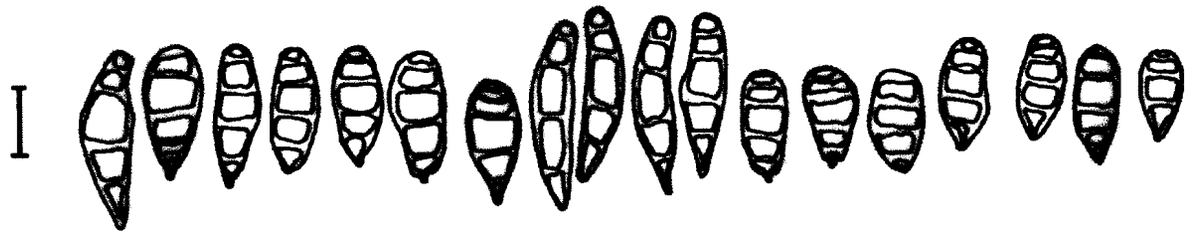
**Figure 13: Line drawings of *E. inaequale* PPRI 6527 spores formed under different cultural conditions. Scale bar = 10 µm; average E=2.98**

Ceres day 5; E=2.09

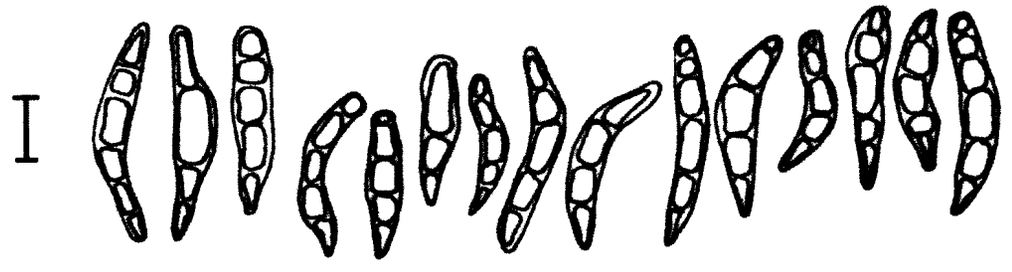


Ceres day 10; E=2.05

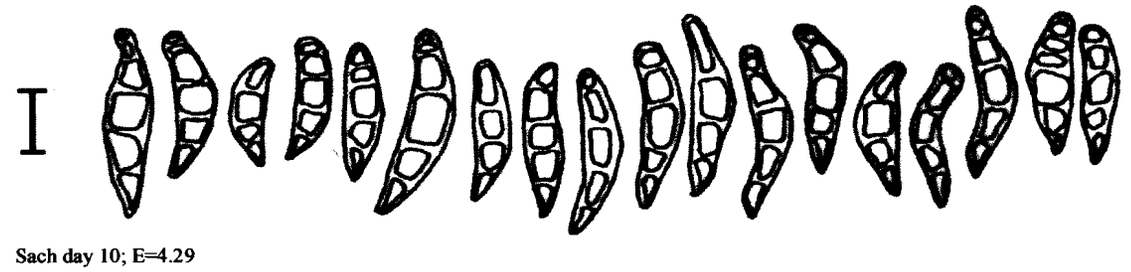
PCA day 5; E=2.45



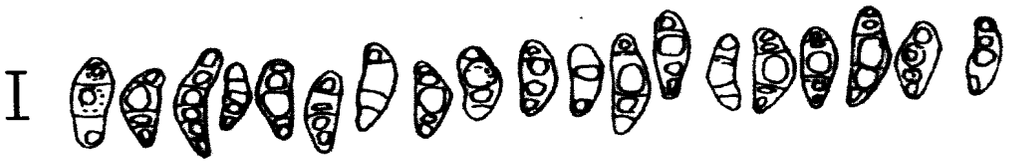
PCA day 10; E=2.85



Sach day 5; E=5.18



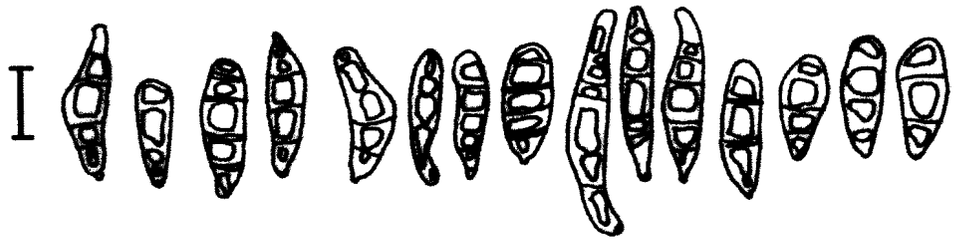
Sach day 10; E=4.29



Sach day 7 at 30°C; E=3.14

WA day 5; E=2.29

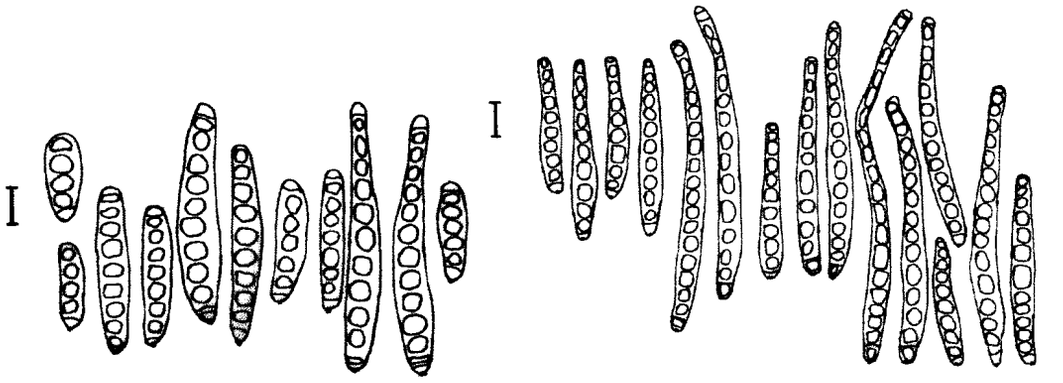
WA day 10; E=3.5



Sorbitol day 10; E=2.71

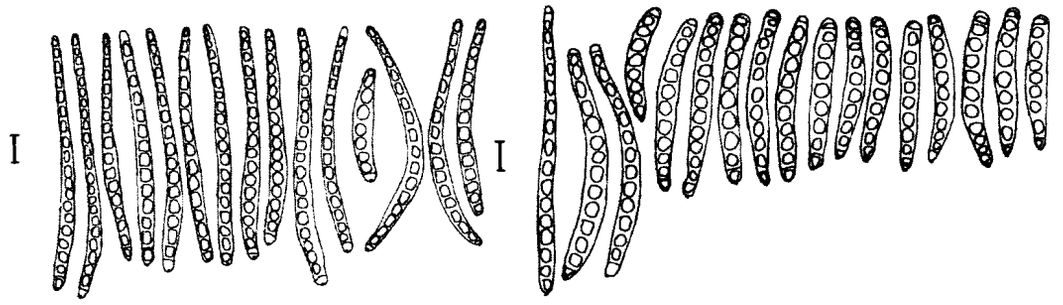
Glucose day 10; E=2.24

**Figure 14: Line drawings of *E. longirostratum* PPRI 6529 spores formed under different cultural conditions. Scale bar = 10 µm; average E=7.82**



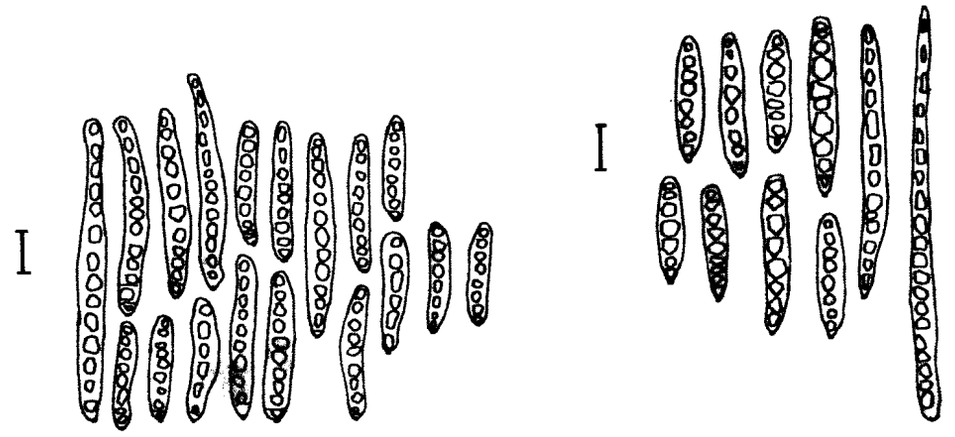
Ceres day 10; E=4.9

Ceres day 5; E=8.38



WA day 5; E=12.41

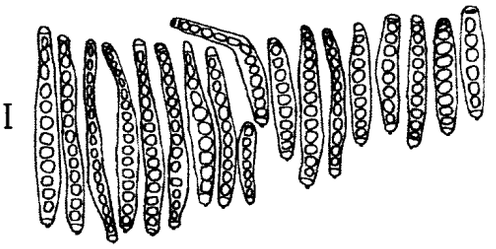
WA day 10; E=8.38



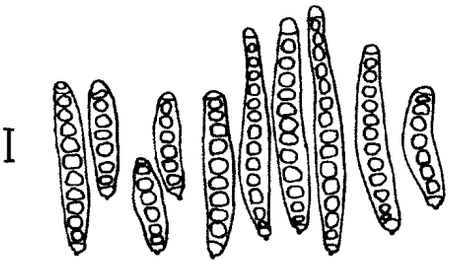
Sorbitol day 10; E=6.2

Glucose day 10; E=6.65

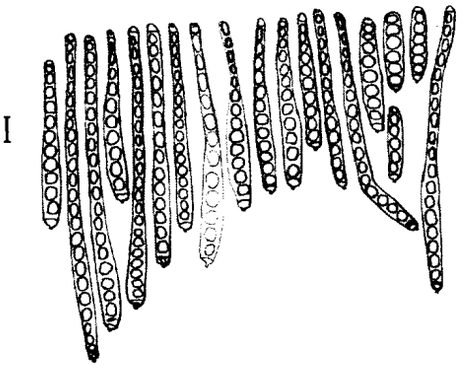
**Figure 15: Line drawings of *E. rostratum* PPRI 6530 spores formed under different cultural conditions. Scale bar = 10 µm; average E=7.95.**

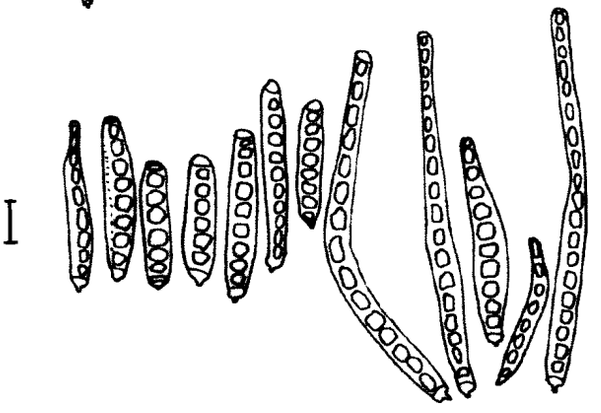


Ceres day 5; E=5.24



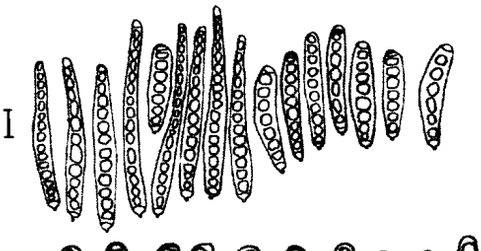
Ceres day 10; E=7.12

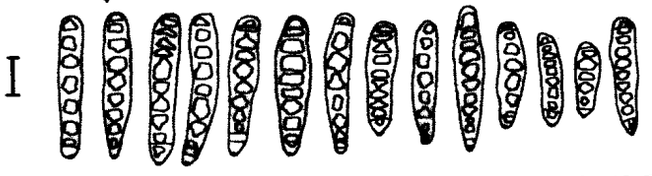




Sach day 5; E=12.29

Sach day 10; E=9.37

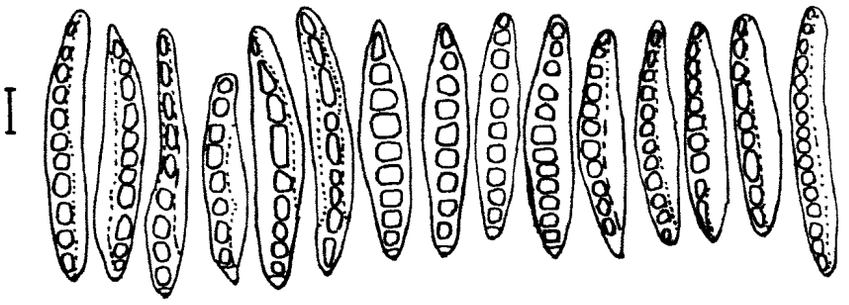




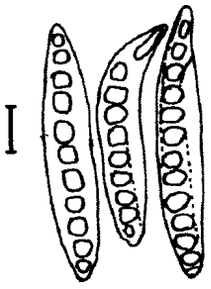
WA day 10; E=13.73

Glucose day 10; E=4.7

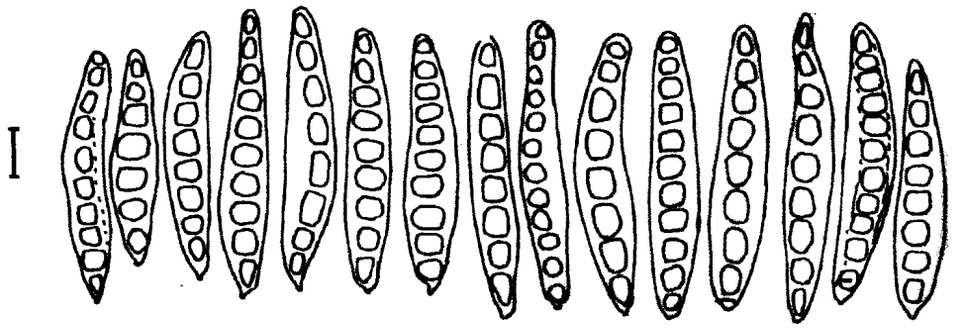
**Figure 16: Line drawings of *E. turcicum* PPRI 6606 spores formed under different cultural conditions. Scale bar = 10 µm. average E=5.44**



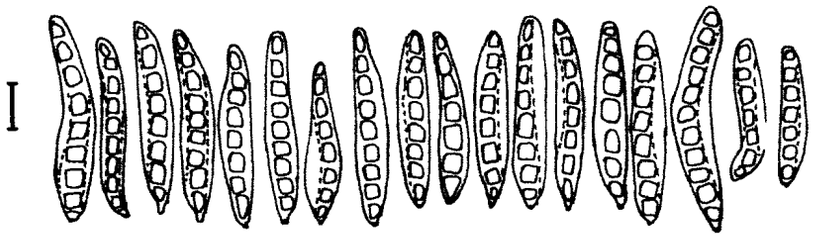
Ceres day 5; E= 5.51



Sach day 5; E= 5.50



Ceres day 10; E= 5.08



PCA day 10; E= 5.69

# Appendix G: RESULTS OF CULTURE-BASED TESTS

**Table 12: Group separation table using DT and culture-based tests 5, 7, 10, 15, 18, 21 and 25.**

| | Total | A | C | D | F | G | J | K | L | M | N | Accuracy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 4 | 4 | | | | | | | | | | 100% |
| **B** | 2 | 2 | | | | | | | | | | 0% |
| **C** | 3 | | 3 | | | | | | | | | 100% |
| **D** | 6 | | | 4 | 1 | | | | | | | 66,6% |
| **E** | 6 | | | 1 | | | 2 | 2 | | | 1 | 0% |
| **F** | 3 | | | | 3 | | | | | | | 100% |
| **G** | 5 | 1 | | 1 | | 2 | | | | | 1 | 40% |
| **H** | 5 | | | | | | | 1 | | | 4 | 0% |
| **I** | 4 | | | | | 1 | 2 | 1 | | | | 0% |
| **J** | 7 | | | | 1 | | 4 | | 1 | | 1 | 57,1% |
| **K** | 6 | | | | | | | 5 | | | 1 | 83,3% |
| **L** | 5 | | | | 1 | | | | 4 | | | 80% |
| **M** | 5 | | | | | | | | | 4 | 1 | 80% |
| **N** | 6 | | | | | | | | | | 6 | 100% |

Total accuracy: 44/65 = 67,7%

**A** = *B. cynodontis;* **B** = *B. ellisii;* **C** = *B. indica;* **D** = *B. maydis;* **E** = *B. papendorfii;* ***F*** = *B. setariae;* ***G*** = *C. affinis;* ***H*** = *C. cymbopogonis;* ***I*** = *C. fallax;* ***J*** = *C. lunata;* ***K*** = *E. inaequale;* ***L*** = *E. longirostratum;* ***M*** = *E. rostratum;* ***N*** = *E. turcicum.*

**Table 13: Group separation table using FOIL and culture-based tests 5, 7, 10, 15, 18, 21 and 25.**

| | Total | A | B | C | D | E | F | G | H | I | J | K | L | M | N | Accuracy |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 4 | 4 | | | | | | | | | | | | | | 100% |
| **B** | 2 | 1 | 1 | | | | | | | | | | | | | 50% |
| **C** | 3 | | | 3 | | | | | | | | | | | | 100% |
| **D** | 6 | | | | 4 | | | | | | | 1 | | | 1 | 66,7% |
| **E** | 6 | | | | | 6 | | | | | | | | | | 100% |
| **F** | 3 | | | | | | 3 | | | | | | | | | 100% |
| **G** | 5 | | 1 | | | | | 3 | | | | | | | 1 | 60% |
| **H** | 5 | | | | | 1 | | | 4 | | | | | | | 80% |
| **I** | 4 | | | | | | | | | 3 | 1 | | | | | 75% |
| **J** | 7 | | 1 | | | 1 | | | | | 5 | | | | | 71,4% |
| **K** | 6 | | | | | 1 | | | | | | 5 | | | | 83,3% |
| **L** | 5 | | | | | | | | 1 | | | | 4 | | | 80% |
| **M** | 5 | | | | | 1 | | | | | | | | 4 | | 80% |
| **N** | 6 | 1 | | | | | | | 1 | | | | | | 4 | 66,7% |

Total accuracy: 54/65 = 83%

**A** = *B. cynodontis;* **B** = *B. ellisii;* **C** = *B. indica;* **D** = *B. maydis;* **E** = *B. papendorfii;* ***F*** = *B. setariae;* ***G*** = *C. affinis;* ***H*** = *C. cymbopogonis;* ***I*** = *C. fallax;* ***J*** = *C. lunata;* ***K*** = *E. inaequale;* ***L*** = *E. longirostratum;* ***M*** = *E. rostratum;* ***N*** = *E. turcicum.*

**Table 14: Rules training set of FOIL for culture based test results.**

IF a04Day5Min>61.5, a23Day6Min>63.5 THEN Organism=*E_rostratum*
IF a04Day6Max>79.0, a15Day7Min>70.0 THEN Organism=*E_longirostratum*
IF a18Day6Min<=43.0, a31Day1Max>3.75 THEN Organism=*B_indica*
IF a15Day5Max>63.5, a31Day1Max>3.75 THEN Organism=*B_setariae*
IF a23Day8Max<=61.5 THEN Organism=*E_turcicum*
IF a23Day9Min<=75.5 THEN Organism=*B_cynodontis*
IF a08Day9Min<=55.5 THEN Organism=*B_papendorfii*
IF a18Day9Min<=83.0 THEN Organism=*C_cymbopogonis*
IF a15Day10Min>72.5 THEN Organism=*B_papendorfii*
IF a18Day3Min<=19.5 THEN Organism=*E_turcicum*
IF a08Day9Min<=66.5, a31Day1Max>3.75 THEN Organism=*E_inaequale*
IF a15Day2Min>23.5, a31Day4Max<=43.5 THEN Organism=*C_lunata*
IF a15Day8Min>72.0, a23Day4Max>39.5 THEN Organism=*C_affinis*
IF a04Day1Min>0.37, a31Day1Max<=7.25 THEN Organism=*B_maydis*
IF a15Day5Max<=42.5 THEN Organism=*E_inaequale*
IF a23Day1Min<=8.75 THEN Organism=*B_cynodontis*
IF a15Day3Min>32.5 THEN Organism=*C_fallax*
IF a23Day2Max<=19.5 THEN Organism=*C_lunata*
IF a13Day3Min>11.5 THEN Organism=*C_cymbopogonis*
IF a31Day5Max>45.0, a31Day2Max>15.5 THEN Organism=*B_papendorfii*
IF a31Day1Max>3.75 THEN Organism=*B_ellisii*
IF THEN Organism=*B_maydis*

# Appendix H: SEQUENCING AND ISSR RESULTS

**Figure 17: Homology tree derived from 28S rRNA encoding gene sequences.**

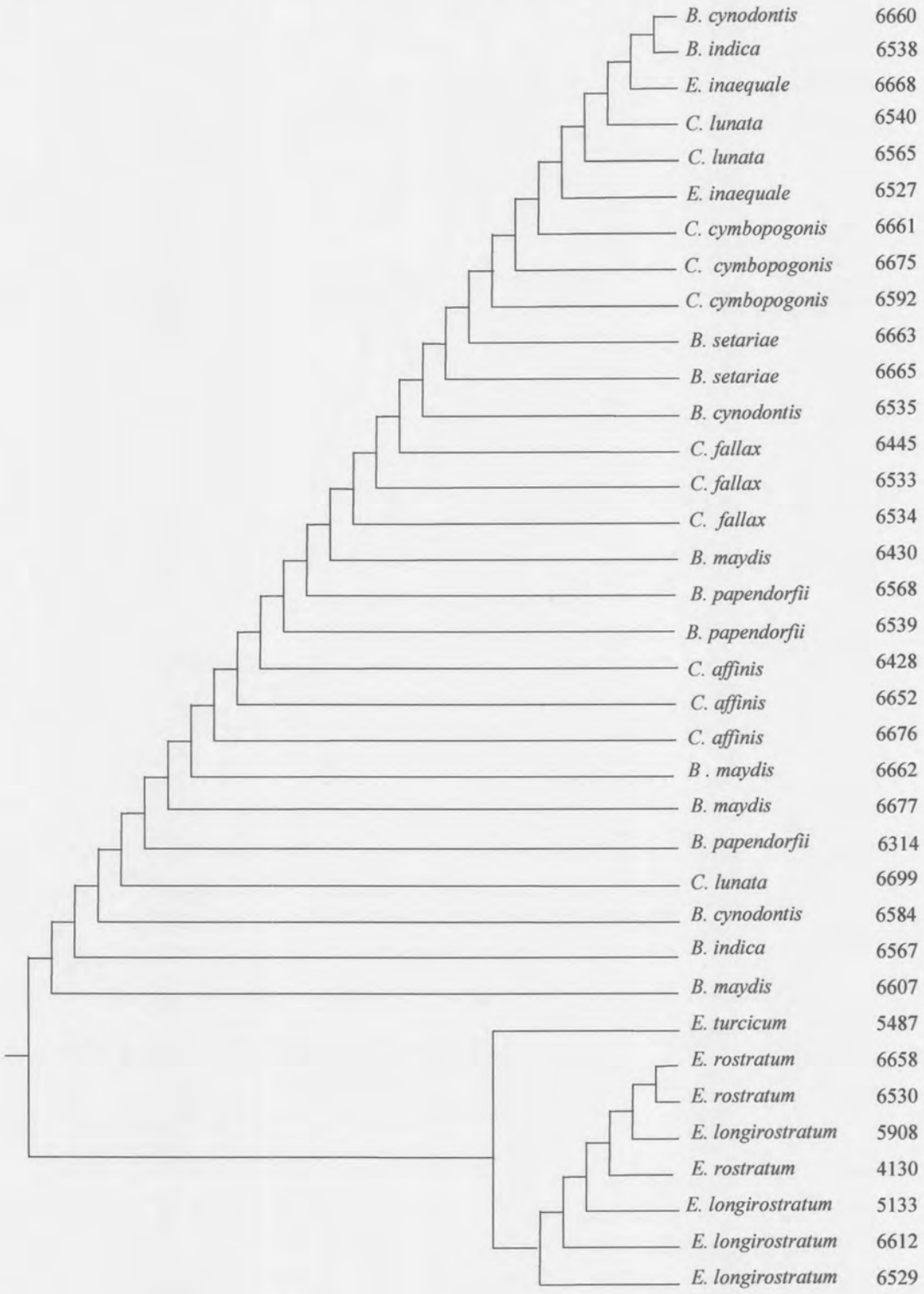
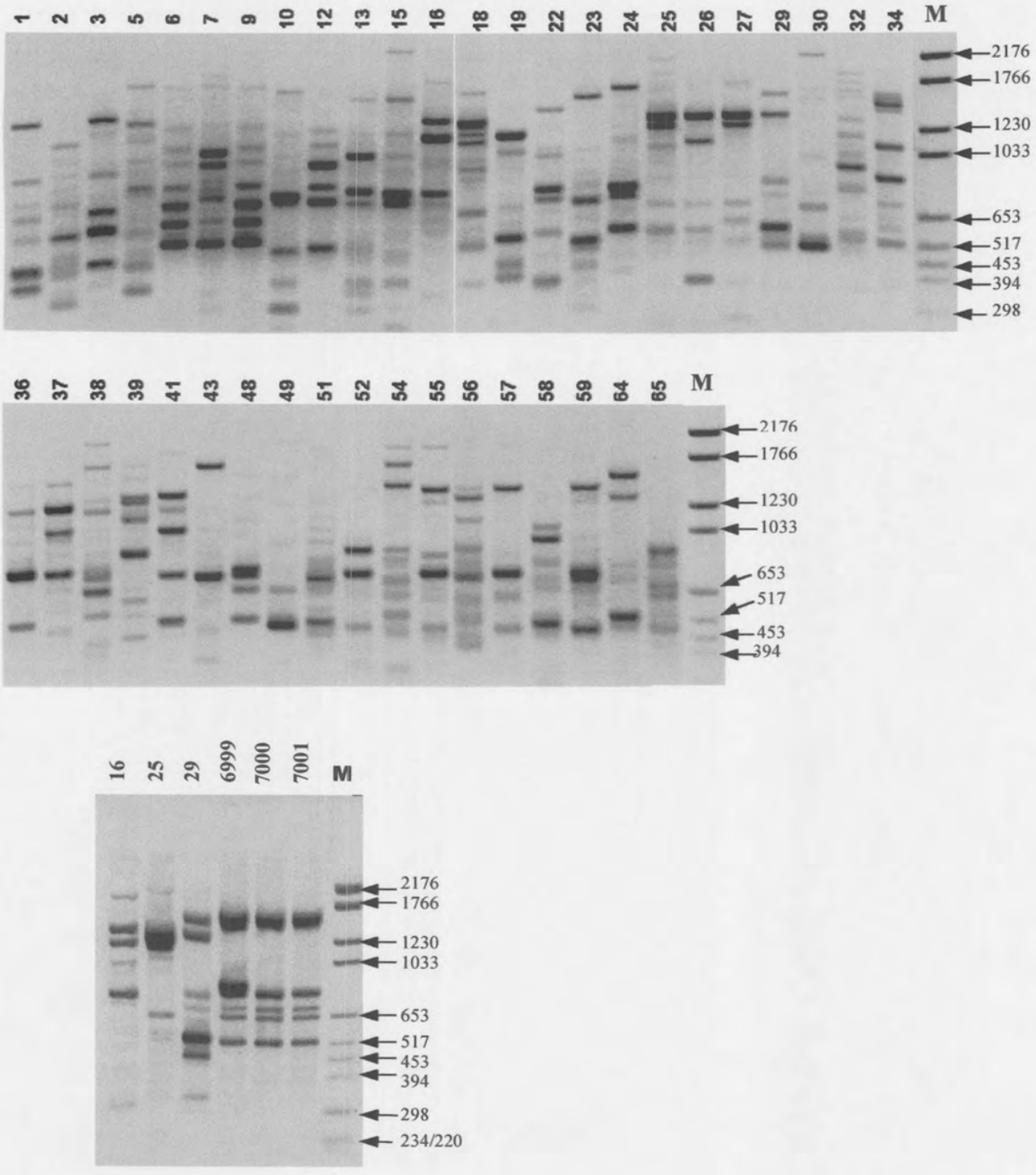
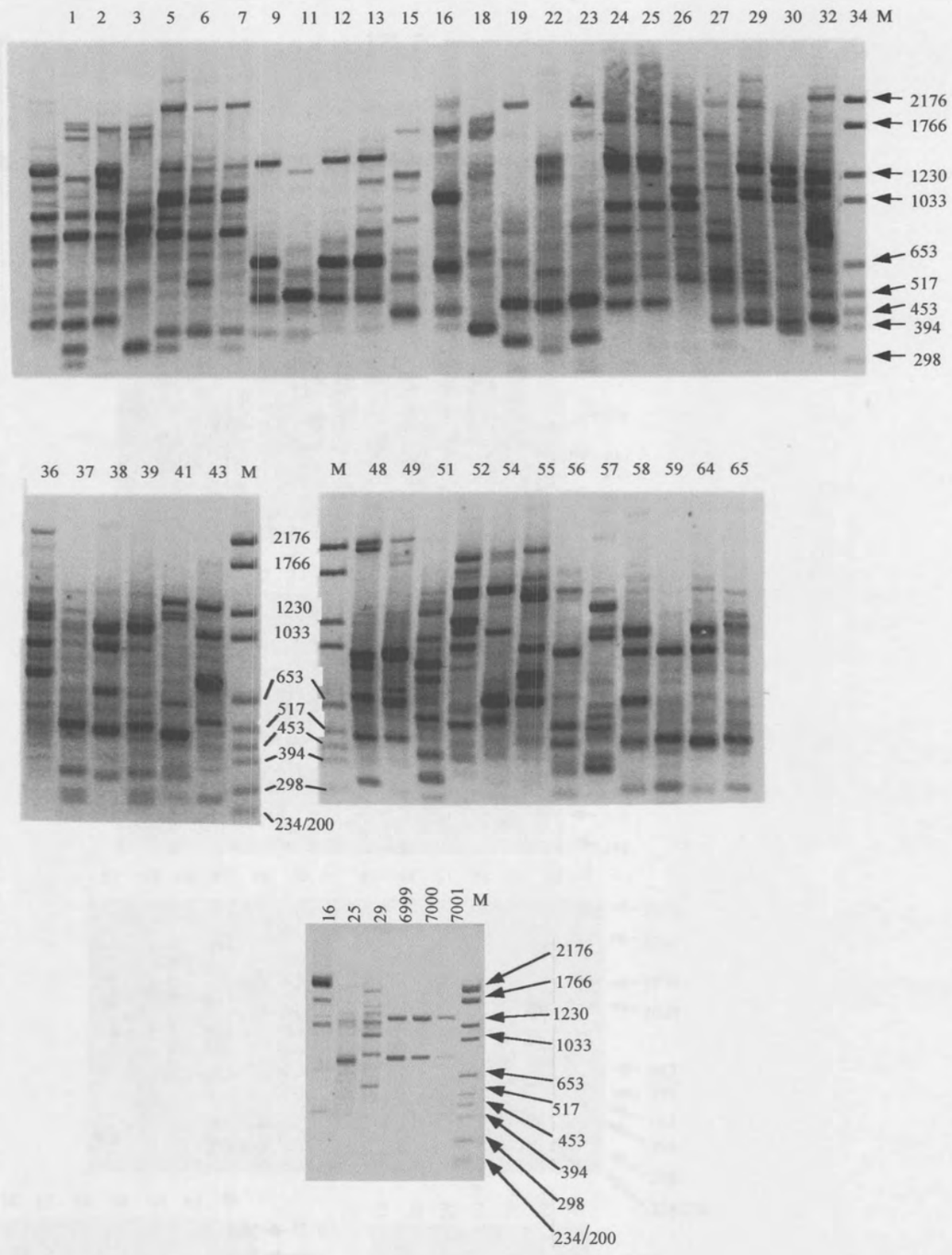


**Figure 18: Agarose gel image of ISSR bands using the primer DBD-$(AC)_7$.**



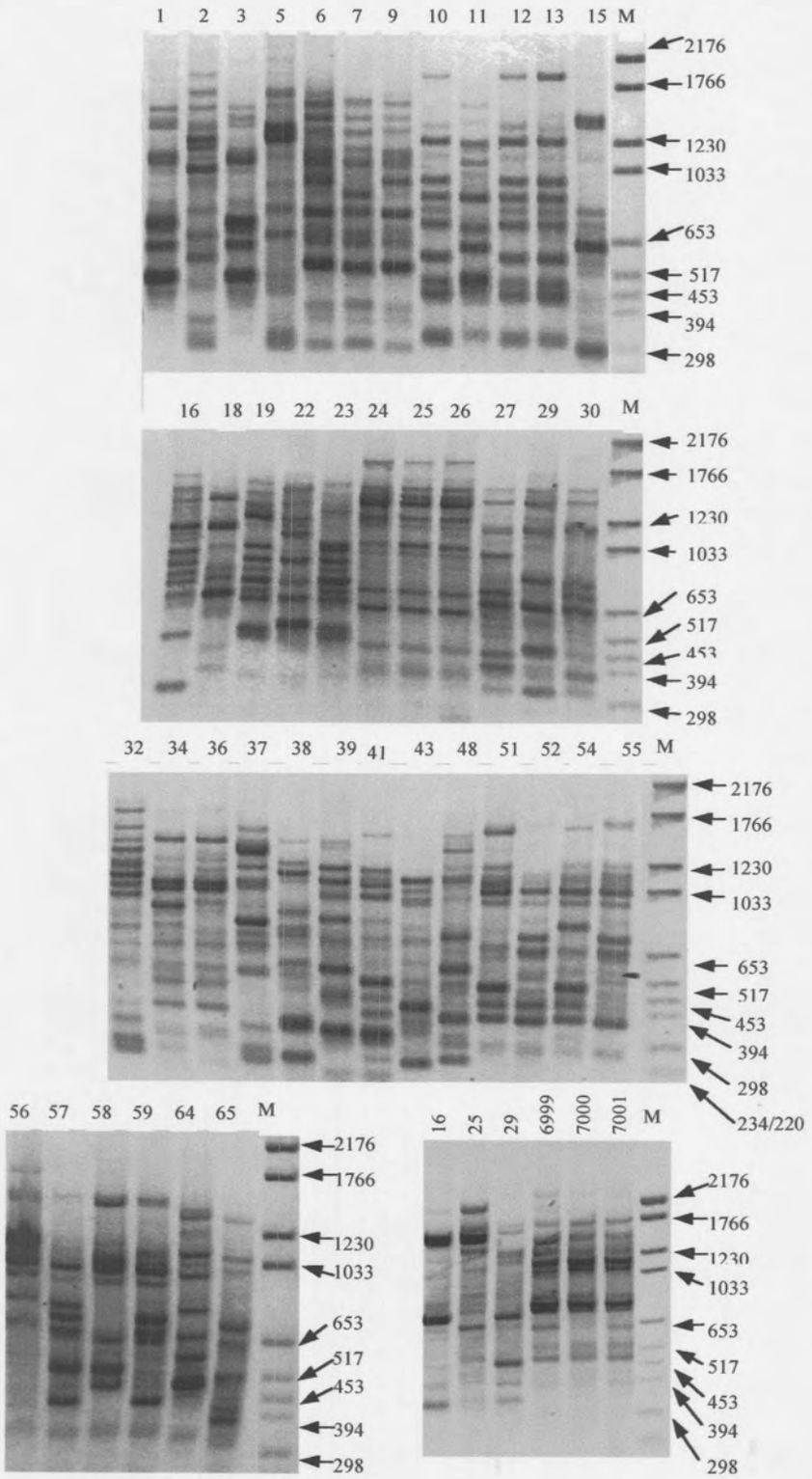

**M**=Molecular weight marker in base pairs

**Figure 19: Agarose gel image of ISSR bands using the primer BDB-$(CAC)_5$.**



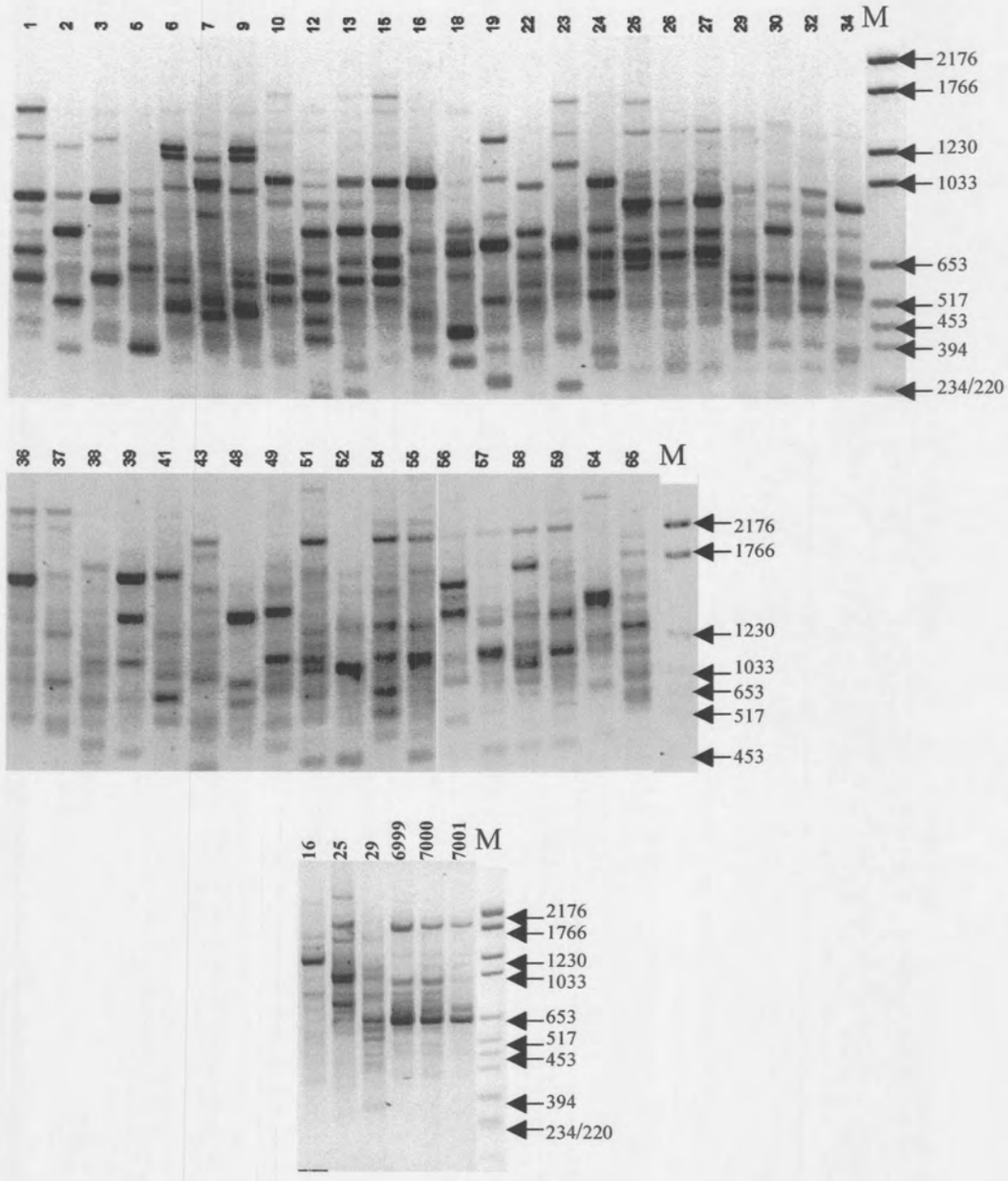

**M**=Molecular weight marker in base pairs

**Figure 20: Agarose gel image of ISSR bands using the primer DHB- $(CGA)_5$**




**M**=Molecular weight marker in base pairs

**Figure 21: Agarose gel image of ISSR bands using the primer VHV-$(GT)_7$-G.**




**M**=Molecular weight marker in base pairs

**Figure 22: Dendrogram using the DICE coefficient illustrating similarity between selected *Bipolaris*-like strains using ISSR fingerprints obtained with the primer BDB-$(CAC)_5$.**

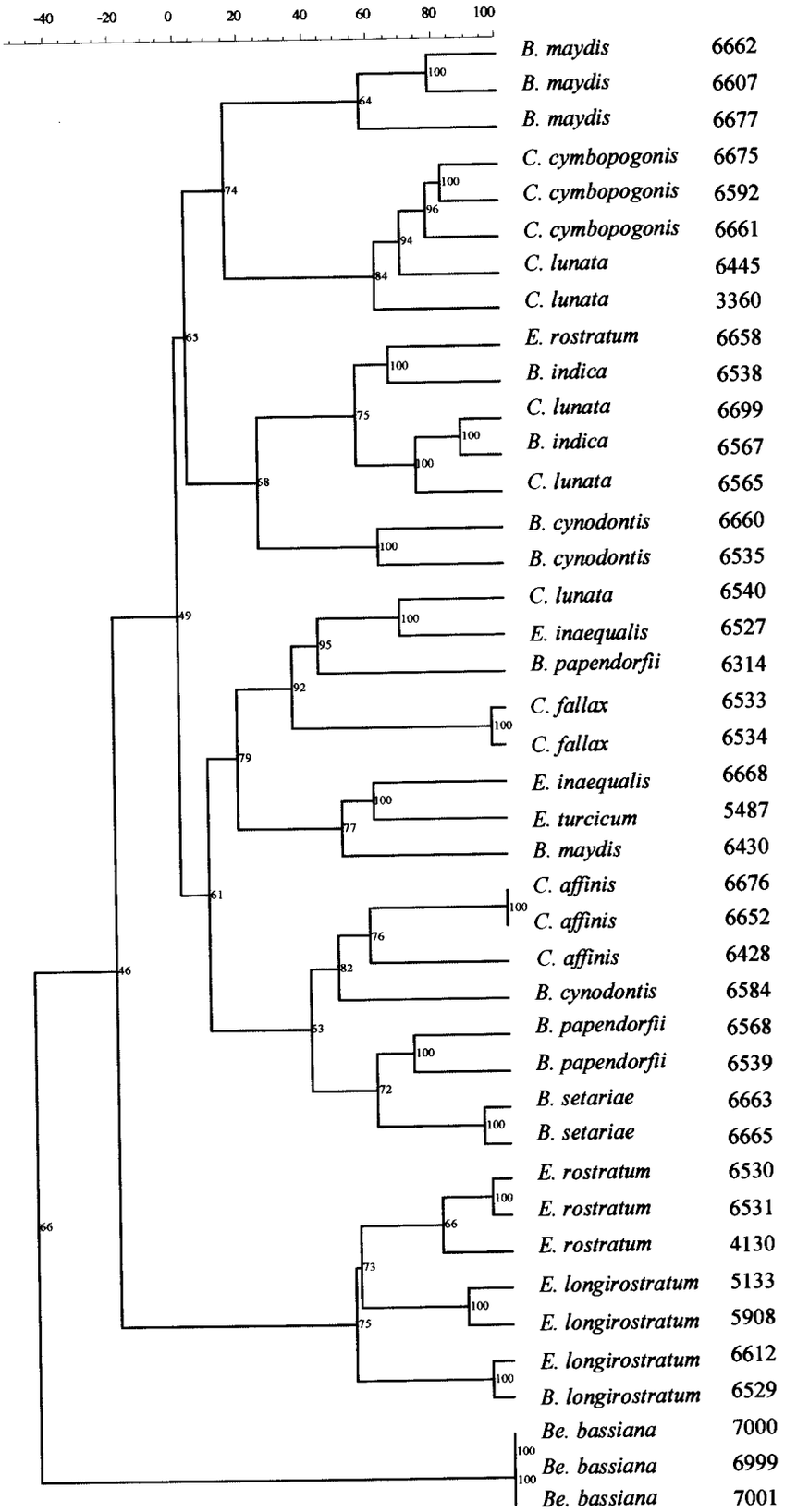


**Figure 23: Dendrogram using the Pearson's correlation coefficient illustrating similarity between selected *Bipolaris*-like strains using ISSR fingerprints obtained with the primer BDB-$(CAC)_5$.**

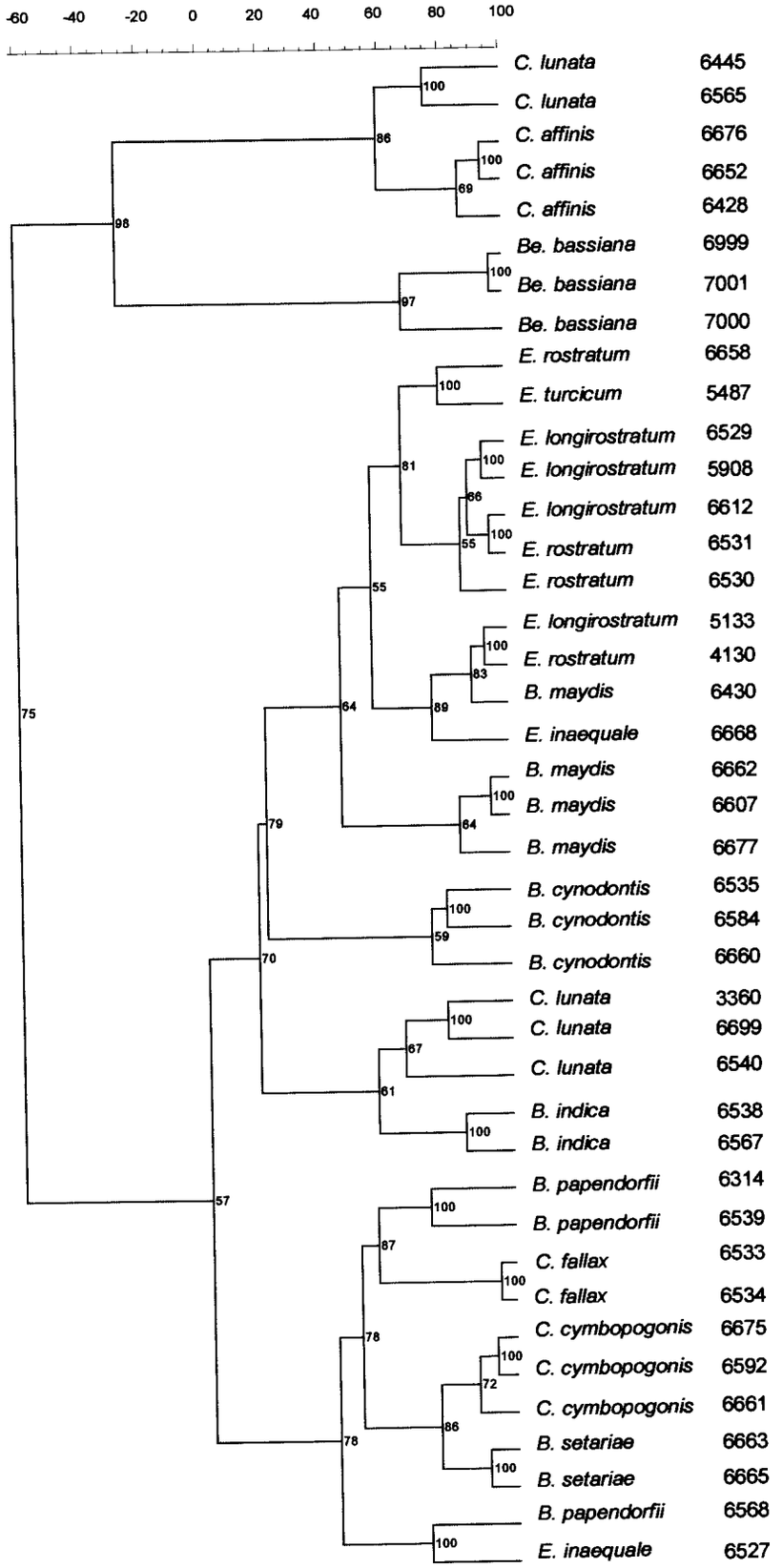


**Figure 24: Dendrogram illustrating similarity between *Bipolaris*-like strains using ISSR fingerprints of all four primers calculated with Pearson's correlation coefficient**

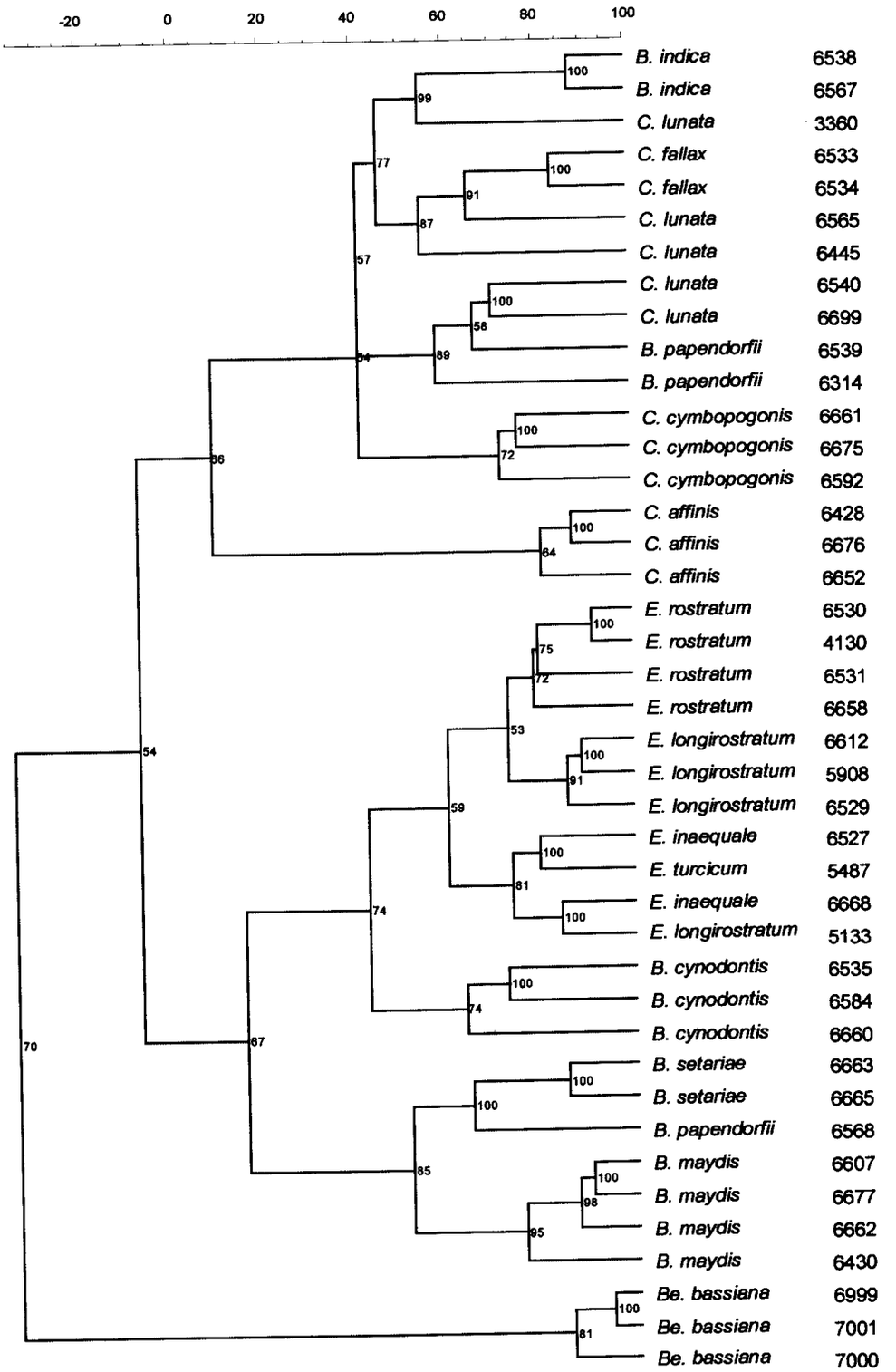


**Figure 25: Dendrogram illustrating similarity between *Bipolaris*-like strains using ISSR fingerprints of all four primers calculated with the Dice coefficient.**

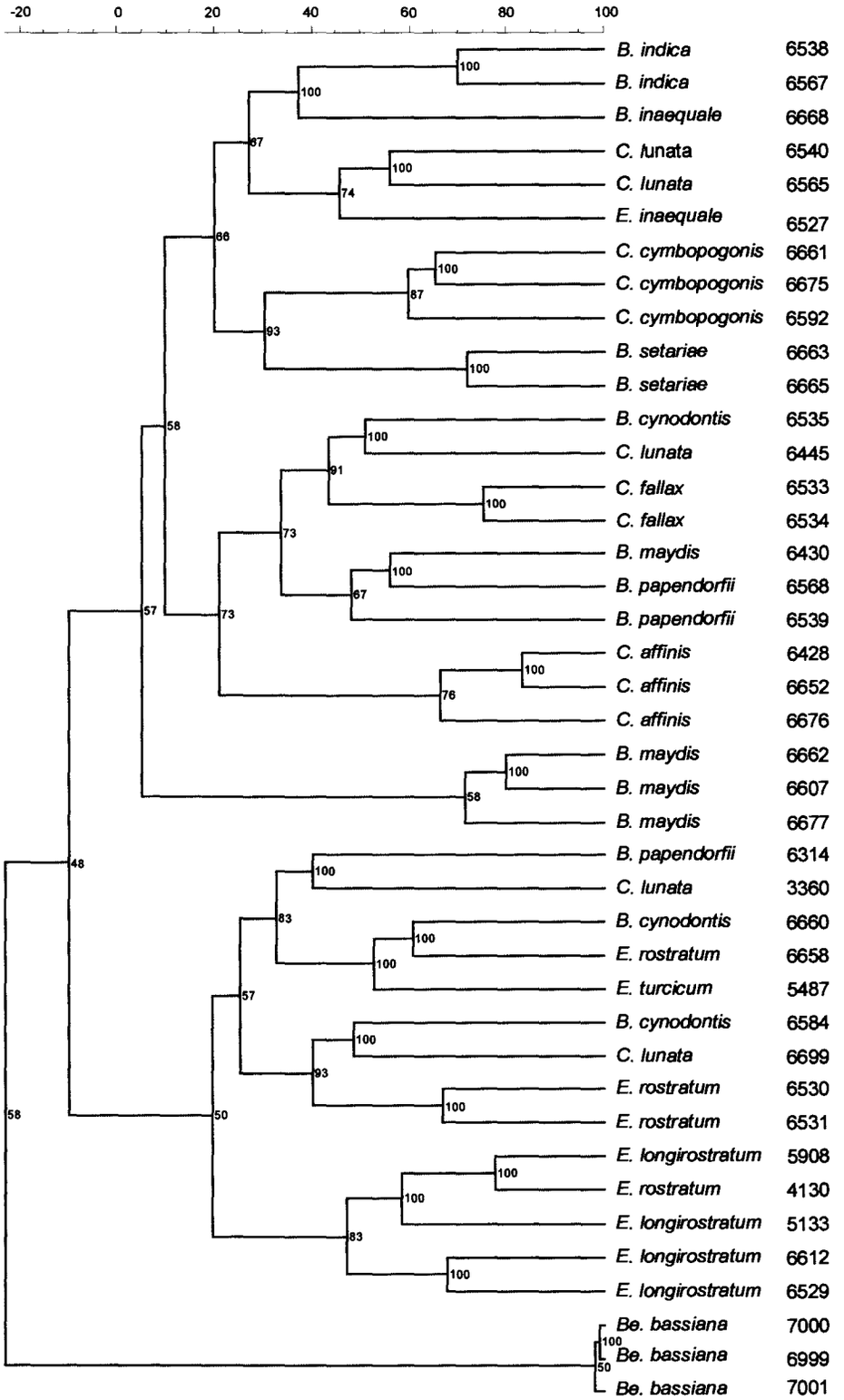


**Table 15: Group separation table of average similarity using banding patterns of all ISSR, Dice similarity coefficient and Ward clustering (Empty cells = 0).**

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 100.00 | | | | | | | | | | | | | |
| **B** | | | | | | | | | 33.33 | | | | 33.33 | 33.33 |
| **C** | | | 100.00 | | | | | | | | | | | |
| **D** | | | 25.00 | 50.00 | 25.00 | | | | 25.00 | | | | | |
| **E** | 33.33 | 33.33 | 33.33 | | | | | | | | | | | |
| **F** | | | | | | 50.00 | | | | | | | | 50.00 |
| **G** | | | 33.33 | | | 33.33 | 33.33 | | | | | | | |
| **H** | | | 100.00 | | | | | | | | | | | |
| **I** | | | | | | | | | 100.00 | | | | | |
| **J** | 20.00 | 20.00 | 20.00 | | 20.00 | | | | | | 20.00 | | | |
| **K** | | | | | | | | | 50.00 | | 50.00 | | | |
| **L** | | 25.00 | | | | | 25.00 | | 50.00 | | | | | |
| **M** | | 25.00 | | | | | | | | | | 50.00 | | 25.00 |
| **N** | | | | | | | | | | | | | | 100.00 |

**A** = *Be. bassiana*; **B** = *B. cynodontis*; **C** = *B. indica*; **D** = *B. maydis*; **E** = *B. papendorfii*; **F** = *B. setariae*; **G** = *C. affinis*; **H** = *C. cymbopoginis*; **I** = *C. fallax*; **J** = *C. lunata*; **K** = *E. inaequale*; **L** = *E. longirostratum*; **M** = *E. rostratum*; **N** = *E. turcicum*.

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**Table 16: Group separation table of maximum similarity using banding patterns of all ISSR, Dice similarity coefficient and Ward clustering (Empty cells = 0).**

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 100.00 | | | | | | | | | | | | | |
| **B** | | | | | 33.33 | | | | | | | | 33.33 | 33.33 |
| **C** | | | 100.00 | | | | | | | | | | | |
| **D** | | | 25.00 | 75.00 | | | | | | | | | | |
| **E** | | | 33.33 | | | | | | 33.33 | | | | 33.33 | |
| **F** | | | | | | 50.00 | | | | | | | | 50.00 |
| **G** | | | | | | | 33.33 | | | | | 33.33 | 33.33 | |
| **H** | | | 33.33 | | | | | | | 33.33 | | 33.33 | | |
| **I** | | | | | | | | | 50.00 | | | 50.00 | | |
| **J** | | | 20.00 | | | | | 20.00 | | 20.00 | | 40.00 | | |
| **K** | | | | | | | | | | 50.00 | | | 50.00 | |
| **L** | | 25.00 | | | | | | | 25.00 | | | | 50.00 | |
| **M** | | 75.00 | | | | | | | | | | 25.00 | | |
| **N** | | 100.00 | | | | | | | | | | | | |

**A** = *Be. bassiana*; **B** = *B. cynodontis*; **C** = *B. indica*; **D** = *B. maydis*; **E** = *B. papendorfii*; **F** = *B. setariae*; **G** = *C. affinis*; **H** = *C. cymbopoginis*; **I** = *C. fallax*; **J** = *C. lunata*; **K** = *E. inaequale*; **L** = *E. longirostratum*; **M** = *E. rostratum*; **N** = *E. turcicum*

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**Table 17: Group separation table of average similarity using banding patterns of all ISSR, Pearson correlation coefficient and Ward clustering (Empty cells = 0).**

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 100.00 | | | | | | | | | | | | | |
| **B** | | | | | | | | | | | 33.33 | | 33.33 | 33.33 |
| **C** | | | 100.00 | | | | | | | | | | | |
| **D** | | | 25.00 | 75.00 | | | | | | | | | | |
| **E** | | 33.33 | | | 33.33 | | 33.33 | | | | | | | |
| **F** | | | | | | 100.00 | | | | | | | | |
| **G** | | | | | | | 100.00 | | | | | | | |
| **H** | | | 100.00 | | | | | | | | | | | |
| **I** | | | | | | | | | 50.00 | | | | 50.00 | |
| **J** | 20.00 | | | | 20.00 | | | | 20.00 | | | 20.00 | | 20.00 |
| **K** | | | | | | | | | | | | | | 100.00 |
| **L** | | | | | | | | | | | | | 25.00 | 75.00 |
| **M** | | | | | | | | | | | | 25.00 | 25.00 | 50.00 |
| **N** | | | | | | | | | | | | | | 100.00 |

**A** = *Be. bassiana*; **B** = *B. cynodontis*; **C** = *B. indica*; **D** = *B. maydis*; **E** = *B. papendorfii*; **F** = *B. setariae*; **G** = *C. affinis*; **H** = *C. cymbopoginis*; **I** = *C. fallax*; **J** = *C. lunata*; **K** = *E. inaequale*; **L** = *E. longirostratum*; **M** = *E. rostratum*; **N** = *E. turcicum*

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**Table 18: Group separation table of maximum similarity using banding patterns of all ISSR, Pearson correlation coefficient and Ward clustering (Empty cells = 0).**

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 100.00 | | | | | | | | | | | | | |
| **B** | | | | | | | | | | | | | 66.67 | 33.33 |
| **C** | | | 100.00 | | | | | | | | | | | |
| **D** | | | 25.00 | 75.00 | | | | | | | | | | |
| **E** | | 33.33 | | | 66.67 | | | | | | | | | |
| **F** | | | | 50.00 | | 50.00 | | | | | | | | |
| **G** | | | | | | | 100.00 | | | | | | | |
| **H** | | | 100.00 | | | | | | | | | | | |
| **I** | | | | | | | | | | 50.00 | | 50.00 | | |
| **J** | 20.00 | | | 20.00 | | | | | 20.00 | 20.00 | 20.00 | | | |
| **K** | | | | | | | | | | | | 100.00 | | |
| **L** | | | | | | | | | | | 25.00 | | 50.00 | 25.00 |
| **M** | | | | | | | | | | | | 75.00 | 25.00 | |
| **N** | | | | | | | | | | | | 100.00 | | |

**A** = *Be. bassiana*; **B** = *B. cynodontis*; **C** = *B. indica*; **D** = *B. maydis*; **E** = *B. papendorfii*; **F** = *B. setariae*; **G** = *C. affinis*; **H** = *C. cymbopoginis*; **I** = *C. fallax*; **J** = *C. lunata*; **K** = *E. inaequale*; **L** = *E. longirostratum*; **M** = *E. rostratum*; **N** = *E. turcicum*

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