Comparative study of *Epicoccum sorghinum* in Southern Africa

by

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Declaration

I, the undersigned, declare that the thesis/dissertation, which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own independent work and has not previously been submitted by me for any degree at this or any other tertiary institution.

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PREFACE

The Coelomycetous genus *Phoma* comprises over 200 true species and has a complex taxonomic background. This genus was regarded as the morphological 'dumping ground' for fungi that produced pycnidia with aseptate conidia and as a result, thousands of species were previously described in this genus. *Phoma* has only been revised in the past thirty years with extensive phylogenetic work done in the 2000s. A prime example of the taxonomic complexities associated with this genus is found in the rich taxonomic history of one of its members, *Phoma sorghina*, which is now known as *Epicoccum sorghinum*. The taxonomy of this genus as well as the significance of *E. sorghinum* is discussed in Chapter 1.

Epicoccum sorghinum was recently moved from the genus *Phoma* to *Epicoccum* when it was warranted based on phylogenetic and morphological evidence. *Epicoccum sorghinum* is described as a plant pathogen, a human pathogen, as well as a phytotoxin and mycotoxin producer (discussed in Chapter 1). Southern African isolates have been isolated from many different substrates and were found to produce different metabolites. These isolates are studied further in Chapter 2 where it is determined whether *E. sorghinum* from Southern Africa represents one ubiquitous species or multiple taxa.

It was suggested before that it is important to follow a polyphasic approach in stabilizing the taxonomy of a genus. Morphology is not an accurate identification method as morphological features are often lacking *in vitro,* or overlapping morphological features are often present in *Phoma.* In Chapter 3, attempts were therefore made to find phenotypic characters that will allow differentiation among *E. sorghinum* groups found in Chapter 2. This was done by testing the carbon source utilization capabilities of various

isolates as this method previously proved successful in distinguishing between other fungal species.

Chapter 1

A review on the complex history of *Phoma* section *Peyronellaea* with special reference to *Epicoccum sorghinum*

ABSTRACT

The Coelomycetous genus, *Phoma*, is defined as filamentous fungi that produce pycnidial conidiomata with monophialidic, doliiform to flask-shaped conidiogenous cells. Host specificity was regarded as an important characteristic in identifying *Phoma* and this Saccardoan system, together with only minor differences in morphological characteristics between species, led to the description of a high number of species with no true taxonomic relevance. Species were extensively revised by Boerema and co-authors in 2004 and reduced to 223 taxa divided into nine sections, although not all species were considered. Experience was still required to accurately differentiate between species. *Phoma* section *Peyronellaea* was characterised by alternarioid dictyochlamydospores, epicoccoid shaped chlamydospores and/or unicellular chlamydospores that looked like pseudosclerotia. This section was later dissolved and the genus *Peyronellaea* re-instated. *Phoma sorghina* belonged to this section, and has a worldwide distribution. It is considered as a weak secondary parasite of plants that produce metabolites such as mycotoxins, phytotoxins and anthraquinones. Since its first description in 1878 by Saccardo as *Phyllosticta sorghina* until 1973, when it was named *Phoma sorghina,* it has been renamed numerous times based on morphological characteristics. It was moved to *Epicoccum* based on phylogenetic and morphological characteristics in 2010. The aim of this review is to discuss the complexity of the taxonomic challenges in the genus, *Phoma*, with special reference to *Epicoccum sorghinum*. In addition, an attempt is also made to demonstrate the importance of *E. sorghinum* as a plant pathogen and the threat it poses to human health.

INTRODUCTION

Fungal species belonging to the genus, *Phoma*, are among the most widespread fungi in the world and occurs in many ecological niches (Montel*, et al.*, 1991). This genus contains approximately 200 true species with relatively simple morphological features (van der Aa*, et al.*, 1990). *Phoma* species have been indicated as plant pathogens (some occurring on economically important crops), human and animal pathogens, mycotoxin producers, degraders of organic materials, pathogens of nematodes and arthropods, parasites of other fungi and oomycetes, as well as bio-control agents (Boerema*, et al.*, 2004, Aveskamp*, et al.*, 2008). Due to the fact that there are only a few distinguishable morphological characteristics among species of *Phoma*, only skilled persons who are familiar with this group of fungi usually have the ability to correctly identify these species.

One of the major issues surrounding this genus is that its taxonomy is not yet clearly resolved. Many of the taxa have been synonymized over the years, for example, Boerema and co-workers (de Gruyter*, et al.*, 2009) attempted to rectify taxonomic discrepancies of the past. Unfortunately, not all species were revised and, therefore, it is difficult to accurately place new species within the genus or determine whether newly discovered ones are truly new.

The aim of this review is to discuss the complexity of the taxonomic challenges in the genus, *Phoma*, with special reference to *Epicoccum sorghinum*, which was, until recently, still identified as *Phoma sorghina*. In addition, an attempt is also made to demonstrate the importance of *E. sorghinum* as a plant pathogen and the threat it poses to human health in order to illustrate why it is important to identify *Phoma* species accurately.

1. THE GENUS *PHOMA*

Saccardo first described the Coelomycetous genus, *Phoma*, in 1880 (Robert, *et al.*, 2005). This genus is currently defined as "filamentous fungi that produce pycnidial conidiomata with monophialidic, doliiform to flask-shaped conidiogenous cells" (Aveskamp, *et al.*, 2008). Approximately 2800 *Phoma* species have been described since the establishment of this genus (Boerema, *et al.*, 2004), using Saccardoan concepts (i.e. concepts introduced in the 1870's by the Italian mycologist Saccardo) that are based mainly on restricted morphological characteristics and host specificity (van der Aa, *et al.*, 1990). Accordingly, fungal species that produce pycnidia with aseptate hyaline conidia were placed in the genus, *Phoma*, when the fungus was isolated from the stem of a plant, and when the fungus grew on the leaves of a plant it was classified as a *Phyllosticta* species (van der Aa, *et al.*, 1990). Fungi with similar characteristics, but with one-septated hyaline conidia, were placed in the genus *Ascochyta* (van der Aa, *et al.*, 1990). At the time, host specificity was regarded as an important characteristic in the classification of these fungi (Sutton, 1980). This, together with minor differences in morphological characteristics, has led to the description of a number of species that had no true taxonomic relevance (Sutton, 1980). Boerema, *et al*., (1965) stated that 'chaos is bound to arise if form-species are based on minor differences only' and indicated that this applied to many *Phoma* species.

Boerema, *et al.* (2004) have extensively studied the taxonomy of *Phoma*. Instead of looking at host specificity, *in vitro* characteristics such as pigmentation, chlamydospore formation, pycnidial features, conidial characteristics and crystal formation were considered and the amount of species was reduced to 223 taxa. In this book it was indicated that the genus, *Phoma*, can be divided into nine sections including *Phoma, Heterospora, Paraphoma,*

Peyronellaea, Phyllostictoides, Sclerophomella, Plenodomus, Macrospora, and *Pilosa* (Boerema*, et al.*, 2004). The different sections are linked to a variety of teleomorphs that include species in *Didymella, Leptosphaeria, Mycosphaerella* and *Pleospora* (Boerema*, et al.*, 2004).

Although *Phoma* species can be divided into distinct sections according to distinguishing characteristics, not all species were considered by Boerema, *et al.,* (2004), and many species from the southern hemisphere were not revised. Furthermore, many of the characteristics of species in different sections are still very similar to each other and a good understanding and experience working with these fungi is still needed to accurately differentiate between species (Aveskamp*, et al.*, 2008).

2. *PHOMA* SECTION *PEYRONELLAEA*

In 1946 the genus, *Peyronellaea*, was proposed by Giodanich (Boerema*, et al.*, 1965). This genus was established to accommodate *Phoma*-like species that produce multicellular chlamydospores. These structures were later renamed dictyochlamydospores by Luedemann (1959) and resembled dictyospores of the genus *Alternaria* (Boerema*, et al.*, 1965, Reddy*, et al.*, 1998). Togliani validly published *Peyronellaea* as a genus in 1952 (Reddy*, et al.*, 1998) with the basionym of *Peyronellaea glomerata* as the type species (Boerema*, et al.*, 1965).

In 1965, it was suggested that *Peyronellaea* should not be a separate genus, but rather a section within the genus, *Phoma* (Boerema*, et al.*, 1965). The reason stated was that the type species of both *Peyronellaea* and *Phoma* resemble each other very closely and can only be

distinguished by differences in their spore morphology and the occurrence of dictyochlamydospores in the genus, *Peyronellaea*. Furthermore, it was mentioned that the ability of strains to produce dictyochlamydospores could be lost in culture and that the production of pycnidia and dictyochlamydospores are dependent on culture medium. Because of this, it was accepted that *Peyronellaea* should be transferred to *Phoma* and regarded as a section within the latter genus instead (Boerema, *et al.*, 1965, Reddy, *et al.*, 1998). The section was referred to as the 'Peyronellaea group' until 1990 when it was formally indicated as one of five sections in *Phoma* (van der Aa, *et al.*, 1990), and in 2004 another four sections were added (Boerema, *et al.*, 2004). *Phoma* section *Peyronellaea* was represented by species that had one or more of the following characteristics (Boerema, *et al.*, 2004): alternarioid dictyochlamydospores, epicoccoid shaped chlamydospores and/or unicellular chlamydospores that look like pseudosclerotia.

In the *Peyronellaea* section, two different asexual forms occur where pycnidia or chlamydospores (as described above) are produced. These two forms rarely occur together and mostly one or the other is formed, depending on the conditions under which growth takes place (Boerema, *et al.*, 2004). The formation of these structures is also dependent on the ratio between the availability of nitrogen and carbon in the substrate.

Although the characteristics to distinguish *Phoma* section *Peyronellaea* from the other sections seemed clear, misidentification is still possible. For instance, two species in *Phoma* section *Heterospora* (*P. clematidina* and *P. narcissi*) produce typical spores characteristic of this section, but also produce alternarioid multicellular chlamydospores that suggests an association with *Peyronellaea* (Boerema, *et al.*, 2004). The pycnidia and conidia in *Phoma* section *Peyronellaea* also resemble those of other sections in *Phoma.* For instance, the one-celled conidia produced by the members of section *Peyronellaea* are indistinguishable from

species in section *Phoma*, two-celled conidia occasionally produced are similar to species within section *Phyllostictoides,* and species in section *Sclerophomella* also resemble some of the species that produce epicoccoid structures (Boerema*, et al.*, 2004). Therefore, in cases where only pycnidia are produced, members of section *Peyronellaea* could easily be misidentified. Two examples are the fungi previously named *Phoma pimprina* and *Phoma sorghina*. Both produce botryoid-alternarioid multicellular chlamydospores, but are differentiated by the broad conidia of *P. pimprina* and variable conidial shapes of *P. sorghina*. If however, only chlamydospore formation is observed with the lack of pycnidial development, misidentification based on morphological characteristics could take place (Boerema*, et al.*, 2004).

In 2010, an extensive morphological and DNA-based phylogenetic analysis of the Didymellaceae was done (Aveskamp*, et al.*, 2010). As a result *Phoma* section *Peyronellaea* was dissolved and the genus *Peyronellaea* reinstated. *Phoma pimprina* and *P. sorghina* were moved to the genus *Epicoccum* and is now known as *Epicoccum pimprina* and *E. sorghinum.*

3. *EPICOCCUM SORGHINUM*

Although *Epicoccum sorghinum* is not regarded to be of major economic significance, it has a remarkable historical and taxonomical background. This ubiquitous fungus (Boerema*, et al.*, 1973) occurs in tropical and subtropical regions (Boerema*, et al.*, 1977) and has a worldwide distribution (Boerema*, et al.*, 2004). It is not only a weak secondary parasite of plants, but is also known to produce various metabolites that can act as mycotoxins (Rabie*, et al.*, 1975, Steyn & Rabie, 1976), phytotoxins (Venkatasubbaiah*, et al.*, 1992) and

anthraquinones (de Souza Borges & Pupo, 2006). Furthermore, the fungus was placed and discussed under various different names since Saccardo initially described it in 1878.

3.1 TAXONOMIC BACKGROUND OF *EPICOCCUM SORGHINUM*

This fungus was initially known as *Phoma sorghina* since Boerema, Dorenbosch and Van Kesteren formally named it in 1973. It was initially renamed *Epicoccum sorghi* based on phylogenetic and morphological characteristics by Aveskamp, *et al.*, in 2010. Since then, this name was indicated as an orthographic variant of *Epicoccum sorghinum*, which is the current accepted name (Robert, *et al.*, 2005). Cultures used as reference strains in the 1973 study included two isolates from sorghum, one isolate from sugarcane and two from wheat, but a type strain was not indicated. In later references (Boerema, 1993, Boerema, *et al.*, 2004), CBS 284.74, deposited at the Centraalbureau voor Schimmelcultures (CBS), was indicated as reference strain. This strain, however, was incorrectly indicated (personal verbal communication with M.M. Aveskamp, CBS) as reference strain.

Index Fungorum (Roskov, *et al.*, 2014) indicates that the accepted name for *E. sorghinum* is *Leptosphaeria sacchari* Breda de Haan 1892. This is incorrect as the basionym of this fungus is *Phyllosticta sorghina* Sacc. (Saccardo, 1878). *Leptosphaeria sacchari* is in fact a completely different fungus and is distinguished from *E. sorghinum* by much larger conidia. In a study on ringspot disease of sugarcane, from which one of the reference strains used by Boerema in 1973 was taken, the author clearly made a distinction between these two fungi (Bourne, 1934)

All the uncertainty in identifying this fungus in the past, as well as numerous name changes that are discussed elsewhere in this document, has resulted in confusion in the literature and a number of synonyms (TABLE 1) for this fungus. Originally, *Epicoccum sorghinum* was described as *Phyllosticta sorghina* by Saccardo when he isolated it from *Sorghum vulgare* (Saccardo, 1878). The fungus underwent a number of name changes before it was named *Phoma sorghina* by Boerema, *et al.* in 1973. As a result its morphology, distribution, hosts and nomenclature are discussed under different taxonomical names in literature (Boerema, *et al.*, 1968, Boerema, *et al.*, 1971, Punithalingam & Holliday, 1972, Boerema, *et al.*, 1973).

Boerema, *et al.,* described *Epicoccum sorghinum* in 1968 as *Phoma indianensis*. In the description, the authors used pycnidia and the red pigment produced by this fungus to distinguish it from other species in *Phoma* section *Peyronellaea*. The authors also suggested that the basionym is *Peyronellaea indianensis* and various plant hosts were associated with this fungus.

In 1971, it was noted that the neotype of *Phoma glumarum* and the holotypes of *Phyllosticta glumarum* and *Phyllosticta glumicola* are identical to pycnidia of *Phoma indianensis* that occurred on rice seed (Boerema, *et al.*, 1971). Therefore, it was suggested that *Phoma glumarum* Ell. & Tracy is the correct name and that *Phyllosticta glumarum*, described in 1888 by Tracy is in fact the basionym (Boerema, *et al.*, 1973). In the former study, *Phoma chartae* was also synonymised with *P. glumarum* as the type material for this species was missing, although notes on the fungus indicated the characteristic reddish pigment.

In 1973, the epithet was changed from '*glumarum*' to '*sorghina*' as the name *Phoma glumarum* was misapplied (Boerema, *et al.*, 1973). This was due to the fact that the authors chose another isolate as neotype as they were under the impression that the original material

did not exist. Furthermore, the original type material for *P. glumarum* represented a species of *Coniothyrium* and not *Phoma* (Boerema, *et al.*, 1973). Therefore, *P. sorghina* was applied as the name with *Phyllosticta sorghina* Sacc. as basionym.

No type strain is indicated in literature for *E. sorghinum*. The closest is a culture deposited at the American Type Culture Collection as ATCC 12115, the type for *Phyllosticta sorghina,* which only produce sterile mycelia. This culture was mentioned in the 1973 paper by Boerema, *et. al.*, and was previously deposited as CBS 288.35 but is no longer available. In 1971, Kuznetzova isolated a strain described as *Peyronellaea stemphylioides* (synonym of *E. sorghinum*) from *Populus nigra* in Novosibirsk. From 1977 it has been used as reference strain for *Phoma sorghina* in literature, although the reason for this is not clear (Boerema, *et al.*, 1977). According to Mycobank (Robert, *et al.*, 2005), the teleomorph of *E. sorghinum* is *Mycosphaerella holci* Tehon. However, no cultural studies have been done to date to support this (Boerema, *et al.*, 2004).

3.2 MORPHOLOGICAL CHARACTERISTICS OF *EPICOCCUM SORGHINUM*

Although *Epicoccum sorghinum* was previously indicated as a member of *Phoma* with many easily recognizable characteristics, it was not always clearly documented or mentioned (White & Morgan-Jones, 1983). This could be attributed to the fact that strains of species in the previously known *Phoma* section *Peyronellaea* often lose their ability to produce multicellular chlamydospores (Boerema, *et al.*, 2004) and that the formation of chlamydospores and pycnidia depends on the nitrogen-carbon ratio of the media. Unfortunately, this leaves the researcher with a lot of uncertainty regarding the

morphological characteristics of *E. sorghinum*. Regarding the description of this fungus in literature the words "mostly", "occasionally", "usually", "highly variable" and "often" are used with all the descriptions of the various characteristics. Therefore, it should be noted that the characteristics discussed below vary in culture and the 'easily recognizable' and 'stable diagnostic characteristics' (White & Morgan-Jones, 1983) are not always present.

The anamorphic fungus is recognized by its distinct red pigment that discolour the medium and forms exudate droplets (White & Morgan-Jones, 1983, Boerema*, et al.*, 2004) which have been identified as anthraquinones (Boerema*, et al.*, 1977). Another distinct characteristic is the mycelium colour, which often have greyish green or whitish to salmon patches and the colonies also often appear tufted (White & Morgan-Jones, 1983, Boerema*, et al.*, 2004).

The conidia of *E. sorghinum* are variable in shape and could be ovoid-ellipsiodal or curved (Boerema*, et al.*, 2004). The pycnidia are subglobose in shape and have long distinct straight necks, but could also be curved. *Epicoccum sorghinum* forms single-celled or alternaria-like chlamydospores that are highly irregular and variable (Boerema*, et al.*, 1965, Boerema*, et al.*, 2004). They mostly occur in intercalary positions (Boerema*, et al.*, 1977), but could also occur terminal-lateral (Boerema*, et al.*, 2004). Sutton (1980) suggested that the chlamydospores are not necessary for identification as the cultural characteristics are sufficiently diagnostic. This could, however, be the cause of misidentification as other *Phoma* species also have the pink colony appearance (e.g. *P. exigua* and *P. minutispora*) (Boerema*, et al.*, 2004). Therefore, the additional characteristics such as pycnidial shape, conidial shape and size, as well as the chlamydospores should be regarded as important, although these characteristics are not always well defined.

3.3 THE IMPACT OF PHYLOGENETICS ON *PHOMA* IDENTIFICATION

In the 1990s, the need to develop methods other than morphology to accurately identify Coelomycetes, especially *Phoma*, was realised and chemical, as well as biological characteristics were suggested as identification tools (van der Aa*, et al.*, 1990, Montel*, et al.*, 1991). These methods were not sufficient when distinguishing between certain species of *Phoma* and it was, therefore, suggested that another method for defining species was necessary (Grondona*, et al.*, 1997).

The first phylogenetic approach to identify *Phoma* species was by Reddy, *et al.* (1998) who used ITS-1 (the first internal transcribed spacer region of the ribosomal RNA cistron) to evaluate a few species in *Phoma* section *Plenodemus,* as well as *Phoma* section *Peyronellaea* and other species with *Leptosphaeria* and *Didymella* teleomorphs. It was also suggested that more work should be done to examine phylogenetic relationships within *Phoma* and to validate whether *Peyronellaea* is a valid section (Reddy*, et al.*, 1998). Torres, *et al.* (2005) also stressed the importance of phylogenetics in the identification of species of *Phoma* and suggested that phylogenetic analysis would improve the taxonomy of this genus. However, a problem that had to be overcome was that up to one fifth of *Phoma* sequences submitted to Genbank and other databases were attributed to incorrect species names (e.g., *P. herbarum*, which is the type species of *Phoma*, was represented by nucleotide sequences of more than one taxon) (Aveskamp*, et al.*, 2008). Another issue was to select correct markers to use in the classification of the genus (Aveskamp*, et al.*, 2008).

These issues were dealt with since 2009 when many authors started to build a base for the classification of the genus *Phoma* by making use of markers such as the small subunit and

large subunit of the nuclear ribosomal RNA genes (SSU and LSU respectively), the internal transcribed spacer regions (ITS), part of the actin gene (ACT) and part of the β-tubulin gene (Aveskamp*, et al.*, 2009, de Gruyter*, et al.*, 2009, Irinyi*, et al.*, 2009, de Gruyter*, et al.*, 2010). Irinyi and Erzsébet (2007) also suggested the large intron of the translation elongation factor gene (TEF) as marker, as it was found that the ITS region is not useful in *Phoma* to infer phylogenetic relationships. Therefore, TEF was included in a study on *P. sojicola* and *P. pinodella* (Irinyi*, et al.*, 2009) but this marker was not used in further studies.

Aveskamp, *et al.* (2009) did the first extensive study on *Phoma* section *Peyronellaea* using phylogenetics together with morphology for classification purposes. At the time of the study, *E. sorghinum* was still recognized as *P. sorghina* and was classified as part of *Phoma* section *Peyronellaea* although other species morphologically classified in this section by Boerema (Boerema*, et al.*, 2004) did not form part of the *Peyronellaea* group when a phylogenetic comparison was done. This section was therefore found to be polyphyletic in nature.

In 2010, an extensive phylogenetic and morphological study was done on the family Didymellaceae (Aveskamp*, et al.*, 2010) in which 159 species of *Phoma* were included. Here, the Boeremaean *Phoma* section *Peyronellaea* was completely re-evaluated. Due to the highly significant phylogenetic support for this group, the section was elevated to genus status and called *Peyronellaea.* This genus included most of the previously known *Phoma* section *Peyronellaea* species, as well as other species that clustered together in this group. Furthermore, *Peyronellaea* species are now morphologically identified by either having unicellular or multicellular alternarioid dictyochlamydospore formations (Aveskamp*, et al.*, 2010). Three species (*Phoma sorghina, P. pimprina* and *Epicoccum nigrum*), previously part of *Phoma* section *Peyronellaea,* were phylogenetically separated from the other *Peyronellaea* species in this study. These species also produced botryoid or epicoccoid chlamydospores

and were therefore moved to a separate genus. This genus was named *Epicoccum* because it is an older generic name than *Phoma* (Aveskamp, *et al.*, 2010), which in turn brought about the most recent name change for the fungus *Epicoccum sorghinum* since 1973 (Boerema, *et al.*, 1973).

Although *E. sorghinum* has been properly described previously, a number of studies have found that there was genetic variation within the species (Aveskamp, *et al.*, 2009). In the first study that looked specifically at genetic variation within *E. sorghinum*, only the two Internal Transcribed Spacer (ITS1 and ITS2) and the 5.8S ribosomal RNA (rRNA) were used (Pažoutová, 2009) and although limited variation was observed, the authors could also not confirm whether this fungus is only one species or whether it possibly represents more than one species. It was suggested that more studies should be conducted to confirm genetic variation or speciation (Aveskamp, *et al.*, 2010).

3.4 DISTRIBUTION AND PLANT HOSTS OF *EPICOCCUM SORGHINUM*

Epicoccum sorghinum occurs worldwide (Boerema, *et al.*, 1965, Boerema, *et al.*, 2004), mainly in tropical and subtropical regions (White & Morgan-Jones, 1983, Boerema, *et al.*, 2004), but also occurs in temperate regions and on plants in glasshouses (Boerema, *et al.*, 2004). Countries where occurrence was reported include Argentina, Brazil, China, France, Ghana, India, Japan, Madagascar, Mali, Namibia, Netherlands, Nigeria, Philippines, South-Africa, Sri Lanka, Tanganyika, Thailand, Turkey, Uganda and USA (Boerema, *et al.*, 1965, Boerema, *et al.*, 1968, Zainum & Parbery, 1974, Rabie, *et al.*, 1975, do Amaral, *et al.*, 2004, Sert & Sümbül, 2005).

This ubiquitous soil- and seed-borne fungus (White & Morgan-Jones, 1983, Boerema, *et al.*, 2004) is regarded as a weak parasite of weakened plants and has been isolated from about 80 different host plant genera, as well as other substrates such as cattle feed and poultry feed (Boerema, *et al.*, 1977, Boerema, *et al.*, 2004). Although it occurs on all kinds of plants and substrates (Boerema, *et al.*, 1973), it is mostly associated with Poaceae such as sorghum and millet (Boerema, *et al.*, 1973, White & Morgan-Jones, 1983).

The importance of *E. sorghinum* as a plant pathogen is not yet determined, but it is known to be associated with many plant diseases. These include leaf spot on dicot crop plants (Venkatasubbaiah, *et al.*, 1992), ring spot disease on sugar cane (Zainum & Parbery, 1974), *Phaeosphaeria* leaf spot (PLS) of maize (do Amaral, *et al.*, 2004) and leaf spot on rice (Boerema, *et al.*, 1965), as well as leaf spot on *Trifolium campestre* (Sert & Sümbül, 2005). The fungus is also a problem on certain seed where it reduces germination (Boerema, *et al.*, 1965) and causes post-emerging death of seedlings (Boerema, *et al.*, 1977). Infected seed will normally develop poorly or not at all, and are dark in colour (Boerema, *et al.*, 1965). Other reported diseases include root rot, dying-off (Boerema, *et al.*, 2004), glume blotch (Boerema, *et al.*, 1965) and glume blight (Boerema, *et al.*, 1965, Venkatasubbaiah, *et al.*, 1992, Boerema, *et al.*, 2004) of various plant hosts.

3.5 METABOLITES

The role of *Phoma* species in the production of toxins that affect human, animal and plant life is not yet fully understood. Because of their generally poor ability to cause food spoilage and disease, these fungi are not regarded economically- or health-wise of great importance.

However, there are certain reports that indicate toxin production by *Phoma* species and also by *E. sorghinum* (Rabie, *et al.*, 1975, Steyn & Rabie, 1976).

3.5.1 PHYTOTOXINS

In 1992 it was reported that *E. sorghinum* could produce phytotoxins such as epoxydon, desoxyepoxydon, phyllostine, 6-methyl-salicylic acid, epoxydon-6-methylsalicylate ester and diphenylether (Venkatasubbaiah, *et al.*, 1992). All of these phytotoxins are biosynthetically related and show broad spectrum toxicity to plants (Venkatasubbaiah, *et al.*, 1992). Although the occurrence of these toxins were not reported elsewhere, it is interesting to note that when tested, the toxins produced necrotic lesions similar to leaf spot symptoms (Venkatasubbaiah, *et al.*, 1992) that were reported by other authors. Examples include *Phaeosphaeria* leaf spot of maize in Brazil (do Amaral, *et al.*, 2004), ring spot diseases of sugarcane reported by Zainum and Parberry (1974) and leaf spot on pokeweed in the USA (Venkatasubbaiah, *et al.*, 1992). There seems thus to be a strong correlation between the disease symptoms experienced in the field and production of these phytotoxins.

Epicoccum sorghinum is not unique in producing phytotoxins. These compounds are also produced by fungal species in genera such as *Penicillium, Phoma, Phyllosticta, Ascochyta* and *Staganospora* (Venkatasubbaiah, *et al.*, 1992). It was also found that phyllostine is an intermediate in the production of the mycotoxin, patulin, which is produced by *Penicillium* species (Wilson & Nuovo, 1973). However, to date, no patulin production by *E. sorghinum* has been reported.

3.5.2 ANTHRAQUINONES

Anthraquinones are yellowish, grey or grey-green organic solids that are used in industry as dyes, in paper pulp production, and as laxatives. De Souza Borges and Pupo (2006), isolated *E. sorghinum* from *Tithonia diversifolia* (Mexican sunflower) and found it to produce anthraquinone derivatives (TABLE 2). These authors also obtained an isolate from *Dendryphiella* species that produced the toxin, dendryol, which is phytotoxigenic to barnyard grass. Anthraquinones, therefore, can also have phytotoxigenic properties.

3.5.3 TENUAZONIC ACID

Tenuazonic acid is a mycotoxin that inhibits protein synthesis in patients that ingest the toxin (Davis, *et al.*, 1977) and leads to growth disorders. This compound also has phytotoxic, cytotoxic, antibiotic and anti-tumour properties (Gitterman, 1965, Lebrun, *et al.*, 1988).

In 1976, Steyn and Rabie isolated magnesium and calcium tenuazonate from *E. sorghinum* cultures isolated from sorghum in Sekukuniland in Southern Africa. Venkatasubbaiah was also aware of the isolation of these toxic constituents from *E. sorghinum* and also tested for tenuazonates in their study on isolates from the USA, but the results were negative (Venkatasubbaiah, *et al.*, 1992).

3.5.4 MYCOTOXIN X

The production of mycotoxin X (possible causal agent of onyalai) is a property that makes *E. sorghinum* occurring in Africa unique. In 1975, Rabie, *et al.,* made a crucial connection between *E. sorghinum* and the disease onyalai. Since the first description of this disease in 1904 (Hesseling, 1992), it was surrounded by mystery and this link could bring researchers one step closer to discovering the true cause of this disease.

Onyalai can be described as an acute purpuric disease closely related to idiopathic thrombocytopenic purpura (ITP) (Wilkinson, 1953a), but with characteristic haemorrhagic bulla in the mouth and under the patients' feet (Rabie*, et al.*, 1975). The disease symptoms are accompanied by a shortened blood platelet lifespan and once patients are submitted to hospitals, they will normally recover within two weeks if they do not succumb to the disease. Onyalai will rarely reoccur in patients and was not found to be infectious or heritable. Patients are normally well-nourished and healthy except for the disease symptoms (Lurie, *et al.,* 1969, Rabie, *et al.,* 1975).

This disease is endemic to Africa. Outside of Africa, only two cases were reported in American patients and one in a Turkish patient (Harris*, et al.*, 1972, Özsoylu & Ertürk, 1991). Over the years many suggestions as to what causes the disease were made such as vitamin deficiencies, plant poisoning, malaria, witchcraft, syphilis, bilharzia, hookworm and malnutrition, but all were refuted (Wilkinson, 1953b, Hesseling, 1992).

It was previously noted that onyalai incidences are higher in areas where millet is ingested as staple diet. When Steyn and Rabie (1976) isolated fungi from millet and sorghum from Ovamboland, *E. sorghinum* occurred in almost all the samples and was isolated together with

three other toxic isolates: *Phoma jolyana, Fusarium fusarioides* and another *Fusarium* species. When fed with these samples, New Hampshire day-old chickens showed haemorrhagic symptoms on the third day and all were dead by the seventh day. Next, the *E. sorghinum* isolates were fed to forty male Wistar-derived rats and all but one died after twenty nine days. These rats also showed the symptoms related to onyalai. From these results the authors deduced that *E. sorghinum* is the causal agent of onyalai. The mycotoxin involved is still unknown.

3.6 *EPICOCCUM SORGHINUM* AS HUMAN PATHOGEN

Epicoccum sorghinum not only produces metabolites that are detrimental to human health, but is also reported to infect and cause erythematous lesions on the skin (Rai, 1989). However, *E. sorghinum* is not the only *Phoma* species that can act as human pathogen. Thirteen cases of *Phoma* infections involving the skin has been reported (Rishi & Font, 2003). Most of the *Phoma* infections were reported in immuno-compromised patients and it is indicated that the infections occur mainly on exposed areas such as the hands. This suggests that the fungus is introduced into the skin by abrasion followed by minor inoculations (Rishi & Font, 2003).

4. CONCLUSIONS

Epicoccum sorghinum is exceptional in that it has multiple plant hosts, produces phytotoxins, anthraquinones, mycotoxins and cause lesions on human skin. It also has a worldwide occurrence and has been associated with many different plant diseases. Based on its history (and even today) the taxonomy of this species is somewhat confusing. The large number of synonyms for this fungus also indicates that many plant diseases were probably either wrongly attributed to another fungal agent or could possibly be wrongly attributed to *E. sorghinum.* Yet, with all of these diseases and toxins attributed to this fungus, its economic importance is still not determined.

The work by Aveskamp, *et al.* (2010), forms a strong phylogenetic basis on which to identify isolates belonging to the Didymellaceae, but the research on this family is not complete. For instance, the morphological and phylogenetic variation within *E. sorghinum* mentioned here has not been dealt with, which leaves room for further investigation. It is clear that morphology alone is not sufficient to clearly identify species within this group and papers such as those by Aveskamp and de Gruyter (Aveskamp*, et al.*, 2009, Aveskamp*, et al.*, 2010, de Gruyter*, et al.*, 2010) indicate a way forward in resolving the taxonomic uncertainty and chaos of the form genus, *Phoma,* by applying molecular techniques.

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TABLE 1. Synonyms of *Epicoccum sorghinum*

Synonym	Described in journal	Reference
Ascochyta arachidis Woron	Notul. Syst. Inst. Crytog. Horti bot. petropol. 3:31. 1924	Boerema, *et al*., 2004
Epicoccum sorghi (Sacc.) Aveskamp, Gruyter & Verkley	Studies in Mycology 65:36. 2010	Aveskamp, *et al*., 2010
Peyronellaea indianensis K.B. Deshp. & Mantri	Mycopath. Mycol. Appl. 30:341. 1966	Boerema, *et al*., 2004
Peyronellaea stemphylioides Kusnezowa	Nov. Sist. Niz. Rast 8:199. 1971	Boerema, *et al*., 2004
Phoma aspidioticola Narendre &V.G. Rao	Mycopath. Mycol. Appl. 54:137. 1974	Boerema, *et al*., 2004
Phoma chartae Verona	Cellulosa 1939:27. 1939	Boerema, *et al*., 2004
Phoma depressitheca Bubák	Annln naturh. Mus. Wien. 28:203. 1914	Boerema, *et al*., 2004
Phoma glumicola Speg.	Revta Mus. La Plata 15:36. 1908	Boerema, *et al*., 2004
Phoma indianensis (K.B. Deshp. & Mantri) Boerema, Dorenb. & Kesteren	Persoonia 5(2):203. 1968	Boerema, *et al*., 1968
Phoma insidiosa Tassi	Boll. R. Orto. Bot. Siena 1:8 1898	Boerema, *et al*., 2004
Phoma saccharicola S. Ahmad	Biologia, Lahore 6:131. 1960	Boerema, *et al*., 2004
Phoma saccharina Syd. & P. Syd. Apud Sydow, Sydow & Butler	Annls mycol 14:187. 1916	Boerema, *et al*., 2004

Synonym	Described in journal	Reference
Phyllosticta arachidis Khokhr.	Bolez. Vredit. Maslich. Kultur 1(2):32. 1934	Boerema, *et al*., 2004
Phyllosticta glumarum Sacc.	Nuovo G. bot. ital. II, 23:207. 1916	Boerema, *et al*., 2004
Phyllosticta glumarum-setariae Henn.	Annls Mus. R. Congo belge Sér. 4to, Bot. V 2:101. 1907	Boerema, *et al*., 2004
Phyllosticta glumarum-sorghi Henn.	Annls Mus. R. Congo belge Sér. 4to, Bot. V 2:101. 1907	Boerema, *et al*., 2004
Phyllosticta glumicola (Speg.) Hara	Dis. Rice Plant 164. 1918	Boerema, *et al*., 2004
Phyllosticta glumicola Speg.	Revta Mus. La Plata 15:36. 1908	Boerema, *et al*., 2004
Phyllosticta hawaiiensis Caum	Hawaii Plrs' Rec. 20:278. 1919	Boerema, *et al*., 2004
Phyllosticta oryzina Padwick	Manual Rice Dis. :163. 1950	Boerema, *et al*., 2004
Phyllosticta penicillariae Speg.	An. Mus. Nac. Hits. Nat. B. Aires 26:129. 1914	Boerema, *et al*., 2004
Phyllosticta phari Speg.	An. Mus. Nac. Hits. Nat. B. Aires III, 13:337. 1910	Boerema, *et al*., 2004
Phyllosticta sacchari Speg.	Revta Fac. Agron. Univ. Naz. La Plata 2:239. 1896	Boerema, *et al*., 2004
Phyllosticta setariae Ferraris	Milpighia 16:18. 1902	Boerema, *et al*., 2004
Phyllosticta sorghina Sacc.	Michelia 1(2):140. 1878	Saccardo, 1878

TABLE 2. Anthraquinones produced by *Epicoccum sorghinum*[1]

Compound name	Colour of amorphous solid
1,7-dihydroxy-3-methyl-9,10-anthraquinone	Orange
Phomarin (1,6-dihydroxy-3-methyl-9,10-anthraquinone)	Orange
Pachybasin (1-hydroxy-3-methyl-9,10-anthraquinone)	Yellow
1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone	Orange
Dentryol E	Pale Yellow
Dentryol F	Pale yellow

1 Adapted from de Souza Borges & Pupo (2006)

Chapter 2

Phylogenetic evidence supports the reclassification of *Phoma* species into the *Epicoccum* genus and analysis of *Epicoccum sorghinum* isolates of Southern Africa reveals genetic diversity

ABSTRACT

Epicoccum sorghinum belongs to the family Didymellaceae. It has a worldwide distribution and is a pathogen on a variety of plant hosts. This fungus is known to produce a variety of metabolites, and is associated with skin infections in humans. *Epicoccum sorghinum* has a perplexing taxonomic history and have been synonymized multiple times. In 1973 it was named *Phoma sorghina* and placed in *Phoma* section *Peyronellaea*, one of the nine sections into which the genus *Phoma* was classified. As *Phoma* species were divided based on morphology, and tend to lose their morphological features *in vitro*, many species were incorrectly described and identified in this genus. With molecular advances, many discrepancies were solved and *Phoma* section *Peyronellaea* was elevated to genus level and *P. sorghina* renamed to *Epicoccum sorghinum*. Isolates of this fungus from different countries has shown significant morphological and phylogenetic variation, and as *E. sorghinum* have been reported on many different hosts in Southern Africa and are known to produce different mycotoxins, the aim of this study was to investigate whether *E. sorghinum* from Southern Africa represents one ubiquitous species or multiple species by utilizing DNA sequencing information of various nuclear encoded regions. Fifty eight *E. sorghinum* isolates were collected from various culture collections and plant material from Southern Africa. Two subsets of these isolates were phylogenetically compared using four molecular markers (the 28S or large subunit of the nuclear ribosomal RNA (LSU), the two Internal Transcribed Spacer regions as well as the 5.8S ribosomal RNA (ITS), part of the Actin gene (ACT), and a portion of the Translation Elongation Factor 1-alpha (TEF) gene. A phylogenetic analysis of LSU and ITS comparing selected samples with a dataset obtained from a previous study, indicates that *E. sorghinum* does not group

solely with the other *Epicoccum* species but also other *Phoma* species that warrants possible recombination of these species into *Epicoccum.* Further analysis with ITS, ACT and TEF revealed that there is high diversity within *E. sorghinum,* but phylogenetic grouping is not consistent throughout all gene region analysis and therefore recognition of new species are not warranted.

1. INTRODUCTION

Epicoccum sorghinum (Sacc.) Aveskamp, Gruyter & Verkley (2010) belongs to the family Didymellaceae (de Gruyter, *et al.*, 2009). It has a worldwide distribution and is regarded as mainly a soil- and seed borne fungus (Boerema, *et al.*, 2004), but also occurs on other substrates such as animal feed and thatch (van der Nest, *et al.*, 2006). It is mostly associated with Poaceae such as sorghum and millet (Boerema, *et al.*, 2004). The importance of this fungus as plant pathogen was discussed by Zainum and Parbery (1974), indicating its involvement in ring spot disease on sugar cane. In addition, it was also reported as the cause of leaf spot in sugar cane and dicot crops (Venkatasubbaiah, *et al.*, 1992), *Phaeosphaeria* leaf spot of maize (do Amaral, *et al.*, 2004), leaf spot on rice (Boerema, *et al.*, 1971), root rot and dying off in sorghum, as well as glume blotch and glume blight in rice (Venkatasubbaiah, *et al.*, 1992, Boerema, *et al.*, 2004).

Epicoccum sorghinum is known to produce a wide variety of metabolites including phytotoxins (epoxydon, phyllostine and diphenylether) (Venkatasubbaiah, *et al.*, 1992), anthraquinones (de Souza Borges & Pupo, 2006), as well as mycotoxins such as tenuazonic acid (Steyn & Rabie, 1976) and an unidentified toxin that is associated with onyalai in humans (Rabie, *et al.*, 1975). Onyalai is a disease closely related to idiopathic thrombocytopenic purpura and is unique to the southern parts of Africa, yet *Epicoccum sorghinum* occurs worldwide. Furthermore, it was also associated with skin infections in humans (Rishi & Font, 2003).

This fungus has a perplexing taxonomic history. It was first described by Saccardo (1878) as *Phyllosticta sorghina* but has been synonymized multiple times over the past century. In 1973 the fungus was named *Phoma sorghina* (Sacc.) Boerema, Dorenb. & v. Kest.

(Boerema, *et al.*, 1973) and it was indicated that previous neotype material used to describe the fungus in 1971 as *Phoma glomarum* (Boerema, et al., 1971) was incorrectly used. Although certain isolates were used to describe the fungus in 1973, a different neotype was not indicated.

In 1990 *Phoma sorghina* was added to *Phoma* section *Peyronellaea*, one of five sections that was described (van der Aa, *et al.*, 1990) and in 2004 another four sections were added to a total of nine sections within *Phoma* (Boerema, *et al*., 2004). *Peyronellaea* was one of the nine sections into which the genus, *Phoma,* was divided based on morphology and comprised *Phoma* species that produce dictyochlamydospores. However, *Phoma* species tend to lose their morphological features when repeatedly plated onto artificial media (Boerema, *et al.*, 1965). In a discussion on the status of *Peyronellaea* it was pointed out that "chaos is bound to arise if form-species are based on minor differences only" (Boerema, *et al.*, 1965). It was made clear that this was the case for *Phoma* as many new species were described and incorrectly identified through the years in this genus. This was because the occurrence of singular chlamydospores, and how dictyochlamydospores are formed, were considered as key elements in describing different species and expert knowledge was needed to accurately identify *Phoma* species.

Many mistakes made in the past through morphological identifications could be solved using DNA as identification tool (Taylor, *et al.*, 2000). Reddy and co-authors (1998) first started to explore phylogenetic methods to identify *Phoma* species using a small fragment of the ITS1-5.8S-ITS2 (ITS) region of the ribosomal RNA (rRNA) cistron (Reddy, *et al.*, 1998). Since then, many advances were made by using the large subunit (LSU) and small subunit (SSU) rRNA genes, as well as the ITS and beta-tubulin (TUB) gene regions to characterize the Didymellaceae (Aveskamp, *et al.*, 2010). Accordingly, the genetically

diverse *Phoma* section *Peyronellaea* was elevated to genus level, while *P. sorghina*, together with *P. pimprina,* were moved to the genus *Epicoccum* and renamed *E. sorghinum* and *E. pimprinum*, respectively (Aveskamp*, et al.*, 2010).

Epicoccum sorghinum has been shown to display significant morphological and phylogenetic variation (Aveskamp*, et al.*, 2010). A study in which *E. sorghinum* isolates from Texas and Southern Africa were phylogenetically compared, using only ITS data, indicated that more information is needed to determine whether this fungus is a monophyletic or polyphyletic group and whether it consist of subspecies (Pažoutová, 2009). Also, *E. sorghinum* have been reported on many different hosts in Southern African countries and some were reported to produce mycotoxins (Rabie*, et al.*, 1975, Rabie*, et al.*, 1997). Therefore, this geographical area is ideal to isolate and study *E. sorghinum* in order to determine its taxonomic status. The aim of this study was to investigate whether *Epicoccum sorghinum* from Southern Africa represents one ubiquitous species or multiple taxa by utilizing the DNA sequence information of various nuclear encoded regions.

2. MATERIALS AND METHODS

2.1 ISOLATES

Epicoccum sorghinum isolates as well as isolates from the previous *Phoma* sect. *Peyronellaea*, were obtained from a number of reference culture collections. These included ATCC 12115, CBS 846.68 and CBS 627.68 that were used in the original description of *P. sorghina* in 1973 (Boerema*, et al.*, 1973) (TABLE 1). All isolates were

kept on malt extract agar (MEA) and oatmeal agar (OA). Malt extract agar was prepared by adding 50 grams of Biolab Malt Extract Agar (Merck, Wadeville, Gauteng) per litre of distilled water and autoclaving as instructed by the manufacturer. Oatmeal agar was prepared by adding 20 grams of oatmeal to one litre of distilled water and autoclaving for 20 minutes. The broth was filtered through cheesecloth and the filtrate was added to 20 grams of Biolab Agar Bacteriological (Merck, Wadeville, Gauteng). The media was made up to one litre with distilled water and autoclaved again.

A wide collection of isolates of *Epicoccum sorghinum* was obtained from plant material collected from various living hosts throughout South Africa and Namibia (TABLE 2). Isolates from these plant tissues were recovered as follows. Seeds were surfaced sterilized using 76% ethanol for one minute and then rinsing twice with sterile distilled water for one minute. The seeds were plated onto malt extract agar and incubated for ten days at 20ºC after which they were examined with a stereomicroscope. Twigs of indigenous trees were surface sterilized with 76% ethanol, aseptically cut open and only plant material from the cambium was isolated and plated onto malt extract agar. Isolates producing dark red pigment or having pycnidium-like fruiting structures were isolated and kept on oatmeal agar as instructed by Boerema *et al.* (2004). Where possible, the morphology of all isolates were compared to those known for *Epicoccum sorghinum* using the characteristics described by Boerema *et al.* (2004). These include colony colour, exudate production, as well as pycnidial shape and size, aerial pycnidia, conidial matrix colour, conidial shape and chlamydospore shape.

2.2 DNA EXTRACTIONS, PCR AND SEQUENCING

Isolates were plated onto MEA and incubated for 10 days at 20ºC. A modified version of the method described by Raeder and Broda (1985) was used to extract DNA. Mycelium was scraped off the agar with a sterile scalpel blade and placed in a 2mℓ eppendorf tube. DNA extraction buffer (prepared according to Raeder and Broda (1985)) was added and the mycelia crushed with a sterile glass rod. The mixture was incubated at 60ºC for one hour after which phenol and chloroform was added. The mixture was centrifuged for one hour at 10 000 rpm and the upper layer transferred to an empty Eppendorf tube. Chloroform was added and the mixture was centrifuged for 10 minutes at 10 000 rpm after which the top layer was again transferred to an empty Eppendorf tube. A volume of 0.1x sodium acetate (pH 5.5) and two volumes of absolute ethanol was added, the tube was inverted a few times and incubated overnight at 4ºC. The tube was centrifuged at 10 000 rpm for 30 minutes after which the ethanol was removed. A total of 1000 µℓ of 70% ethanol was added and the tube centrifuged at 10 000 rpm for five minutes, after which the ethanol was removed. This step was repeated once. The tube was then placed into a dryer to evaporate all remaining ethanol. The DNA was resuspended in 50 µℓ Sabax water. Five µℓ RNAse (5 mg/mℓ) was added and the tube incubated at 37ºC for 60 minutes. The quality and quantity of DNA extracted was determined using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Rockland, DE, USA). All DNA samples were diluted to contain a concentration of 2 ng/µℓ and were stored at -20°C.

The extracted DNA was used to amplify four gene regions with the polymerase chain reaction (PCR). The two Internal Transcribed Spacer (ITS1 and ITS2) and the 5.8S ribosomal RNA (rRNA) gene was amplified using primers ITS1 and ITS4 (White*, et al.*,

1990). Part of the Actin gene (ACT) was amplified with the primers ACT-512F and ACT-783R (Carbone & Kohn, 1999). The 28S or Large Subunit rRNA gene (LSU) was amplified with LR0R (Rehner & Samuels, 1994) and LR7 (Vilgalys & Hester, 1990). A portion of the Translation Elongation Factor 1-alpha (TEF) gene was amplified using the primer pair EF1-728F and EF1-986R (Carbone & Kohn, 1999). Each 25 µℓ reaction mixture contained 2 – 10 ng DNA, 2.5 µℓ 10x PCR reaction buffer, 2.5mM $MgCl_2$, 400ηM of each primer, 200µM of each dNTP and 3.5 U Faststart *Taq* DNA Polymerase (Roche Applied Science, Mannheim, Germany).

PCR reactions were carried out on a Biorad i-Cycler. The program for all four gene regions included an initial denaturation step at 95°C for four minutes, followed by 10 cycles consisting of 94°C for 20 seconds (denaturation), 56°C (ITS, ACT, TEF) or 48°C (LSU) for 48 seconds (annealing) and 72°C for 45 seconds (elongation) followed by a further 25 cycles of 94°C for 20 seconds, 56°C (ITS, ACT, TEF) or 48°C (LSU) for 40 seconds with an five second extension step per cycle followed by 72°C for 45 seconds. This was followed by a final step of 72°C for 10 minutes. An aliquot of five µℓ of each of the PCR products were stained with GelRed™ nucleic acid gel stain (Biotium, Hayward, CA, USA), separated on a 2% agarose gel for 20 minutes at 90 Volts and viewed under UV light.

PCR products were cleaned with 0.067 g/mℓ Sephadex G-50 (Sigma-Aldrich, Amersham Biosciences Ltd., Sweden) according to the manufacturer's instructions. The concentrations of the cleaned PCR products were determined using a NanoDrop ND-1000 spectrometer (NanoDrop Technologies, Rockland, DE, USA) and 60 – 100 ng of DNA was added to each sequencing reaction. The same primer pairs were used to sequence the amplified gene regions as were used in the PCR reactions, and products were sequenced in

both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 3100 DNA sequencer (Applied Biosystems). Sequence results were viewed manually and consensus sequences were assembled with Vector NTI Advanced 10 (Vector NTIH Software, 1600 Faraday Avenue, Carlsbad, California 92008).

2.3 PHYLOGENETIC ANALYSIS

Sequences for isolates and species that were closely related to the *Epicoccum* clade, as well as *Epicoccum* species used by Aveskamp *et al.* (2010), were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) to allow comparisons with the Southern African isolates (TABLE 3). Sequences were aligned with the online version of the alignment program MAFFT 6 (Katoh, *et al.*, 2002). Aligned data was imported into MEGA version 4 (Tamura, *et al.*, 2007) to manually check and to edit the alignments by indicating missing data as 'n'.

Maximum parsimony (MP) analysis was performed with the software package PAUP* 4.0b10 (Swofford, 2003). One thousand random stepwise addition heuristic searches were performed with tree-bisection-reconnection (TBR) as the branch-swapping algorithm. Uninformative characters were excluded and the consistency index (CI), homoplasy index (HI), rescaled consistency index (RC), retention index (RI) and tree length (TL) were determined for the resulting trees. Max trees were set to auto increase, but because analyses took inordinately long the analysis was repeated with Max trees set to 10 000. The confidence levels were estimated by performing 1000 bootstrap replicates. Partition

homogeneity tests (PHT) were conducted to test the congruence of trees obtained from the different gene regions with PAUP* 4.0b10 (Swofford, 2003) to determine the possibility to combine data from the different gene regions.

In order to perform Maximum likelihood (ML) analysis, the best fit substitution models for each of the data combinations were determined using jModeltest 0.1.1 (Posada, 2008). The models derived for each dataset is indicated in TABLE 4. Maximum likelihood (ML) analysis was performed with the program PhyML version 3.0 (Guindon & Gascuel, 2003). The confidence levels were estimated with 1000 bootstrap replicates.

3. RESULTS

3.1 ISOLATES

Thirty three isolates were collected from various culture collections (TABLE 1) to represent *Epicoccum sorghinum* isolates from different countries, toxicity levels and substrates. ATCC 12115 was used to describe *Phoma sorghina* in 1973 by Boerema *et al.* and was, therefore, also included. The remainder of isolates (26) were donated or collected from plant material in Southern Africa (TABLE 2). These isolates were confirmed to be *Epicoccum sorghinum* based on morphological characteristics given in the *Phoma* identification manual by Boerema *et al.* (2004). In some cases not all characteristics were typical and tended to vary when cultures were sub-cultured. Therefore, identifications were based on available characteristics present in each individual culture.

3.2 PHYLOGENETIC ANALYSIS

Two different datasets were analysed. First, a random selection of *E. sorghinum* isolates that were previously deposited in culture collections, and representing different hosts and locations (from TABLE 1), were selected together with isolates that grouped together with the *Epicoccum* clade as determined by Aveskamp *et al.* (2010) (TABLE 3), as well as the isolates that previously belonged to *Phoma* section *Peyronellaea* (TABLE 3). All the *E. sorghinum, E. nigrum* and *E. pimprinum* isolates (TABLE 3) that were included in the study by Aveskamp *et al.* (2010) were also included. This was done to establish whether the *E. sorghinum* isolates that were collected fit within the existing *Epicoccum* genus as described by these authors. For these analyses, the ITS and LSU alignments consisted of 44 taxa each and contained 475 and 1327 aligned nucleotides respectively (TABLE 5), while the combined ITS and LSU alignment contained 1802 characters (TABLE 5). When testing congruence for the regions of this dataset, a p-value of 0.011 was obtained indicating that the gene regions were congruent and could therefore be combined. One combined tree is shown for this dataset (FIGURE 1).

The position of *E. sorghinum* isolates were shown to be separate from other species in the Didymellaceae based on the combined ITS and LSU sequence data (FIGURE 1, Group A). Sequences representing *E. nigrum* and *E. pimprinum* also grouped separately from *E. sorghinum* and not together in one clade as was previously indicated by Aveskamp *et al.* (2010) but grouped together in a clade with other *Phoma* species (FIGURE 1, Group B). The *Peyronellaea* species previously known as *Phoma* section *Peyronellaea* grouped together in Group C. The outgroup of *Phoma paspali* and *Ascochyta hordei* var. *hordei*

were selected for the sake of uniformity as these cultures were used in previous taxonomic papers analysing the Didymellaceae (Aveskamp*, et al.*, 2009, Aveskamp*, et al.*, 2010).

The second dataset that was analysed was for the ITS, ACT and TEF regions. This was done to establish whether there was variability within *E. sorghinum*. As the TEF region is not available for all isolates used in previous phylogenetic papers of the Didymellaceae, this dataset consisted of only the *Epicoccum sorghinum* isolates. The alignments for the single regions included 60 taxa of 262 to 465 nucleotide positions (TABLE 5). When the three regions of this dataset were tested for congruence, however, the partition homogeneity test (PHT) indicated that the p-value was 0.001 indicating that they represent non-homogenous partitions. This probably resulted from the switching of internal nodes, and branches indicating certain phylogenetic groups collapsing between gene regions. Therefore, the sequence data was not combined and only the results for all the individual gene regions are shown (FIGURES 2 – 4). The statistics for each tree derived with PAUP as well as the models used for Maximum likelihood analysis is summarized in TABLES 4 - 5.

The phylogenetic trees obtained for the second dataset revealed that ITS (FIGURE 4) had only high bootstrap support for one clade within the *E. sorghinum* group. The Actin gene region (FIGURE 3) showed more variability with bootstrap support higher than 75% for four different clades. The portion of the Translation Elongation Factor 1-alpha gene (FIGURE 2) showed the most variability with nine groups within the *E. sorghinum* clade with bootstrap support higher than 75%. Phylogenetic trees obtained for the individual loci (ITS, ACT and TEF) did not consistently support the same groupings of isolates, but did not show contradictions. One group that was consistent throughout (with strong bootstrap support) contained four isolates collected from South Africa of which three were from sorghum and one from maize (Clade K, FIGURES 2-4).

The strain CBS 284.76 that is indicated as the morphological reference strain for *E. sorghinum* in the manual by Boerema (Boerema *et al*., 2004) has a basal grouping to the rest of the *E. sorghinum* isolates where the *Phyllosticta sorghina* isolate (ATCC 12115), which was initially used to describe *P. sorghina* by Boerema et al. (1973), was part of the *E. sorghinum* clades. This isolate grouped together with an isolate from India and an isolate from Martinique when comparing TEF sequences (Clade H, FIGURE 2), but this grouping was not consistent when comparing ITS and ACT. The other two isolates that were used to describe *P. sorghina* in 1973 are CBS 846.68 from *Coffea* in India and CBS 627.68 from *Citrus* in France. Although these two isolates grouped together with a bootstrap support of 79% in the TEF analysis (Clade G, FIGURE 2) other gene regions did not support the same resolution (FIGURES 3-4).

4. DISCUSSION

In 2010 Aveskamp, *et al*. did a comprehensive study in which the Didymellaceae was looked at from an evolutionary perspective. In this paper many of the species in the Didymellaceae were moved to other genera such as *Peyronellaea* and *Epicoccum.* However, when the same isolates that grouped basally with *Epicoccum* in that study were compared to the isolates collected for this study, it was evident that the three *Epicoccum* species did not group together in their own clade but with the other *Phoma* species included in the analysis (FIGURE 1). Additionally, these authors used one morphological feature – the fact that these three species produce epicoccoid or botryoid shaped chlamydospores – to warrant the recombination of these species into the *Epicoccum* genus. Other *Phoma* species are also known to produce botryoid-like chlamydospores (e.g.,

Phoma clematidina) (Boerema, et al., 2004) and this characteristic is therefore not exclusive to these three species. *Phoma draconis, P. henningsii, P. plurivora* and *P. brasiliensis* were separated from the *Epicoccum* species by Aveskamp, *et al.* (2010) because these *Phoma* species do not produce chlamydospores. *Phoma crystallifera* was treated as a residual species in the previous study on the Didymellaceae as it could not be assigned to a group based on phylogenetic evidence (Aveskamp, *et al.*, 2010). In the present study, however, *P. crystallifera* grouped together with *Epicoccum,* as well as the four aforementioned *Phoma* species. These five *Phoma* species, like the three *Epicoccum* species, produce unicellular conidia and have pseudoparenchymatous pycnidial walls (de Gruyter, *et al.*, 1993, Boerema, *et al.*, 2004, Aveskamp, *et al.*, 2010). It is therefore proposed that these morphological characteristics, in combination with phylogenetics, should rather be used to identify these species. As *Epicoccum* is the oldest generic name in this group, it is proposed that these five *Phoma* isolates could possibly be recombined in the genus *Epicoccum* but further investigation is necessary.

When the morphology for initial identification was examined, not all structures were present for all isolates and identifications were made based on structures that were present at the time. Furthermore, isolates are inconsistent in their colony characteristics when subcultured. Therefore, it is deduced that morphology is not an accurate identification tool for *E. sorghinum.* This could also be demonstrated by the erroneous use of a *P. glomerata* strain (CBS 284.76) by Boerema *et al.* (2004) as the reference strain for *E. sorghinum*. This was clearly confirmed by the phylogenetic analysis of the second dataset in this study (FIGURES 2 – 4). In 1973 various isolates were used for the description of *P. sorghina*. These isolates include ATCC 12115 (Clade H, FIGURE 2), CBS 846.68 and CBS 627.68 (Clade G, FIGURE 2), of which the CBS cultures are still available. In terms of geographical distribution, Clade G and H (FIGURE 2) contained all the isolates not

collected from Southern Africa, with the exception of clade A that also contained an isolate from Singapore. The Southern African isolates did not group according to their geographic origins and isolates from different countries were mixed in the other clades. Most of the species described previously in the form genus *Phoma* were isolated in Europe (Boerema, *et al.*, 2004). No isolates are currently indicated as type strains for *E. sorghinum*. As there are no other reference strains indicated and ATCC 12115 is sterile, it is proposed that CBS 846.68 and CBS 627.68 should be typified.

When examining the different clades formed by phylogenetic analysis of TEF (FIGURE 2), it was found that the correlation between clade formation and fungal host was not consistent. Although Clade I and J both only contained isolates collected from Poaceae, other clades such as Clade A - F contained isolates collected from various hosts of different plant families. The same was observed in the analysis of the other gene regions (FIGURES 3 and 4). Therefore, the host does not appear to be an important driver of their divergence or population biology. Furthermore, isolates that were tested for toxicity (TABLE 1) were distributed in all clades except Clades G and H and therefore toxicity and tenuazonic acid production is not correlated to clade formation. Clade F (FIGURE 2) consisted of 16 isolates from Namibia and South Africa that were isolated from various hosts. This clade did not have a bootstrap support of higher than 75% for either of the phylogenetic analysis conducted. If additional samples should be collected and sequenced, more diversity could be detected or better-supported clades could be formed in further analysis.

The weak bootstrap support for the tree inferred for the ITS region (FIGURE 4) supports the finding that the ITS region is not an ideal marker to use for phylogenetic analysis (Balmas, *et al.*, 2005). The only consistency within ITS, ACT and TEF was one clade (group K)

with four species from South Africa. Pažoutová (2009) studied *E. sorghinum* from South Africa and Texas but only used a small subset of samples and only analyzed the ITS region. The final outcome suggested that further studies are required to establish if there is a subspecies structure in *E. sorghinum* or if this variation warrants the description of new species. It was also confirmed in this study that other gene regions could provide more in-depth and accurate distinction between species and subspecies.

Results indicated that the TEF gene region showed the most variability, and could, therefore, be used to identify variation within *Phoma* species. However, this gene region has not been sequenced across the *Phoma* genus and, therefore, a more comprehensive dataset could not be compiled in this study. Irinyi, *et al.* (2007) suggested TEF as a molecular marker for the classification of *Phoma* species, but also suggested that further studies are required to confirm this. In 2009, this marker was also used to synonymise *P. pinodella* and *P. sojicola,* and also to synonymise *P. exigua* and *Phyllosticta sojicola* (Irinyi, et al., 2009). This study is, therefore, consistent with their findings that TEF is a valuable marker in distinguishing *Phoma* species and the phylogenetic analysis of this region has successfully provided proof that there is high diversity within *E. sorghinum*.

This study could not identify clades within the *E. sorghinum* group based on toxicity, or host specificity and geographically only distinguished between non-Southern African and Southern African isolates but not more specifically. Pažoutová (2009) used rep-PCR data to draw a UPGMA dendogram of *E. sorghinum* isolates that showed distinct geographical separation of samples. This method could be considered as a more accurate approach of distinguishing between isolates or could give clear answers on how this fungus was distributed through Southern Africa.

This study indicated that, although multiple species could not be identified based on all the markers used, there is extensive diversity within *E. sorghinum* based on the phylogenetic analysis of the TEF marker. It is suggested that this marker should be used in the future for further identification of *Phoma* and to resolve taxonomic difficulties regarding this genus. As it was indicated before, morphological identification of this genus is not always possible and therefore a phylogenetic or phenotypic identification method is vital, especially for pathogenic *Phoma* species. It is also suggested that a standardized selection of markers should be used for identification as different authors use different subsets of gene regions, which makes comparability and identification problematic. Although no correlation was found regarding clade formation, host specificity, toxicity or geographical distribution of the *E. sorghinum* isolates, one should not rule out the importance of metabolite production and utilization of resources as possible indicators of variation within this species.

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TABLE 1. ***Epicoccum sorghinum*** **isolates collected from culture collections**

Fungal species	Collection number	Host plant or substrate	Location	Country and Region	Known toxicity[1]	Tenuazonic acid production[1]
Epicoccum sorghinum	CGM 1620	millet	Ovamboland	Namibia	Fed to ducklings, 8/8 died	3 g/kg
Epicoccum sorghinum	CGM 1621	boerhavia	Not available	India	Fed to chickens, 0/4 died	not detected
Epicoccum sorghinum	CGM 1622	millet	Ovamboland	Namibia	Fed to ducklings, 4/4 died	290 mg/kg
Epicoccum sorghinum	CGM 1623	sorghum malt	Potchefstroom	South Africa	Fed to ducklings, 4/4 died	not detected
Epicoccum sorghinum	CGM 1624	wheat	Free State	South Africa	Fed to ducklings, 4/4 died	not tested
Epicoccum sorghinum	CGM 1625	wheat	Ficksburg	South Africa	Fed to ducklings, 4/4 died	not tested
Epicoccum sorghinum	CGM 1626	sorghum	Bethal	South Africa	Information not available	80 mg/kg
Epicoccum sorghinum	CGM 1627	millet	Ovamboland	Namibia	Fed to ducklings, 4/4 died	220 mg/kg
Epicoccum sorghinum	CGM 1628	sorghum	Ovamboland	Namibia	Information not available	not detected
Epicoccum sorghinum	CGM 1629	oats	Clarens	South Africa	Fed to ducklings, 4/4 died	not tested
Epicoccum sorghinum	CGM 1630	barley	Caledon	South Africa	Fed to ducklings, 4/4 died	not tested
Epicoccum sorghinum	CGM 1631	millet	Settlers	South Africa	Information not available	not tested
Epicoccum sorghinum	CGM 1632	millet	Settlers	South Africa	Fed to ducklings, 4/4 died	not tested

Fungal species	Collection number	Host plant or substrate	Location	Country and Region	Known toxicity[1]	Tenuazonic acid production[1]
Epicoccum sorghinum	CGM 1633	millet	Ovamboland	Namibia	Fed to ducklings, 8/8 died	290 mg/kg
Epicoccum sorghinum	CGM 1634	millet	Ovamboland	Namibia	Fed to ducklings, 8/8 died	not tested
Epicoccum sorghinum	CGM 1635	sorghum	Settlers	South Africa	Fed to ducklings, 0/4 died	not tested
Epicoccum sorghinum	CGM 1636	groundnuts	Not available	Mozambique	Fed to ducklings, 4/4 died	not tested
Epicoccum sorghinum	CGM 1637	millet	Ovamboland	Namibia	Information not available	200 mg/kg
Epicoccum sorghinum	CGM 1638	sorghum	Lichtenburg	South Africa	Fed to ducklings, 1/8 died	not tested
Epicoccum sorghinum	CGM 1639	millet	Windhoek	Namibia	Fed to chickens, 4/4 died	not tested
Epicoccum sorghinum	CGM 1640	sorghum	Sekukuniland	South Africa	Fed to chickens, 4/4 died	1.5 g/kg
Epicoccum sorghinum	CGM 1641	sorghum	Estancia	South Africa	Fed to ducklings, 4/4 died	660 mg/kg
Epicoccum sorghinum	CGM 1642	millet	Ovamboland	Namibia	Information not available	not tested
Epicoccum sorghinum	CGM 1643	sorghum	Ventersdorp	South Africa	Fed to ducklings, 4/4 died	100 mg/kg
Epicoccum sorghinum	CGM 1644	sorghum	Settlers	South Africa	Fed to ducklings, 4/4 died	not tested
Epicoccum sorghinum	CGM 1645	sorghum	Estancia	South Africa	Fed to ducklings, 4/4 died	not tested
Epicoccum sorghinum	CGM 1646	rice	Not available	Singapore	Information not available	not tested

Fungal species	Collection number	Host plant or substrate	Location	Country and Region	Known toxicity[1]	Tenuazonic acid production[1]
Phyllosticta sorghina	ATCC 12115	sugar cane	Florida	USA	not tested	not tested
Epicoccum sorghinum	CBS 985.95	soil	Brahman	Papua New Guinea	not tested	not tested
Epicoccum sorghinum	CBS 846.68	fruit of *Coffea*	Not available	India	not tested	not tested
Epicoccum sorghinum	CBS 218.81	*Gossypium hirsutum*	Dharwad	Karnataka	not tested	not tested
Epicoccum sorghinum	CBS 627.68	*Citrus* twig	Antibes	France	not tested	not tested
Peyronellaea glomerata	CBS 284.76	*Populus nigra*	Novosibirsk	Russia	not tested	not tested

1 Information obtained from the culture collection of the CSIR, Pretoria, South Africa

TABLE 2. ***Epicoccum sorghinum*** **isolates collected from plant material in Southern Africa**

Isolate number	Fungal species	Host or substrate	Location	Country	Collected by	Isolated by
CGM 1647	*Epicoccum sorghinum*	*Acacia erioloba*	Not available	South Africa	Elsie de Meyer	Elsie de Meyer
CGM 1649	*Epicoccum sorghinum*	*Pennisetum glaucum*	Not available	South Africa	Annelie Lubben	Ariska van der Nest
CGM 1650	*Epicoccum sorghinum*	*Eragrostis tef*	Not available	South Africa	Annelie Lubben	Ariska van der Nest
CGM 1651	*Epicoccum sorghinum*	*Zea mays*	Not available	South Africa	Annelie Lubben	Ariska van der Nest
CGM 1652	*Epicoccum sorghinum*	*Eucalyptus grandis*	Mtubatuba	South Africa	Marieka Gryzenhout	Marieka Gryzenhout
CGM 1653	*Epicoccum sorghinum*	*Acacia karroo*	Bloemfontein	South Africa	Fahimeh Jami	Fahimeh Jami
CGM 1654	*Epicoccum sorghinum*	*Acacia karroo*	Kuruman	South Africa	Fahimeh Jami	Fahimeh Jami
CGM 1655	*Epicoccum sorghinum*	*Zea mays*	Not available	South Africa	Ariska van der Nest	Ariska van der Nest
CGM 1656	*Epicoccum sorghinum*	*Zea mays*	Not available	South Africa	Ariska van der Nest	Ariska van der Nest
CGM 1657	*Epicoccum sorghinum*	*Zea mays*	Not available	South Africa	Ariska van der Nest	Ariska van der Nest
CGM 1658	*Epicoccum sorghinum*	*Zea mays*	Not available	South Africa	Ariska van der Nest	Ariska van der Nest
CGM 1659	*Epicoccum sorghinum*	*Eucalyptus grandis*	Mtubatuba	South Africa	Marieka Gryzenhout	Marieka Gryzenhout
CGM 1660	*Epicoccum sorghinum*	*Rhus lancea*	Northern Cape	South Africa	Francois van der Walt	Francois van der Walt
CGM 1661	*Epicoccum sorghinum*	*Acacia mellifera*	Not available	Namibia	Francois van der Walt	Francois van der Walt
CGM 1662	*Epicoccum sorghinum*	thatch	Shelly Beach	South Africa	Gert J. Marais	Annelie Lubben

Isolate number	Fungal species	Host or substrate	Location	Country	Collected by	Isolated by
CGM 1663	*Epicoccum sorghinum*	thatch	Elliot	South Africa	Gert J. Marais	Annelie Lubben
CGM 1664	*Epicoccum sorghinum*	thatch	Aliwal North	South Africa	Gert J. Marais	Annelie Lubben
CGM 1665	*Epicoccum sorghinum*	*Acacia mellifera*	Northern Cape	South Africa	Gert J. Marais	Violet Simataa
CGM 1666	*Epicoccum sorghinum*	*Acacia mellifera*	Northern Cape	South Africa	Gert J. Marais	Violet Simataa
CGM 1667	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia	Violet Simataa	Violet Simataa
CGM 1668	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia	Violet Simataa	Violet Simataa
CGM 1669	*Epicoccum sorghinum*	*Acacia mellifera*	Ondangwa	Namibia	Violet Simataa	Violet Simataa
CGM 1670	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia	Violet Simataa	Violet Simataa
CGM 1671	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia	Violet Simataa	Violet Simataa
CGM 1672	*Epicoccum sorghinum*	*Acacia mellifera*	Ondangwa	Namibia	Violet Simataa	Violet Simataa
CGM 1673	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia	Violet Simataa	Violet Simataa

TABLE 3. Strains of the Didymellaceae for which Genbank data was downloaded for use in the phylogenetic analysis

Culture collection number	Name	Genbank accession numbers	
		LSU	ITS
CBS 185.85	*Peyronellaea americana*	GU237990	FJ426972
CBS 109.92	*Peyronellaea calorpreferens* Type	GU238002	FJ426983
CBS 464.97	*Peyronellaea glomerata*	GU238009	FJ427012
CBS 528.66	*Peyronellaea glomerata*	EU754184	FJ427013
CBS 285.76	*Peyronellaea pomorum* var. *circinata* Type	GU238025	FJ427053
CBS 388.80	*Peyronellaea pomorum* var. *cyanea* Type	GU238027	FJ427055
CBS 110.92	*Peyronellaea subglomerata*	GU238032	FJ427080
CBS 448.83	*Phoma calidophila* Type	GU238052	FJ427059
PD 84/109	*Phoma calidophila*	GU238053	FJ427060
CBS 463.69	*Peyronellaea musae*	GU238011	FJ427026
CBS 558.81	*Phoma plurivora* Type	GU238132	GU237888
CBS 284.93	*Phoma plurivora*	GU238131	GU237822
CBS 123395	*Phoma infossa* Type	GU238089	FJ427025
CBS 123394	*Phoma infossa*	GU238088	FJ427024
CBS 390.93	*Phoma huancayensis*	GU238085	GU237857
CBS 105.80	*Phoma huancayensis* Type	GU238084	GU237732
CBS 104.80	*Phoma henningsii*	GU238081	GU237731
CBS 527.66	*Phoma eupyrena*	GU238073	FJ427000
CBS 374.91	*Phoma eupyrena*	GU238072	FJ426999
CBS 186.83	*Phoma draconis*	GU238070	GU237795
CBS 193.82	*Phoma crystallifera* Type	GU238060	GU237797
CBS 108.79	*Phoma clematidina* Type	FJ515632	FJ426989

Culture collection number	Name	Genbank accession numbers	
		LSU	ITS
CBS 102.66	*Phoma clematidina*	FJ515630	FJ426988
CBS 120105	*Phoma brasiliensis* Type	GU238049	GU237760
PD 77/1028	*Epicoccum pimprinum*	GU237977	FJ427050
CBS 246.60	*Epicoccum pimprinum* Type	GU237976	FJ427049
CBS 173.73	*Epicoccum nigrum* Type	GU237975	FJ426996
CBS 125.82	*Epicoccum nigrum*	GU237974	FJ426995
CBS 627.68	*Epicoccum sorghinum*	GU237979	FJ427072
CBS 179.80	*Epicoccum sorghinum*	GU237978	FJ427067
CBS 560.81	*Phoma paspali* Type	GU238124	FJ427048
CBS 561.81	*Phoma paspali*	GU238125	GU237889
CBS 544.74	*Ascochyta hordei* var. *hordei*	EU754134	GU237887

TABLE 4. Maximum likelihood models and parameter statistics[2]

	FIGURE 1	FIGURE 2	FIGURE 3	FIGURE 4
Number of taxa	44	60	60	60
Number of aligned characters	1802	371	262	465
The best fit substitution models[3]	TIM2+I+G	TIM2+G	HKY+G	HKY+G
Gamma	0.204	0.461	0.519	0.01
P-invar	0.565	0	0	0
Kappa	-	-	8.5804	4.3187
freqA	0.2562	0.2022	0.2086	0.2329
freqC	0.2189	0.2776	0.3303	0.2467
freqG	0.2763	0.2294	0.2529	0.2217
freqT	0.2486	0.2909	0.2082	0.2987
ti/tv	-	-	4.1959	2.183
Number of substitution categories	6	6	2	2

2 The best fit substitution models and parameter statistics for all data combinations were determined using jModeltest 0.1.1 (Posada, 2008)

3 The models determined for Figure 1 and Figure 2 were the Transitional model (TIM) (Posada, 2008) that included invariable sites (+I) as well as rate variations among sites (+G) respectively, models for Figure 3 and Figure 4 were the Hasegawa, Kishino, Yano 85 (HKY) models (Hasegawa, Kishino and Yano, 1985) and included rate variations among sites (+G)

TABLE 5. Statistics from maximum parsimony analysis[4]

	FIGURE 1	FIGURE 2	FIGURE 3	FIGURE 4
Aligned characters	1802	371	262	465
P-score (PHT)	0.011	-	-	-
Constant characters	-	215	183	445
Parsimony uninformative characters	1656	51	28	9
Parsimony informative characters	146	105	51	11
Tree length	257	219	80	13
Consistency index (CI)	0.642	0.684932	0.75	0.846154
Retention index (RI)	0.856	0.872458	0.919679	0.958333
Rescaled consistency index (RC)	0.55	0.597574	0.689759	0.810897
Homoplasy index (HI)	0.358	0.315068	0.25	0.153846

4 Maximum parsimony (MP) statistics were determined with the software package PAUP* 4.0b10 (Swofford, 2003)

FIGURE 1. The most parsimonious tree of the combined LSU and ITS gene regions of *Epicoccum sorghinum* isolates as well as isolates previously known as *Phoma* section *Peyronellaea* and isolates that clustered together with *Epicoccum* in the work by Aveskamp *et al.* 2010. The outgroup consists of *Phoma paspali* and *Ascochyta hordei* var. *hordei* and was chosen as it was used as outgroup in previous works on *Phoma.* Only bootstrap support of 75% and higher is shown with bootstrap support derived from maximum parsimony indicated on the top of each branch, and bootstrap support derived from maximum likelihood indicated at the bottom of each branch.

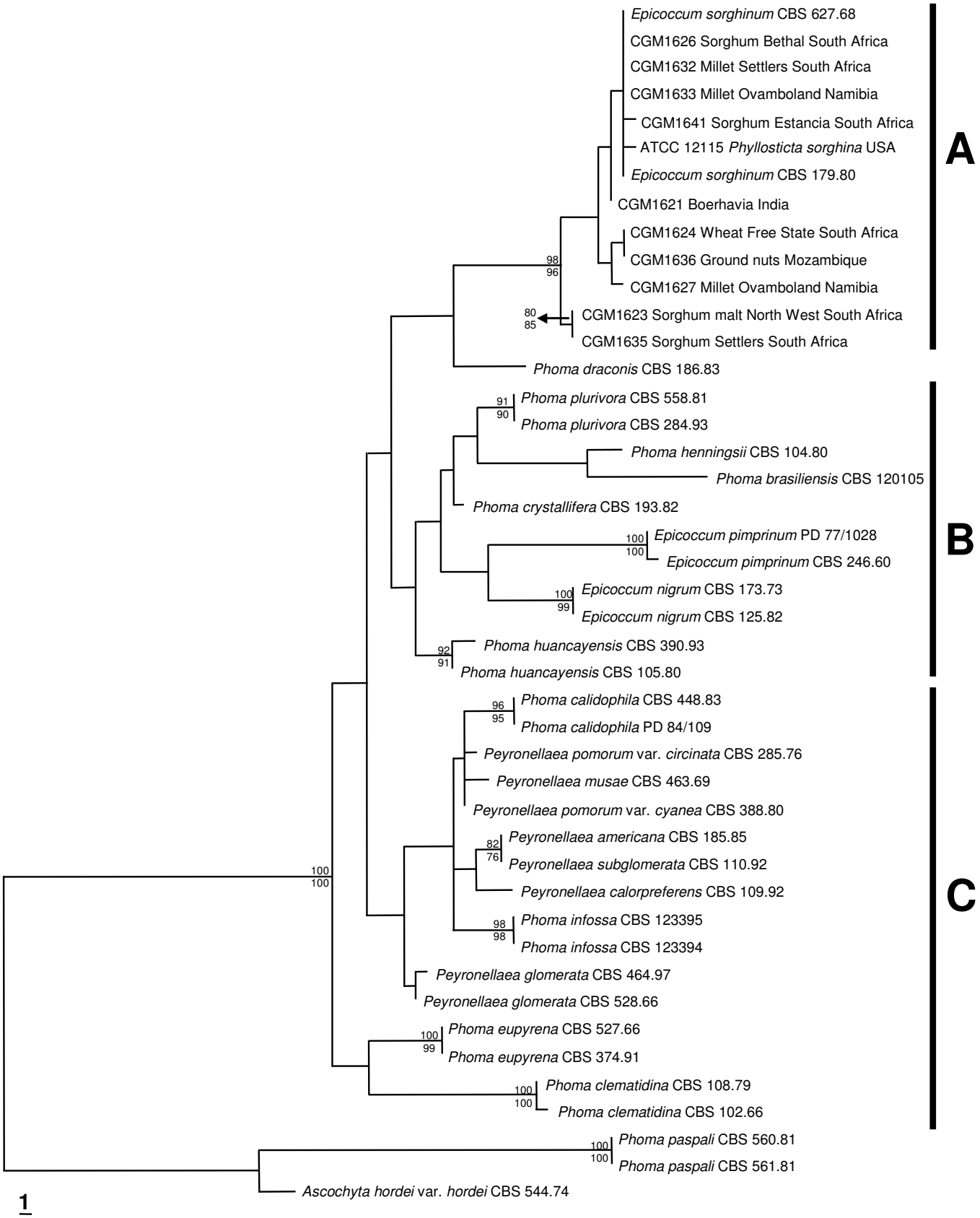


FIGURE 2. The most parsimonious tree for the TEF region of 58 *Epicoccum sorghinum* isolates, including a *Phoma pinodella* as well as *Phoma glomerata* (the strain previously indicated as the reference strain for *E. sorghinum*) as outgroup. Only bootstrap support of 70% and higher is shown with bootstrap support derived from maximum parsimony indicated on the top of each branch, and bootstrap support derived from maximum likelihood indicated at the bottom of each branch.

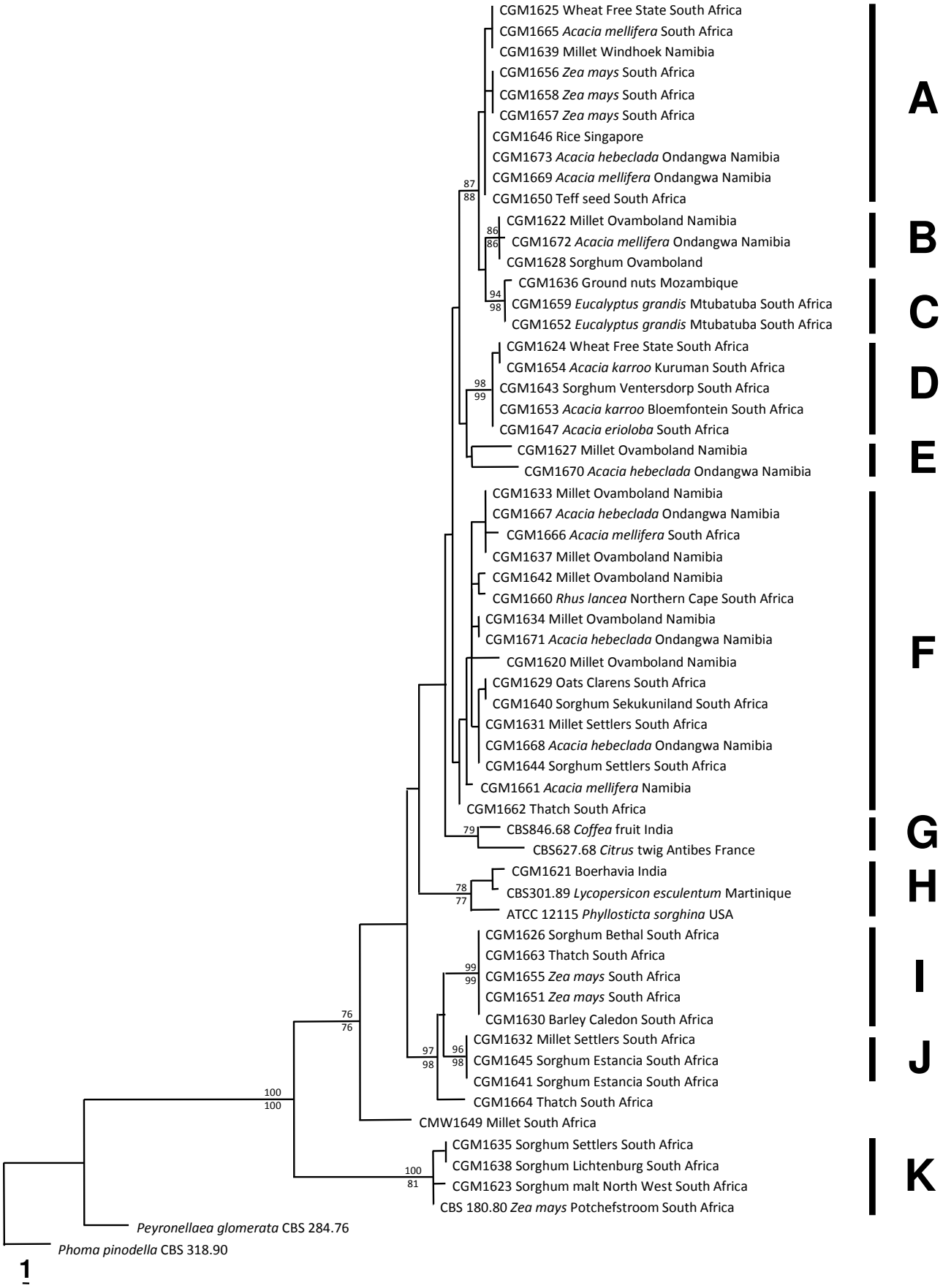


FIGURE 3. The most parsimonious tree for the ACT region of 58 *Epicoccum sorghinum* isolates, including *Phoma pinodella* as well as *Phoma glomerata* (the strain previously indicated as the reference strain for *E. sorghinum*) as outgroup. Only bootstrap support of 70% and higher is shown with bootstrap support derived from maximum parsimony indicated on the top of each branch, and bootstrap support derived from maximum likelihood indicated at the bottom of each branch.

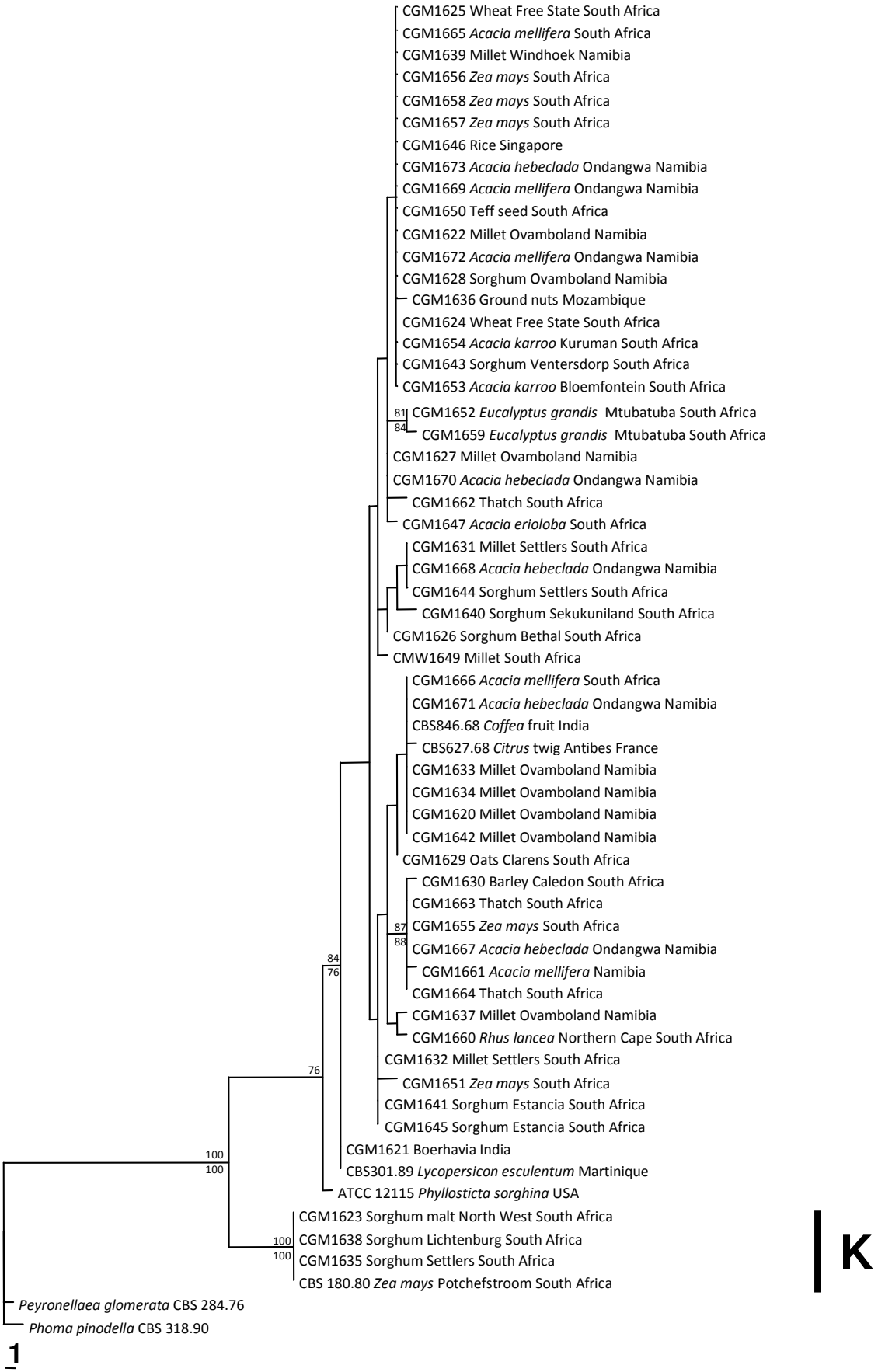
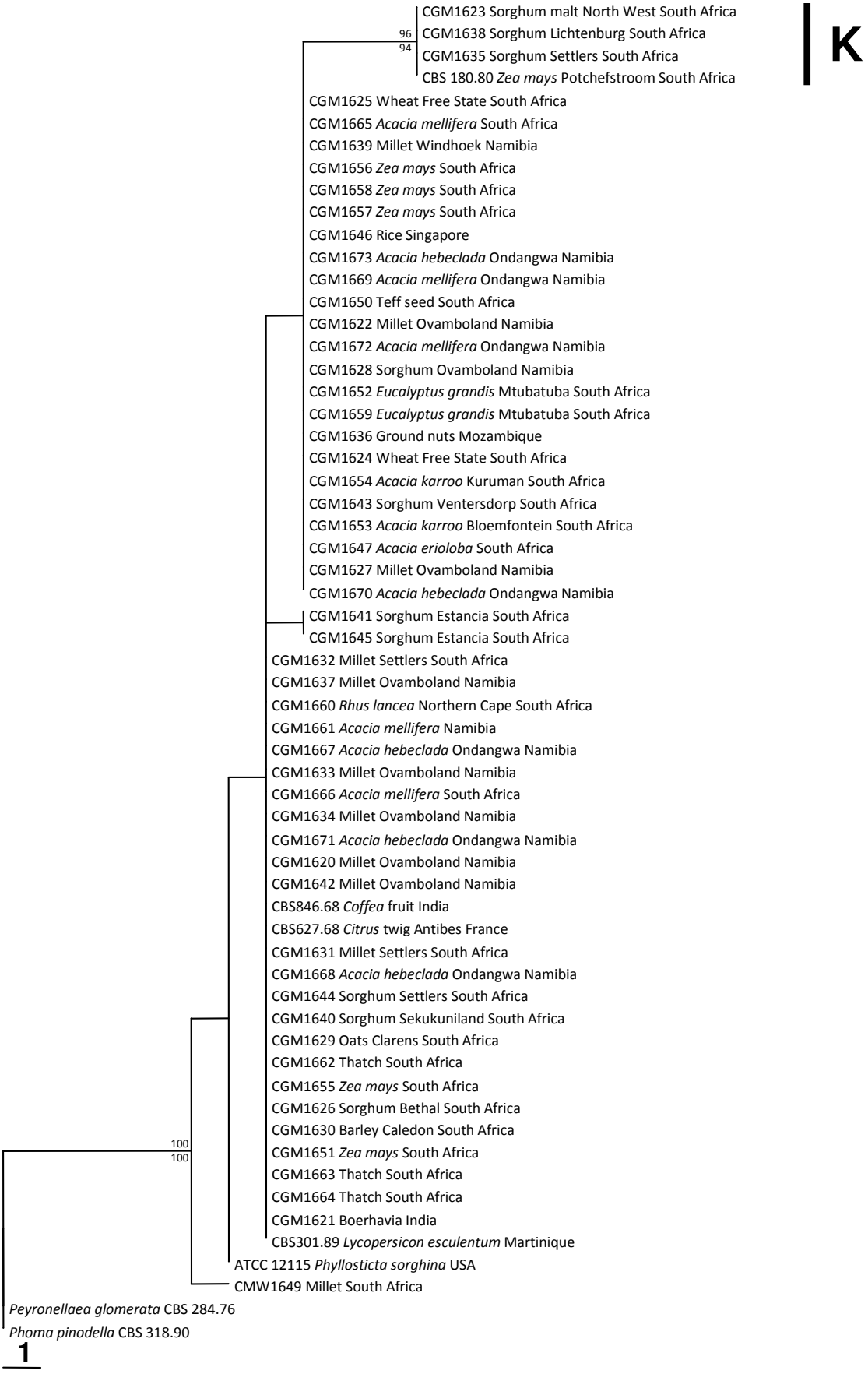


FIGURE 4. The most parsimonious tree for the ITS region of 58 *Epicoccum sorghinum* isolates, including a *Phoma pinodella* as well as *Phoma glomerata* (the strain previously indicated as the reference strain for *E. sorghinum*) as outgroup. Only bootstrap support of 70% and higher is shown with bootstrap support derived from maximum parsimony indicated on the top of each branch, and bootstrap support derived from maximum likelihood indicated at the bottom of each branch.



Chapter 3

Carbon source utilization by *Epicoccum sorghinum*

ABSTRACT

Epicoccum sorghinum is a tenuazonic acid and phytotoxin producing fungus belonging to the Didymellaceae. This Coelomycete is a soil-borne fungus that occurs globally on a wide range of hosts and is known as a weak plant pathogen. It has also been documented as a human pathogen and as the causal agent of onyalai, a bleeding disorder found in the southern parts of Africa. Morphology-based identification of this, and other species belonging to the Coelomycetes are challenging as only a few characteristics that often overlap are used to differentiate between these fungi. Subculturing also often results in the loss of identifiable characteristics. DNA-based studies demonstrated that *E. sorghinum* is represented by genetically diverse groups. It was previously suggested that a polyphasic approach is necessary to stabilize the taxonomy of a genus. An alternative to morphological identification could be the use of carbon source utilization profiles. The aim of this study was to determine whether carbon source utilization could be used to differentiate between genetically diverse groups found within *E. sorghinum.* Thirty seven carbon source solutions were prepared and tested using 25 isolates of *E. sorghinum* that were previously shown to be genetically diverse. Culture growth, pycnidium formation, and colour formation in the media were visually observed. Very little variation in the utilisation of carbon sources between the selected isolates was observed, and most of the isolates were able to utilize the majority of the tested carbon sources. All isolates, except for one, produced pycnidia on certain carbon sources and these carbon sources could be used to promote fruiting structure formation in order to study morphological characteristics of Coelomycetous fungi on artificial media. Colour pigmentation production in the different solutions was not widely observed. No particular patterns were

observed regarding carbon source utilization between the different genetic groups previously identified and carbon source utilization does not seem to be useful as a taxonomic tool to distinguish among diversity within *E. sorghinum.* Further studies are needed to investigate if carbon source utilization could be used at a higher taxonomic level in order to distinguish between different species of the Didymellaceae.

1. INTRODUCTION

Epicoccum sorghinum is a tenuazonic acid (Steyn & Rabie, 1976) and phytotoxin producing (Venkatasubbaiah, *et al.*, 1992) fungus belonging to the Ascomycota (family Didymellaceae). This soil-borne fungus occurs globally on a wide range of hosts and is known as a secondary invader or weak pathogen (Zainum & Parbery, 1974, Boerema, *et al.*, 2004, do Amaral, *et al.*, 2004). It has also been documented as a human pathogen (Rishi & Font, 2003). In 1975, it was demonstrated that this fungus is the causal agent of onyalai, a bleeding disorder mainly found among people living in Southern Africa (Rabie, *et al.*, 1975).

Morphology-based identifications of this and other species belonging to the Coelomycetes are generally challenging (Aveskamp, *et al.*, 2010). This is because only a few characteristics that sometimes overlap are used to differentiate between these fungi (de Gruyter, *et al.*, 2010). For example, the same conidial size and shape, or the size of the chlamydospores, or colour of the mycelial mat could often occur in more than one species (Boerema, *et al.*, 2004). It was also found that subculturing often results in the loss of identifiable characteristics (Boerema, *et al.*, 1965).

DNA-based studies (CHAPTER 2) have demonstrated that *Epicoccum sorghinum* may be represented by genetically diverse groups. However, Cai, *et al.* (2009) suggested in a study on *Colletotrichum* that it is necessary to use a polyphasic approach to stabilize the taxonomy of a genus. *Colletotrichum* identification poses the same difficulties as *Epicoccum* in that its species are not easily identified morphologically, they also occur on many different hosts, and their classification seems to be problematic. Such an approach could prove to be a useful tool in the classification of *Epicoccum sorghinum* as individual

isolates are not necessarily morphologically distinguishable from one another. An alternative to the use of morphological traits and DNA-based markers could be the use of carbon source utilization profiles.

Carbon source utilization has been successfully used as a phenotypic identification method for yeasts (Barnett & Pankhurst, 1974) and bacteria (Miller & Rhoden, 1991). Although it is not widely used for the identification of mycelial fungi, carbon source utilization has been shown to be useful to distinguish between some fungal species (Botha*, et al.*, 1997, Campbell*, et al.*, 1997, Khalil & Alsanius, 2009). The aim of this study was to determine whether carbon source utilization could be used to differentiate between genetically diverse groups found within *E. sorghinum*.

2. MATERIALS AND METHODS

A solution was prepared for each of the 37 carbon sources selected (TABLE 1), which contained 67 g/L Difco ™ Yeast Nitrogen Base and 50 g/L of each of the respective carbon sources. Each solution was then filter sterilized using 47 mm Nalgene cellulose acetate filters with a 0.45 µm pore size. Of these solutions, 0.5 mℓ was added to sterile McCarthy bottles containing 4.5 mℓ sterile distilled water. Bottles were prepared in duplicate for each carbon source tested.

Twenty five isolates (TABLE 2) of *E. sorghinum* used in CHAPTER 2 were tested for carbon source utilization. These isolates are deposited in the CGM culture collection at the University of the Free State. Water agar plates, containing 15 g of Biolab Agar Bacteriological (Merck, Wadeville, Gauteng) per litre of distilled water, were prepared.

All cultures were grown in duplicate for one week in the dark at 25ºC. The cultures were cut into 5 x 5 millimetre disks that were used to aseptically inoculate each of the carbon source bottles. The bottles were loosely closed to allow airflow to promote culture growth. Each carbon source was tested in duplicate for each isolate using separately inoculated water agar plates for each duplicate. As negative control, a water agar disk containing mycelium of each isolate was used to inoculate individual McCartney bottles that contained only 67 g/L Difco ™ Yeast Nitrogen Base without any carbon source.

After four weeks of incubation in the dark at 25ºC and at a 45º-angle, culture growth was observed visually in each McCartney bottle relative to the control. The density of the hyphal growth in the control treatment was compared to those McCartney bottles containing the respective carbon sources. In addition, pycnidium and colour formation relative to the control were also noted.

3. RESULTS

Results indicated that the tested isolates of *Epicoccum sorghinum* showed very little variation in the utilisation of carbon sources (TABLES 3-5). In total, 36 of the 37 carbon sources tested could be utilised by most of the isolates tested. However, none of the isolates could utilise starch (TABLE 3). All duplicates responded the same to each carbon source with minimal differences in colour or growth observed. Strain ATCC 12115 utilised the least number of carbon sources tested.

All isolates, except ATCC 12115, produced pycnidia on certain carbon sources tested (TABLE 4). The lack of pycnidia formation by this isolate is consistent with the fact that it

is a sterile culture. There was notable variation between the isolates for which pycnidium formation was observed. Each isolate appeared to have its own set of carbon sources that showed pycnidium formation without any particular pattern. However, D-arabinose, adonitol, arabitol, xylitol and dulcitol seemed to promote pycnidial formation in more than 80% of the isolates tested where inulin, D-gluconid acid lactone, D-gluconic acid and lactic acid promoted pycnidial formation in more than 65% of the isolates tested. Most of the isolates had the capability to produce pycnidia on less than 50% of the carbon sources tested, but seven isolates (CGM 1626, CGM 1634, CGM 1638, CGM 1641, CGM 1644, CGM 1667 and CBS 180.80) produced pycnidia on more than 50% of the carbon sources tested.

Production of colour pigmentation that diffused into the carbon source solutions were not widely observed (TABLE 5). However, some carbon sources seemed to promote the formation of yellow to orange discolouration of the solutions. These included arbutin where 18 of the 25 tested isolates discoloured the medium. Other carbon sources that seemed to also promote discolouration of the media to some extent included erythritol (10 of 25 isolates) and glucuronic acid (8 of 25 isolates).

4. DISCUSSION

It was previously demonstrated that in a natural soil environment, different carbon sources are utilized by different fungal taxa in order for species to coexist (Hanson*, et al.*, 2008). As *E. sorghinum* is a known soil-borne pathogen, one would expect that it would also have a certain carbon source niche. In this study however, there was very little variation in the

utilisation of carbon sources and most of the isolates used in this study were able to utilise the vast majority of carbon sources tested. One could speculate that the ability of this ubiquitous fungus to utilize a vast array of carbon sources, is the reason why it has the ability to colonise more than 80 plant host species and have the ability to utilise a wide range of substrates (Boerema, *et al.*, 2004).

In previous studies, carbon source utilization proved to be a useful method in distinguishing between different species, and even between different strains of a particular species (Marais, 1996, Derso & Waller, 2003, Bridge, *et al.*, 2008, Prihastuti, *et al.*, 2009). No particular patterns were observed regarding carbon source utilisation between the different genetic groups identified in CHAPTER 2. Carbon utilisation, therefore, does not reflect the genetic variation observed in CHAPTER 2.

Fungi need hydrolytic enzymes that hydrolyse complex substrates into smaller molecules that can be utilized by the cells (Garraway & Evans, 1984). For instance, α-amylase is needed to break down starch (Gupta, *et al.*, 2003). Many fungal species have the ability to produce α-amylase such as *Fusarium* sp., *Phytophtora* sp., *Aspergillus* spp., and *Trichoderma viride* among others (Hankin & Anagnostakis, 1975, Abouzied & Reddy, 1986, Carlsen & Nielsen, 2001). None of the isolates of *Epicoccum sorghinum* tested could utilise starch. Whether this is a unique characteristic among isolates in *Epicoccum sorghinum* compared to *Epicoccum nigrum* and *E. pimprinum* is not known. Ironically, many isolates of *E. sorghinum* used in this study have been isolated from starch-based crops such as sorghum, millet, maize, wheat, barley, and rice. The dominance of this fungus in crops such as sorghum, millet and maize in Southern Africa can, therefore, not be attributed to starch as a carbon source. This implies that isolates of *E. sorghinum* either

obtain their carbon-based nutrients from elsewhere, or they rely on other microorganisms to convert the starch into utilisable forms.

Different fungal species, and even strains, have different abilities to independently utilize substrates for vegetative growth or sporulation (Griffin, 1981). This is also clear in this study where four isolates were capable of producing pycnidia on starch, although no obvious vegetative growth was observed. Although no particular patterns regarding the formation of pycnidia in comparison with the genetic clades were observed, it seems that a number of carbon sources have the ability to induce fruiting although the mechanism is not clear. Due to the fact that many isolates of *Epicoccum sorghinum* take considerable time (months) to fruit, or do not easily sporulate on culture media, the use of these sporulation promoting carbon sources could proof to be useful in the study of the fruiting structures and morphology of this fungus. Kumara & Rawal (2008) found that fructose induced heavy sporulation by *Colletotrichum gloeosporioides,* but that the fungus sporulated poorly on other substrates such as mannitol and lactose. In this study, fructose was not investigated, but lactose also induced poor sporulation, where mannitol induced sporulation in 50% of isolates tested. Further investigation to determine the value of using D-arabinose, inulin, adonitol, xylitol, arabitol, dulcitol, D-gluconic acid lactone, D-gluconic acid, and lactic acid as fruiting promoting agents for the members of *Epicoccum* and *Phoma* is needed.

Overall, the discolouration of media by the utilisation of carbon sources was very limited. None of the carbon sources tested showed any patterns of discolouration that resembles the genetic clades in CHAPTER 2. Arbutin was the only carbon source that promoted the yellow to orange discolouration in the majority of isolates tested. Interestingly, arbutin is used in standardised tests for β-glucosidase activity, as this enzyme has the ability to split

arbutin into glucose and a coumarin derivative (Sangeetha & Thangadurai, 2013). In another pathway, it splits arbutin into glucose and a hydroquinone derivative (Levin, 1976). This pathway is often found in nature and sometimes is used by plants as a natural defence mechanism where either the plant or the fungus can provide the enzyme. For instance, in pear trees the pathogen, *Erwinia amylovora*, often hydrolyse arbutin in the plant which yields high levels of hydroquinone, killing the fungus (Levin, 1976). Although this pathway could negatively influence fungal growth, β-glucosidase forms part of the fungal cellulase pathway (Parry*, et al.*, 2001) that acts on soluble substrates to degrade cellulose (Silva, 2013). As both coumarin and hydroxyquinone are reported to have an orange or tan colour (Honda*, et al.*, 1996, Anonymous, 2013) it is not possible to determine which pathway was simulated in the carbon source utilization test and further investigation is needed.

In conclusion, carbon source utilisation does not seem to be useful as a taxonomic tool among isolates of *E. sorghinum*. However, some carbon sources seem to be useful in the promotion of fruiting structures and can likely be used to study morphological characteristics of these fungi in artificial media, and possibly also other fungal species in the Coelomycetes. Further studies are, however, needed to realise the full potential of using these carbon sources for this purpose. As was suggested by Prihastuti (2009), carbon source utilization can be a useful tool in taxonomic studies and, although this study was not useful in distinguishing among phylogenetically different clades of *E. sorghinum*, further studies are needed to investigate whether this methodology can be used at a higher taxonomic level to distinguish between different species of the Didymellaceae.

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TABLE 1. Carbon sources used to test utilization by *Epicoccum sorghinum*

Reference Number	Carbon Source	Reference Number	Carbon Source
C1	α-D(+)-Glucose	C20	Starch
C2	D(+)-Galactose	C21	i-Erythritol
C3	D(+)-Glucosamine	C22	Adonitol
C4	D(+)-Xylose	C23	Xylitol
C5	L(+)-Arabinose	C24	L(-)Arabitol
C6	D(-)-Arabinose	C25	D-Sorbitol
C7	α-L-Rhamnose	C26	D-Mannitol
C8	Sucrose	C27	Dulcitol
C9	Maltose	C28	Myo-Inositol
C10	D(+)-Trehalose	C29	D-Gluconic Acid Lactone
C11	1-0-Methyl-α-D-Glucopyranoside	C30	D-Gluconic acid
C12	D(+)-Cellobiose	C31	D-Glucuronic acid
C13	Salicin	C32	D(+)-Galacturonic acid
C14	Arbutin	C33	DL-Lactic acid
C15	α-D-(+)-Melibiose	C34	Succinic acid
C16	α-Lactose	C35	Citric acid
C17	D(+)-Raffinose	C36	1,2-Propylenglycol
C18	D(+)-Melezitose	C37	Gulonic Lactone
C19	Inulin		

TABLE 2. ***Epicoccum sorghinum*** **isolates tested for carbon source utilization**

Culture collection number	CHAPTER 2 (FIGURE 2) Clade	Fungal species	Host or substrate	Location	Country
CMG 1665	A	*Epicoccum sorghinum*	*Acacia mellifera*	Northern Cape	South Africa
CMG 1673	A	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia
CMG 1669	A	*Epicoccum sorghinum*	*Acacia mellifera*	Ondangwa	Namibia
CMG 1672	B	*Epicoccum sorghinum*	*Acacia mellifera*	Ondangwa	Namibia
CMG 1628	B	*Epicoccum sorghinum*	sorghum	Ovamboland	Namibia
CMG 1670	E	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia
CMG 1667	F	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia
CMG 1666	F	*Epicoccum sorghinum*	*Acacia mellifera*	Northern Cape	South Africa
CMG 1634	F	*Epicoccum sorghinum*	millet	Ovamboland	Namibia
CMG 1671	F	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia
CMG 1640	F	*Epicoccum sorghinum*	sorghum	Sekukuniland	South Africa
CMG 1631	F	*Epicoccum sorghinum*	millet	Settlers	South Africa

Culture collection number	CHAPTER 2 (FIGURE 2) Clade	Fungal species	Host or substrate	Location	Country
CMG 1668	F	*Epicoccum sorghinum*	*Acacia hebeclada*	Ondangwa	Namibia
CMG 1644	F	*Epicoccum sorghinum*	sorghum	Settlers	South Africa
CMG 1661	F	*Epicoccum sorghinum*	*Acacia mellifera*	not available	Namibia
CMG 1662	F	*Epicoccum sorghinum*	thatch	Shelley Beach	South Africa
CMG 1629	F	*Epicoccum sorghinum*	oats	Clarens	South Africa
ATCC 12115	H	*Phyllosticta sorghina*	sugar cane	Florida	United States of America
CMG 1626	I	*Epicoccum sorghinum*	sorghum	Bethal	South Africa
CMG 1663	I	*Epicoccum sorghinum*	thatch	Elliot	South Africa
CMG 1630	I	*Epicoccum sorghinum*	barley	Caledon	South Africa
CMG 1641	J	*Epicoccum sorghinum*	sorghum	Estancia	South Africa
CMG 1664	J	*Epicoccum sorghinum*	thatch	Aliwal North	South Africa
CMG 1638	K	*Epicoccum sorghinum*	sorghum	Lichtenburg	South Africa
CBS180.80	K	*Epicoccum sorghinum*	maize	Potchefstroom	South Africa

TABLE 3. Vegetative growth observed on 37 carbon sources tested[1]

Culture collection number	CHAPTER 2 FIG 2 clade	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36	C37	Control
CGM 1665	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	+	-	-	+	+	+	+	+	-
CGM 1673	A	+	+	+	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+	-	-	+	-
CGM 1669	A	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
CGM 1672	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	+	-	-	+	-	+	+	-	-	+	-
CGM 1628	B	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	-
CGM 1670	E	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-
CGM 1667	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	++	+	+	+	+	-	+	+	+	+	+	+	++	-
CGM 1666	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	++	-
CGM 1634	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	-	-	+	+	+	+	+	+	+	-
CGM 1671	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	++	+	++	+	+	+	+	+	+	+	+	+	+	++	-
CGM 1629	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CGM 1640	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-
CGM 1631	F	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	-	+	-	+	+	+	+	-
CGM 1668	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-	-	-
CGM 1644	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	++	-
CGM 1661	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-
CGM 1662	F	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
ATCC12115	H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	-	-	-	+	+	-	+	+	+	+	-
CGM 1626	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CGM 1663	I	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CGM 1630	I	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CGM 1641	J	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	++	++	+	+	+	+	-	-	-	+	+	+	+	+	-
CGM 1664	J	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CGM 1638	K	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-
CBS 180.80	K	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-

1 The ability to utilize a specific carbon source is indicated with: – (no additional vegetative growth in comparison with the negative control), + (more vegetative growth observed in comparison with the negative control), ++ (abundant vegetative growth in comparison with other carbon sources tested for the isolate in question). C1 to C37 correlates with the carbon sources listed in TABLE 1.

TABLE 4. Pycnidium formation observed on 37 carbon sources tested[2]

Culture collection number	CHAPTER 2 FIG 2 clade	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36	C37	Control
CGM 1665	A						pp	p	p		p				p								p	p		p	p	p		p	p			p		p			
CGM 1673	A	p		p			p	p						p						p		p	pp	p	p	p		pp	p	p	p		p				p		
CGM 1669	A			p			p	p			p			p						p			p	p		p		p	p	p				p			p		
CGM 1672	B						p													p			p	p	p			pp	pp	pp	pp		pp	p	p	p	p		
CGM 1628	B						p													p	p		p	p	p	p	pp	pp			p	p		pp	pp		p	p	
CGM 1670	E		p				p																p	p	p		p	p	p				p					p	
CGM 1667	F			p	p	p	p			p		p			p				p	p				p	pp	pp	pp	p	p	pp	p					p		p	
CGM 1666	F						p				p								p	p			pp	p	pp	pp	pp	p		p	p			p					
CGM 1634	F			p		p	p	p		p	p		p		p		p		p	p			pp	p	pp	p	p	pp	p	pp	p	p	p	p	p	p	p	p	
CGM 1671	F						p				p									p	p	p	p		pp	p	pp	pp			p		p			p			
CGM 1629	F		p	p			p	p		p					p					p			pp	p	p			pp		p	p	p		pp					
CGM 1640	F			p			p							p	p								pp			p		pp	p		p		p	p					
CGM 1631	F			p			p				p								p	p	p		p	p	p	p		p				p	p	p		p		pp	
CGM 1668	F			p			p					p								p			pp	p	p			pp		p	p			p				pp	
CGM 1644	F			p	p		pp	pp			pp		p				p			p			p	p	p	p	p	pp	p	p	p	pp			p		p	p	
CGM 1661	F		p		p	p	p																pp					pp	p	p	p			p					
CGM 1662	F						p																p	p	p			p			p							p	
ATCC12115	H																																						
CGM 1626	I	p					pp	p		p	p		p	p				p	p	pp			pp	pp	pp	pp	p	pp	p	p	pp	p		pp			p	p	
CGM 1663	I			p			pp												p	p			p	p	pp			pp	p	p				p				p	
CGM 1630	I	p			p							p	p		p								p	p	pp			p							p				
CGM 1641	J	p	pp		pp	pp	p	pp		p	pp	pp	pp	p	p	pp	pp	p	pp	p		pp	p	p	pp	pp	p	p	pp	p	p			p	pp	p	pp	p	
CGM 1664	J			p			p																p	p	p	p	p	p	p	p			p		p			p	
CGM 1638	K		p	p	p	p	p				p	p	p	p						p		p	p	p	p	p	p	pp	pp	p	pp		p	p	p	p	p	p	
CBS 180.80	K		p	p	p	p	p	p	p	p	pp	pp	pp	pp			p			p	p		pp	pp	pp	pp	pp	pp	pp	pp	p	p	p	p	p	p	pp	pp	

2 The ability to produce pycnidia on the carbon sources evaluated: p = few pycnidia observed, pp = abundant pycnidia observed. C1 to C37 correlates with the carbon sources listed in TABLE 1.

TABLE 5. Colour formation observed on substrates containing the 37 carbon sources tested[3]

Culture collection number	CHAPTER 2 FIG 2 clade	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24	C25	C26	C27	C28	C29	C30	C31	C32	C33	C34	C35	C36	C37	Control
CGM 1665	A														ly																								
CGM 1673	A													ly																									
CGM 1669	A														do																								
CGM 1672	B													ly				ly																					
CGM 1628	B	ly							ly	ly						ly	ly	ly	ly			ly																	
CGM 1670	E														ly															ly	ly								
CGM 1667	F	ly						y	ly		y		ly		ly	ly	y		ly													ly							
CGM 1666	F				ly			y	y						ly			ly	ly			y		ly					y	ly	ly							
CGM 1634	F								ly							ly						dy																
CGM 1671	F																												y			ly						
CGM 1629	F			ly										y	y							ly																
CGM 1640	F														do							ly		ly	ly		ly											
CGM 1631	F														ly																							
CGM 1668	F													ly	do	ly						y																
CGM 1644	F							y							ly				ly			y																
CGM 1661	F														do							ly																
CGM 1662	F														ly																	ly							
ATCC12115	H				ly									ly	ly				ly													ly			ly				
CGM 1626	I	lp		ly					ly	ly		y	y	y	lp	y	ly	ly	ly			ly																
CGM 1663	I					ly					ly			ly	ly				ly	y				ly			ly					ly					ly		
CGM 1630	I	lp	lp	lp	ly	ly		ly	ly	ly	ly		y		y	ly	ly	ly	ly			ly					ly	ly	y	ly				y		ly		
CGM 1641	J														dy																							
CGM 1664	J													ly	do					ly												ly			ly				
CGM 1638	K													ly																								
CBS 180.80	K	lp													do																								

3 Colouration in the substrate is depicted as follows: ly = light yellow colouration in substrate; lp = light peach colouration in substrate; y = yellow colouration in substrate; dy = dark yellow colouration in substrate; do = dark orange colouration in substrate. C1 to C37 correlates with the carbon sources listed in TABLE 1.

SUMMARY

Thesis title: Comparative study of *Epicoccum sorghinum* in Southern Africa

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The genus *Phoma* has a complex taxonomic history. More than 2000 species were described with no true taxonomic value since its first description in 1880. These species were extensively revised and reduced to 223 taxa in 2004, dividing them into nine sections based on morphological characteristics. However, not all species were considered. Advances in molecular identification led to the delineation of many sections within the Didymellaceae but its taxonomy is not yet clearly resolved. One of the nine sections, *Phoma* section *Peyronellaea*, among others, was dissolved and the genus *Peyronellaea* re-instated. *Epicoccum sorghinum* belonged to this section before it was moved to the genus *Epicoccum* based on morphological and phylogenetic evidence. *Epicoccum sorghinum* has a worldwide distribution and is considered a weak secondary pathogen of plants. Furthermore, it produces a variety of metabolites and is a known human pathogen. Although *Epicoccum sorghinum* is not regarded to be economically significant, it serves as a good example to illuminate the taxonomic complexities of *Phoma.*

A phylogenetic study was conducted on Southern African isolates of *E. sorghinum* to determine whether this fungus resembles multiple taxa or only a single species in this region, as this fungus was isolated from multiple hosts and is known to cause a disease called onyalai in the southern parts of Africa. Although clades within the *E. sorghinum* group were not specifically based on toxicity, host specificity, or geography, different clades did form between non Southern African and Southern African isolates. This study indicated that there is extensive diversity within *E. sorghinum* based on the phylogenetic analysis of the Translation Elongation Factor 1-alpha marker. It is suggested that this marker should be used in the future for further identification of *Phoma* and to resolve taxonomic difficulties in the Didymellaceae.

When the morphology for initial identification was examined, it was found that cultural characteristics were inconsistent. Therefore, we deduced that morphology is not an accurate identification tool for *E. sorghinum.* It was suggested before that it is important to follow a polyphasic approach in stabilizing the taxonomy of a genus. An attempt was made to validate the phylogenetic diversity found in *Epicoccum sorghinum* by testing the carbon source utilization capabilities of various isolates. This method previously proved successful in distinguishing between other fungal species. In this study, however, there was very little variation in the utilisation of carbon sources. Although no particular patterns were observed regarding carbon source utilisation between the different genetic groups identified, other interesting patterns were observed. For instance, none of the isolates of *Epicoccum sorghinum* tested could utilise starch, a number of carbon sources stimulated fungal fruiting although the mechanism was not clear, and some isolates had the ability to produce discolouration in arbutin, suggesting that *E. sorghinum* has the potential to produce either coumarin or hydroxyquinone. In conclusion, carbon source utilisation does not seem to be useful as a taxonomic tool to distinguish between

genetically diverse isolates of *E. sorghinum*. However, some carbon sources seem to be useful in the promotion of fruiting structures and can likely be used to study morphological characteristics of these fungi in artificial media, and possibly also other fungal species in the Didymellaceae.