Endophytic seed mycobiome of six sorghum (*Sorghum bicolor*) cultivars from commercial seedlots using an Illumina sequencing approach

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Abstract

Pathogen-free seeds are important for the establishment of young seedlings, prevention of health problems upon consumption by animals and livestock, and inadvertent movement of pathogens into and out of countries. However, testing for the presence of fungi, including pathogens from seeds is a time consuming and difficult process. In this study, we characterized the seed fungal microbiome (mycobiome) of six commercial sorghum cultivars from South Africa using a deep amplicon next generation sequencing approach based on the Internal Transcribed Spacer (ITS) region of the ribosomal operon. Sorghum is the fifth most important crop in the world, and widely used by African farmers. We found that the fungi present in each of the seedlots were similar to those reported by cultural studies. By comparing phylotypes of certain key families and genera to phylotypes used in established phylogenies and reputed sequences from public databases, the diagnostic value of the NGS method was also investigated. We showed that a number of molecular operational taxonomic units (MOTUs) could be identified at the species level and established that certain known pathogens are not present in the tested seeds, for instance in the *Aspergillus* group. Other groups could not be identified, not even to genus level. While acknowledging the shortcomings of using partial ITS data, we demonstrated that deep amplicon sequencing is a valuable diagnostic tool for seed disease control and prevention in some cases.

Key words – Deep amplicon sequencing – Diagnostics – Endophytic Fungi – Pathogens – Seed-borne fungi

Introduction

Pathogen-free seeds are one of the most important factors for successful crop production. Many plant pathogens are seed-borne and can cause failed establishment of plants, enormous crop losses, the reduction in plant growth and productivity, and high costs to eradicate the pathogens once established (Dawson & Bateman 2001, Islam et al. 2009). For instance, when untreated seeds

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are grown in the field, seed-borne pathogens of sorghum are responsible for variation in plant morphology and yield reduction of up to 90% (Wiese 1984). Seed-borne pathogens also cause spoilage when seeds are used as food, or contamination of grain through the productions of toxins that can negatively affect humans and livestock that consume the seeds (González et al. 2008).

The seed microbiome consists of obligate and facultative endophytes (ubiquitous microbes occurring inside living plant tissues that do not cause any apparent symptoms in the plant) inside the seed, and those organisms occurring on the exterior (Baldani et al. 1997, Ferreira et al. 2008, Kaga et al. 2009). Vertically-transmitted endophytes represent the seed core microbiome, usually occurring in seeds over many generations (Hodgson et al. 2014). Horizontally-transmitted microorganisms from the environment constitute the accessory microbiome (Hodgson et al. 2014). Endophytes can be mutalistic symbionts that, for instance, enhance growth, and confer tolerance to abiotic stress, or resistance to pests, herbivores and pathogens (Hyde & Soytong 2008, Oldrup et al. 2010, Ralphs et al. 2011, Cook et al. 2013, Wani et al. 2015). Phytopathogenic and rotting organisms can also have latent endophytic phases, but these do not always have vertical transmission, as in the case of members of the genera *Alternaria* and *Cladosporium* (Oliver et al. 2001, Galperin et al. 2003, Sowley et al. 2010). The majority of pathogenic fungi in seeds are probably derived from the environment such as surrounding soil and air in either the field or storage places, when fungal propagules land on the exterior of the seed coat before germination (Hodgson et al. 2014, Parsa et al. 2016).

Sorghum (*Sorghum bicolor*) is the fifth most important grain crop in the world after wheat, maize, rice and barley. In semi-arid regions of Africa, Asia and Latin America, it is one of the major staple food crops (Rooney & Waniska 2004, Dicko et al. 2006), especially in many rural communities. This is especially true in the more drought prone areas of South Africa where this hardy crop provides better household food security than maize (Patil et al. 2014). Seventeen of the twenty-five species of sorghum are native to Australia, with the range of some extending to Africa, Asia, Mesoamerica, and certain islands in the Indian and Pacific oceans (Spenceley et al. 2005). *Sorghum bicolor* is native to Ethiopia (Adugna 2014) while other species of sorghum native to Africa include *Sorghum arundinaceum*, *Sorghum drummondii* and *Sorghum halapensis* (Royal Botanic Gardens Kew & Missouri Botanic Garden 2017).

Despite the economic importance of sorghum, only a limited number of studies have focused on diseases and food problems linked to sorghum seeds, and mainly on grain mold disease, which is caused by different fungi in the genera *Fusarium*, *Alternaria*, *Cochliobolus* and *Cladosporium* (Frederiksen & Odvody 2000, Tarekegn et al. 2006, Sharma et al. 2010). Seed-borne pathogens can be responsible for deteriorating seed quality during storage and can be viable even after seeds have been treated with fungicides. Reported species include *Alternaria alternata*, *A. tenuissima, A. longissimi*, *Fusarium culmorum*, *F. moniliforme*, the *F. incarnatum-equiseti* species complex, *F. andyazi*, *F. thapsinum,* the *F. oxysporum* species complex and the *F. chlamydosporum* species complexes, *Curvularia penniseti*, *C. lunata*, *Drechslera rostrata*, *Exserohilum rostratum Aspergillus fumigatus, A. niger*, *A. flavus*, *Phoma* spp., *Epicoccum sorghinum*, *Gonatobotrys* spp., *Mucor* spp., *Cladosporium* spp. *Aureobasidium pullulans, Rhizopus stolonifera, R. nigricans*, and *Phytophthora* spp. (Kassim 1985, Thakur et al. 2003, Leslie & Summerell 2006, Patil et al. 2008, Zida et al. 2008, Abdulsalaam & Shenge 2011, Panchal & Dhale 2011, Yago et al. 2011, Stokholm et al. 2016). In South Africa, the dominant fungal genera in sorghum seeds are *Alternaria*, *Phoma*, *Epicoccum, Fusarium, Cladosporium* and *Curvularia* spp. (Tesfaendrias et al. 2011, Beukes et al. 2017).

More research has been done in sorghum seeds to study contamination by mycotoxins and fungi that produce mycotoxins. Sorghum is susceptible to infection by mycotoxigenic fungi such as *Aspergillus flavus*, *A. parasiticus* (producing aflatoxins), *Fusarium thapsinum* (fumonisin), *F. verticillioides* and *F. proliferatum* (fumonisins) (Leslie & Summerell 2006, Beukes et al. 2017). Mycotoxins are of great public health and agro-economic importance because the economic impact of mycotoxins includes loss of human and animal life, increased health and veterinary care costs, reduced livestock production, disposal of contaminated foods and feeds, and investment in research

and applications to reduce severity of the mycotoxin problems (Zain 2011). Factors contributing to the presence or production of mycotoxins in foods or feeds include storage, environmental and ecological conditions favorable to the growth of fungi or production of toxins (Zain 2011) and are often beyond human control (Hussein & Brasel 2001).

Traditionally, the components of the plant and seed microbiomes have been characterized by isolating and culturing microbes on different media and growth conditions. These time and effort consuming culture based techniques can miss unculturable microbial diversity, omit some species during subculturing and accurate morphological identifications require expertise (Hugenholtz et al. 1998, Jeewon & Hyde 2007). Microbial communities are now detectable by modern culture independent techniques such as fatty acid/ protein/enzyme extractions, environmental sequencing and whole environmental metagenomes and metaproteomics (Hugenholtz et al. 1998, Mendes et al. 2011, Knief et al. 2012, Lakshmanan et al. 2014). Furthermore, environmental sequencing of communities, also named mini- or metabarcoding, or deep amplicon sequencing, gives a level of identification for the components of the communities (Li et al. 2019). Understanding community structure, dominance and latent occurrence of microbial pathogens in seeds improves epidemiological studies and strategies for disease management (Peay et al. 2016). Diagnosis of microbiomes in seeds can avoid uncontrolled propagation and quarantine breaches of pathogens through long-distance exchange of such material, will prevent economic losses more efficiently, and diminish unnecessary use of fungicides (Mancini et al. 2016). Pre-knowledge of the infection levels of potential mycotoxin-producing fungi in seeds can prevent risks associated with consumption of such contaminated foods, while a rapid technique to characterize the variation in composition of seed-borne communities between different sorghum cultivars or production areas, will significantly aid breeding efforts. Therefore, the use of a high throughput method to characterize seed microbiomes is greatly advantageous when a large number of samples or collections need to be screened.

To the best of our knowledge, the seed microbiome of sorghum has not hitherto been investigated using a non-culture-based method. The objective of this study was to characterize the seed mycobiome of six commercial sorghum cultivars from specific seedlots of sorghum by means of Ilumina sequencing of the ITS2 region. Using additional phylogenetic analyses, we also assessed the diagnostic feasibility of the method.

Materials & Methods

Plant Material

Seed samples, pre-treated with fungicides (not disclosed by the manufacturer) of six commercial cultivars of sorghum (NS5511, PAN8816, PAN8420, PAN8625, PAN8906 and PAN8706W) were obtained from the seed company Pannar (South Africa). The cultivars were chosen based on their colour and tannin level. PAN8816, PAN8420, PAN8625 and PAN8906 are cultivars of a red variety with a low tannin and high malt content, whereas PAN8706W and NS5511 have tan coloured seeds. PAN8706W has a low tannin and malt content, and NS5511 has a high tannin and malt content.

Illumina sequencing

Twenty seeds were randomly collected from a seedlot of each of the six cultivars. Seed were surface sterilized (immersion in 3% sodium hypochlorite for 3 min, sterile distilled H_2O for 1 min, 70% ethanol for 2 min, rinsed with sterile distilled water for 1 min), freeze dried in 50 ml Falcon tubes, ground, bashed with 2-mm-diameter metal beads in a Qiagen Tissue Lyser II cell disrupter (Whitehead Scientific, Cape Town, South Africa) and the powder transferred to 2 ml Eppendorf tubes for DNA extraction. DNA was extracted using the Nucleospin® Plant II mini Kit (Macherey Nagel, Germany). DNA concentrations were determined using Nanodrop LITE spectrophotometer (Thermo Scientific, USA) and diluted to 10ng/ul.

The ITS2 region (internal transcribed spacer) of the ribosomal operon was amplified using the primers ITS3F (5'-GCATCGATGAAGAACGCAGC-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3'), with overhang Illumina adapters (Nilsson et al. 2009, Caporaso et al. 2012). These primers are universal and result in amplicons that are variable enough to efficiently distinguish between closely related species or to identify operational taxonomic units for most fungi (Op De Beeck et al. 2014). The PCR mixture consisted of 2 μL template DNA (10ng/ Ml), 1.5 μL ITS 3 primer (10 μM), 1.5 μL ITS 4 primers (10 μM), 12.5 μL 2U HiFi ReadyMix DNA Polymerase (KAPA Biosystems, Lasec, SA) and 9 μL nuclease free water (total reaction volume of 25 μL). The negative control replaced DNA with PCR-grade water to evaluate the presence of contaminants. All materials used for the preparation of reactions were sterile and nuclease free. PCR was done using a G-Storm GS04822 thermal cycler (Somerton Biotechnology Centre, United Kingdom- UK) programmed for an initial denaturation at 95 ºC for 3min, followed by 25 cycles at 98 ºC for 20 s, 65 ºC for 30 s and 72 ºC for 30 s, with a final extension at 72 ºC for 5 min. After amplification, the PCR products were visualized under UV light by 2% agarose gel electrophoresis.

The amplicons were sent for sequencing library construction and sequencing at the Next Generation Sequencing Facility at the University of the Free State, South Africa on an Illumina MiSeq platform (Sigma-Aldrich, U.S.A). The quality and quantity assessment for the PCR products were performed using a 2100 Bioanalyzer (Agilent Technologies, Inc.), using a DNA 12000 Chip. Sample quantification was performed using the Picogreen assay (Invitrogen). Finally, the amplified PCR products, after gel purification and recovery using the Agencourt AMPure XP Bead Clean-up kit (Beckman Coulter, Inc.), was normalized, pooled and denatured before being subjected to the MiSeq system for deep sequencing using paired 300 bp reads and MiSeq v3 reagents.

Cluster analysis

The quality of the ITS sequences was performed using PrinSeq-lite v0.20.4 (Schmieder & Edwards 2011) and trimmed to obtain an average quality score of ≥ 20 using a 5 nt window with a 3 nt step. Sequences shorter than 200 bp were filtered out and paired end reads merged using PEAR 0.9.6 (Zhang et al. 2014). Paired end reads were analysed using QIIME v. 1.9.1 as described by Caporaso et al. (2010). Chimeric sequences were identified, using usearch 6.1.544 (Edgar 2010) as the chimera detection method (Edgar 2010), and filtered out of the quality trimmed reads by using identify_chimeric_seqs.py and filter_fasta.py commands, respectively.

Molecular Operational Taxonomic Units (MOTUs) were defined at 99% sequence identity (Nilsson et al. 2011). Taxonomic affiliations of representative MOTUs were performed with the pick_open_refrenece_otus.py script using the UNITE ITS reference database (alpha version 12_11) (Kõljalg et al. 2013). Prior to the analysis, the OTU-table was normalized using normalize_table.py in QIIME with the CSS normalization option (Paulson et al. 2013). Fungal alpha diversity (i.e. Shannon diversity) was obtained with alpha_rarefaction.py. Beta-diversity, obtained with Bray-curtis distances, was visualized using a Principal Coordinates Analysis (PCoA) plot in R (www.r-project.org) with the "plot_ordination" function in the "Phyloseq" package (McMurdie & Holmes 2013). Tentative ecological guilds were assigned to prominent MOTUs that could be assigned to genera using FUNGuild (Nguyen et al. 2016) The DNA reads were deposited under the bioproject accession no. SAMN09831275, SAMN09831276, SAMN09831277, SAMN09831278, SAMN09831279, SAMN09831280.

Phylogenetic analyses

Potentially important pathogenic MOTUs were tentatively identified with the UNITE database to genus level and were further verified using phylogenetic analyses with sequences obtained from datasets of publications and the NCBI database. This was done since species and even generic identities of a number of fungal groups are inconclusive based only on ITS data, and assignment of the generic names of MOTUs may also be incorrect (Nilsson et al. 2006, 2009).

Special attention was given to MOTUs related to grain mold of sorghum, that were dominant in analyses or that were known to be important as pathogens or mycotoxin producers in plants.

Detailed phylogenetic analyses were done for *Alternaria* (dataset from Woudenberg et al. 2013), *Fusarium* (ITS sequences were obtained from the Multilocus Sequence Type database (http://www.westerdijkinstitute.nl/fusarium/), the Didymellaceae including genera such as *Phoma* and *Epicoccum* (dataset from Chen et al. 2015), Cladosporiaceae including genera such as *Cladosporium* and *Toxicocladosporium* (dataset from Bensch et al. 2012), *Colletotrichum* (dataset from Damm et al. 2009 supplemented with the type sequence option of GenBank developed by Schoch et al. 2014), *Cochliobolus* (dataset from Manamgoda et al. 2012, Tan et al. 2014), *Erysiphe*, *Aspergillus*, *Aureobasidium* and *Cryptococcus* (using the type sequence function of GenBank). Sequences were aligned using MAFFT (Kuraku et al. 2013, Katoh et al. 2017) using the gap option and manually checked. The curated datasets were analyzed using MEGA 7 with Maximum Likelihood (ML), using the appropriate evolutionary model per dataset determined with the model function of MEGA (indicated in phylogenetic trees included in figures). A 1000 bootstrap replication was performed to determine the statistical support of branches.
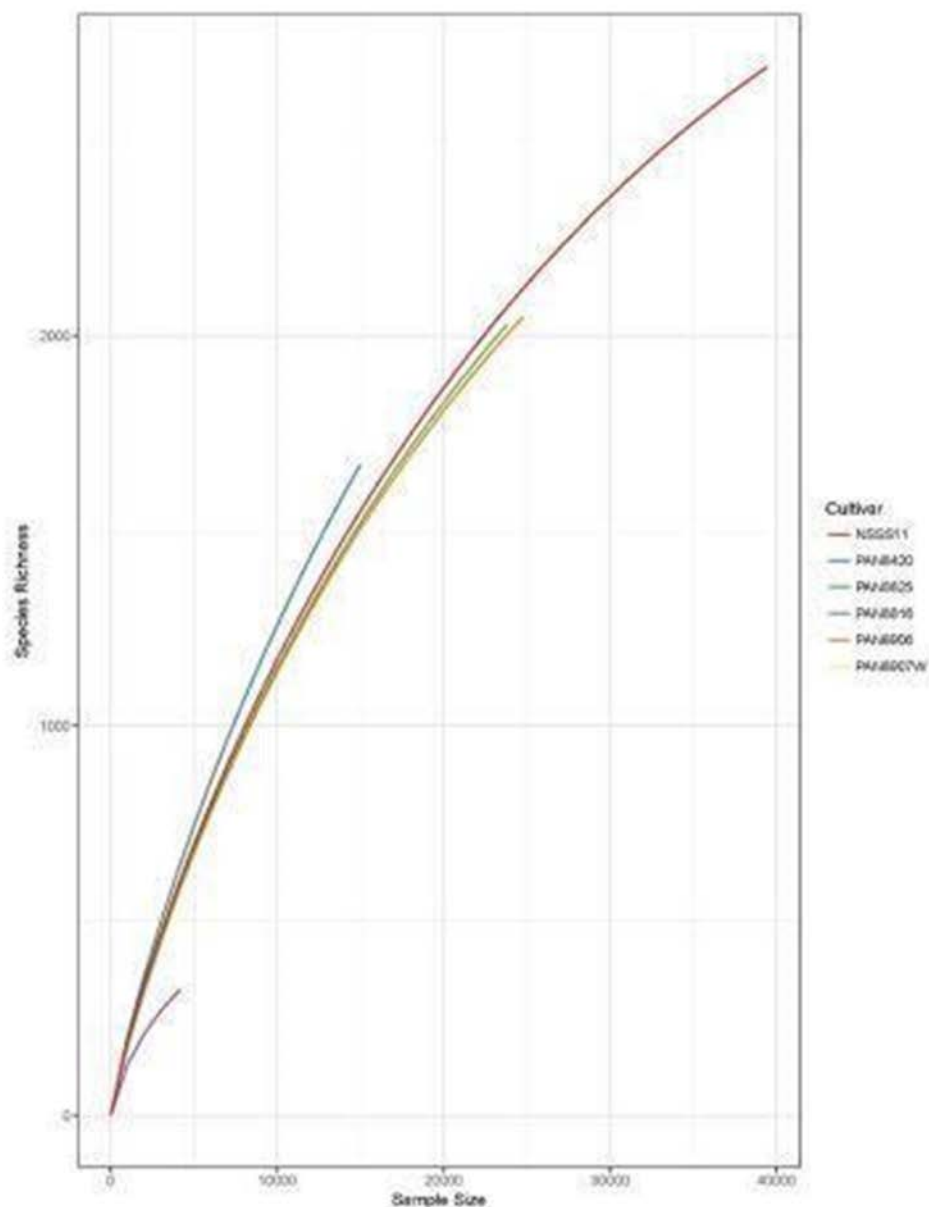
Figure 1 – Rarefaction curves of seed mycobiome for six cultivars of sorghum.

Results

Illumina sequencing

After quality checking and data filtering the read lengths ranged from 200 to 251 bp. A minimum of 27,224 to a maximum of 94,999 sequences were retained for the different seed cultivars (Table 1). After rarefying to 27,224 sequences per sample, the number of unique MOTUs (99% similarity cut-off) across all the seed cultivars was 5,718. These sequences represented a minimum of 834 MOTUs to a maximum of 3397 MOTUs per cultivar (Table 1). Rarefaction curves showed that deeper sequencing is needed to completely resolve the fungal diversity of the samples (Fig. 1). Percentage Relative Abundance plots (Fig. 2, Table 2) showed that the phylum Ascomycota accounted for the largest (93 % average of total number of reads) fraction of the fungi in the six sorghum cultivars. At the class, order and family level, seeds were dominated by Dothideales (41 %, Dothideomycetes), Pleosporales (26 %, Dothideomycetes) and Pleosporaceae (16 %, Dothideomycetes, Pleosporales), respectively (Table 2). A total of thirteen different genera (Table 2) were found. "*Alternaria*" (family Pleosporaceae, order Pleosporales) was present in all cultivars at high levels. "*Phoma*" and "*Epicoccum*" (both Dydimellaceae, Pleosporales), were also relatively high, but were assigned under *incertae sedis*. "*Aureobasidium*" (Dothioraceae, Dothideales) accounted for the largest proportion of the Dothideales but it was only present at a very high level in cultivar PAN8816, and negligible or absent in the others. The only other genera present in higher abundance than 1% were "*Cladosporium*" (Cladosporiaceae, Capnodiales, Dothideomycetes) and "*Fusarium*" (Nectriaceae, Hypocreales), with the latter only present in cultivar NS5511. The high dominance of sequences belonging to the Pleosporales was mostly due to the presence of "*Alternaria*", and to a lesser degree, taxa in the Didymellaceae. Most sequences affiliated to the Dothioraceae resulted from the abundance of the genus "*Aureobasidium*" in the cultivar PAN8816.

MOTUs assigned to "*Alternaria*" were shared by seeds from all cultivars (Fig. 2). Other MOTUs that were shared included what was assigned as a "*Podospora*" and "*Leptosphaerulina*" species, and 13 MOTUs that could not be named (data not shown). Two groups of unknown MOTUs were dominantly present in all six cultivars (Fig. 2). The two principal coordinates explained the variations (differences in the six cultivars), with principal component 1 and principal component 2 showing 59.2.9% and 32.1% of the variation, respectively. The analysis separated the six cultivars into four groups (Fig. 3). Cultivar PAN8625 and PAN8906, and PAN8420 and PAN8907W, respectively, grouped together. PAN 8816 and NS551 grouped separately. Guild analyses (Fig. 4) showed that fungi with complex ecological roles inhabited the various seeds. Within the 16 assigned groups, roles ranged from pathogens of both animals and plants, to endophytes, to saprophytes, as for the most dominant group. This can be correlated with the presence of prominent genera such as *Alternaria* and *Aureobasidium* (Fig. 2) that have complex ecologies. Whereas endophytes were expected to be prevalent, some of the guilds did not include known endophytic roles. The patterns of assigned guilds also were quite variable between the various seeds.

Table 1 Statistical summary of number of reads and Molecular Operational Taxonomic Units (MOTUs) for the six sorghum cultivars investigated.

Cultivars	Number of Reads before QC	Number of Reads after QC	Number of MOTUs before taxon assignment
NS5511	123618	94999	3397
PAN8816	51892	27224	834
PAN8420	55090	31686	1848
PAN8625	78379	61298	2342
PAN8906	63629	47383	2245
PAN8706W	49142	28741	3397

Table 2 Comparative Percentage Abundance from Taxa plots of fungal in different taxonomic ranks in seeds of six cultivars of sorghum measured by Illumina Sequencing of the ITS2 Region.

Division[a]	NS5511	PAN8816	PAN8420	PAN8625	PAN8906	PAN8706W	Average
Ascomycota	**88.6**	**98.3**	**87.6**	**96.3**	**95.1**	**92.4**	**93.1**
Basidiomycota	0	0.4	0.1	0.3	0.3	0.2	0.2
Total percentage abundance	*88.6*	*98.7*	*87.6*	*96.6*	*95.4*	*92.6*	*93.3*
Class							
Dothideomycetes	**53.9**	**78.3**	**31.5**	**22.9**	**22.2**	**36.7**	**40.9**
Eurotiomycetes	0	0	0	0	0.1	0	0.0
Leotiomycetes	0	0.1	0	1.0	0	0.2	0.2
Saccharomycetes	0	0	0	0.1	0	0	0.0
Sordariomycetes	**2.6**	0	0	0.1	0	0.1	0.5
Tremellomycetes	0	0.4	0.1	0.1	0.2	0	0.1
Total percentage abundance	*56.5*	*78.8*	*31.6*	*24.1*	*22.5*	*37.1*	*41.8*
Orders							
Capnodiales	1.5	0.2	0.9	0.3	0.1	0.2	0.5
Dothideales	**0.0**	71.4	0	0.2	0	0	11.9
Pleosporales	**45.5**	**6.5**	**28.3**	**22.5**	**22.**	**32.6**	**26.2**
Eurotiales	0	0	0	0.0	0.1	0	0.0
Erysiphales	0	0	0	1.0	0	0.2	0.2
Saccharomycetales	0	0	0	0.1	0	0	0.0
Hypocreales	**2.6**	0	0	0	0	0.1	0.5
Phyllachorales	**0**	0	0	0	0	0.1	0.0
Filobasidiales	0	0.4	0	0.1	0.1	0	0.1
Total percentage abundance	*49.6*	*78.6*	*29.3*	*24.1*	*22.5*	*33.3*	*39.6*
Families							
Incertae sedis	**22.1**	0.5	**7.6**	0.1	0.6	**11.1**	7.0
Mycosphaerellaceae	1.5	0.2	0.6	0.3	0.1	0.2	0.5
Dothioraceae	0	**53.0**	0	0.1	0	0	8.9
Phaeosphaeriaceae	0	0	0.1	0	0	0	0.0
Pleosporaceae	**14.4**	**5.9**	**17.3**	**22.4**	**21.2**	**17.1**	**16.4**
Trichocomaceae	0	0	0	0	0.1	0	0.0
Erysiphaceae	0	0.1	0	1.0	0	0.2	0.2
Nectriaceae	**2.4**	0	0	0	0	0.1	0.4
Phyllachoraceae	0	0	0	0	0	0.1	0.0
Filobasidiaceae	0	0.4	0	0.1	0.1	0	0.1
Total percentage abundance	*40.4*	*60.1*	*25.7*	*24.0*	*22.2*	*28.9*	*33.6*
Genera							
Toxicocladosporium	0.0	0.0	0.4	0.0	0.0	0.0	0.1
Cladosporium	1.4	0.2	0.5	0.3	0.1	0.2	0.5
Aureobasidium	0.0	**53.0**	0.0	0.1	0.0	0.0	**8.9**
Phoma	**22.1**	0.5	**7.2**	0.0	0.6	**11.1**	**6.9**
Ampelomyces	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Alternaria	**8.6**	**5.8**	**14.6**	**22.4**	**21.0**	**15.3**	**14.6**
Epicoccum	**5.7**	0.1	**2.7**	0.0	0.2	1.7	1.7
Aspergillus	0.0	0.0	0.0	0.0	0.1	0.0	0.0
Erysiphe	0.0	0.1	0.0	0.8	0.0	0.2	0.2
Kodamaea	0	0.0	0.0	0.1	0.0	0.0	0.0
Fusarium	**2.4**	0.0	0.0	0.0	0.0	0.0	0.4
Colletotrichum	0.0	0.0	0.0	0.0	0.0	0.1	0.0
Cryptococcus	0.0	0.4	0.0	0.1	0.1	0.0	0.1
Total percentage abundance	*40.2*	*60.0*	*25.6*	*23.7*	*22.1*	*28.8*	*33.4*

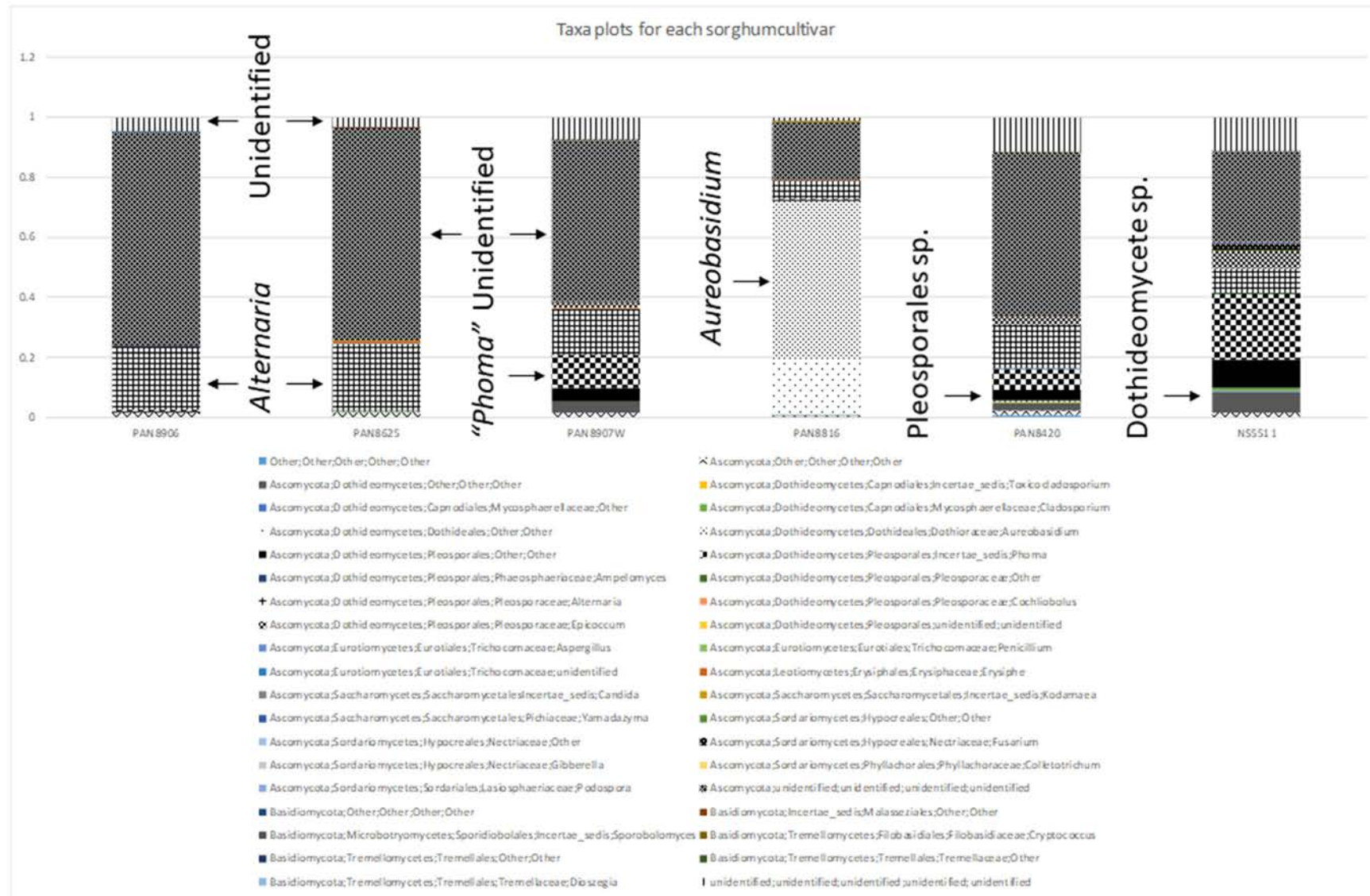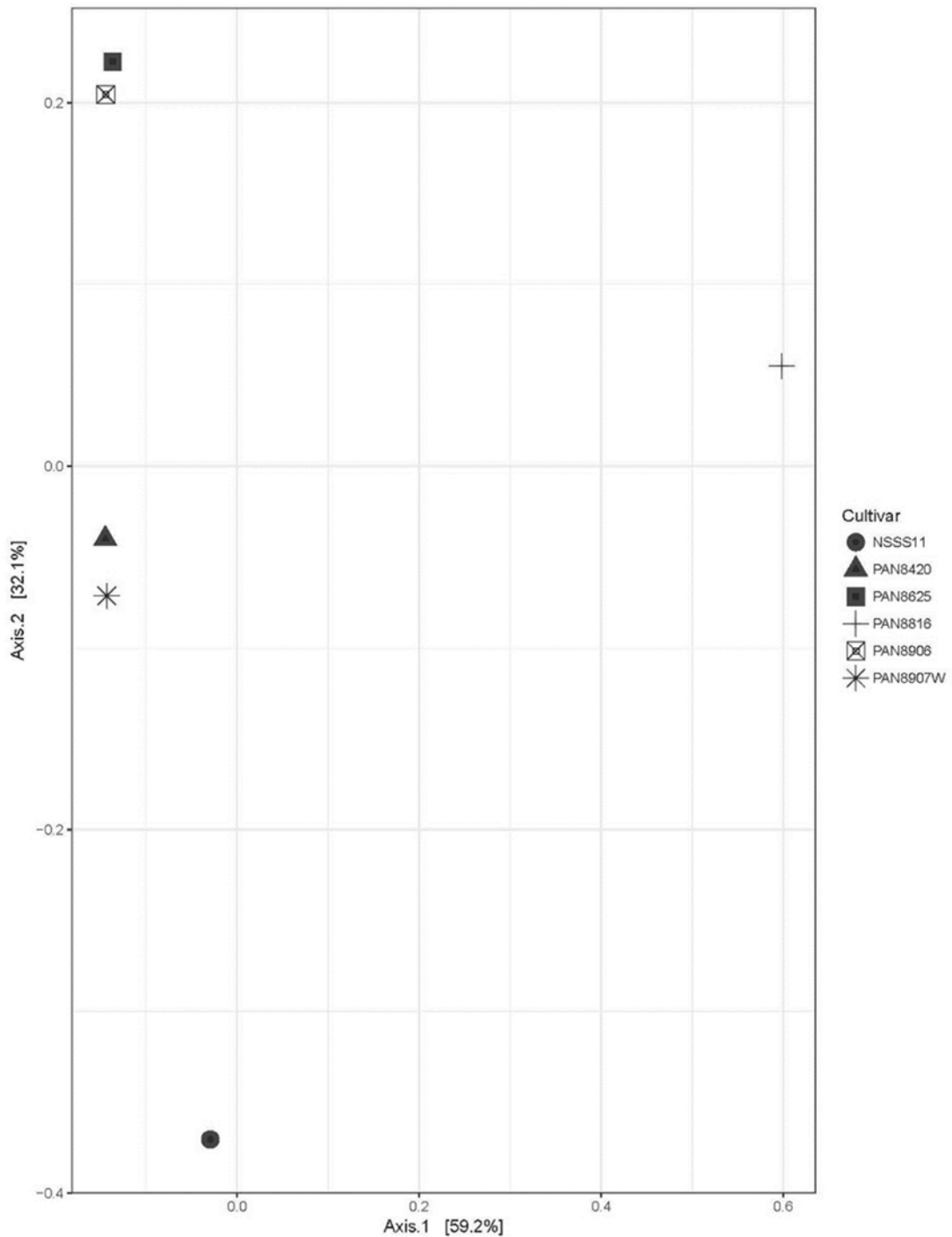[a] Names are used as assigned by the UNITE database used in lthe pipeline.

Figure 2 – Percentage relative abundance of fungi (up to genus level) in seeds of six sorghum cultivars at 99% level of sequence similarity.

Figure 3 – Principal Coordinate Analysis of mycobiome in seeds of six sorghum cultivars obtained with Bray-curtis distances in R. The graph was based on MOTUs defined at a 99% similarity cutoff.

Phylogenetic analysis

Phylogenetic analyses of Illumina reads yielded more precise placements for MOTUs than the automated hits. Reads were either placed in sections or species complexes for some genera, or names given to others were discounted. Putative species positions could also be assigned to some MOTUs. Phylogenetic analysis of the ITS2 region for *Alternaria* sequences placed MOTUs in the *Alternata alternata* complex (Bootstrap support 98%), with MOTUs having a great deal of sequence variation (Fig. 5a). MOTUs of *Fusarium* were genetically diverse and grouped in the

Fusarium incarnatum-equiseti species complex (bootstrap support 99%), *F. chlamydosporum* species complex (bootstrap support 98%), *F. graminearum* species complex (bootstrap support 98%), *Bisifusarium* (bootstrap support 100%) and possibly in the collapsed *F. fujikuroi* species complex (Fig. 5b). The phylogenetic tree for *Colletotrichum* (Fig. 5c) showed that a sequence read grouped in a clade containing *C. truncatum*, *C. aciculare* and *C. jasminigenum* (weakly supported based on bootstrap). Species could, unfortunately, not be distinguished with confidence for these genera.
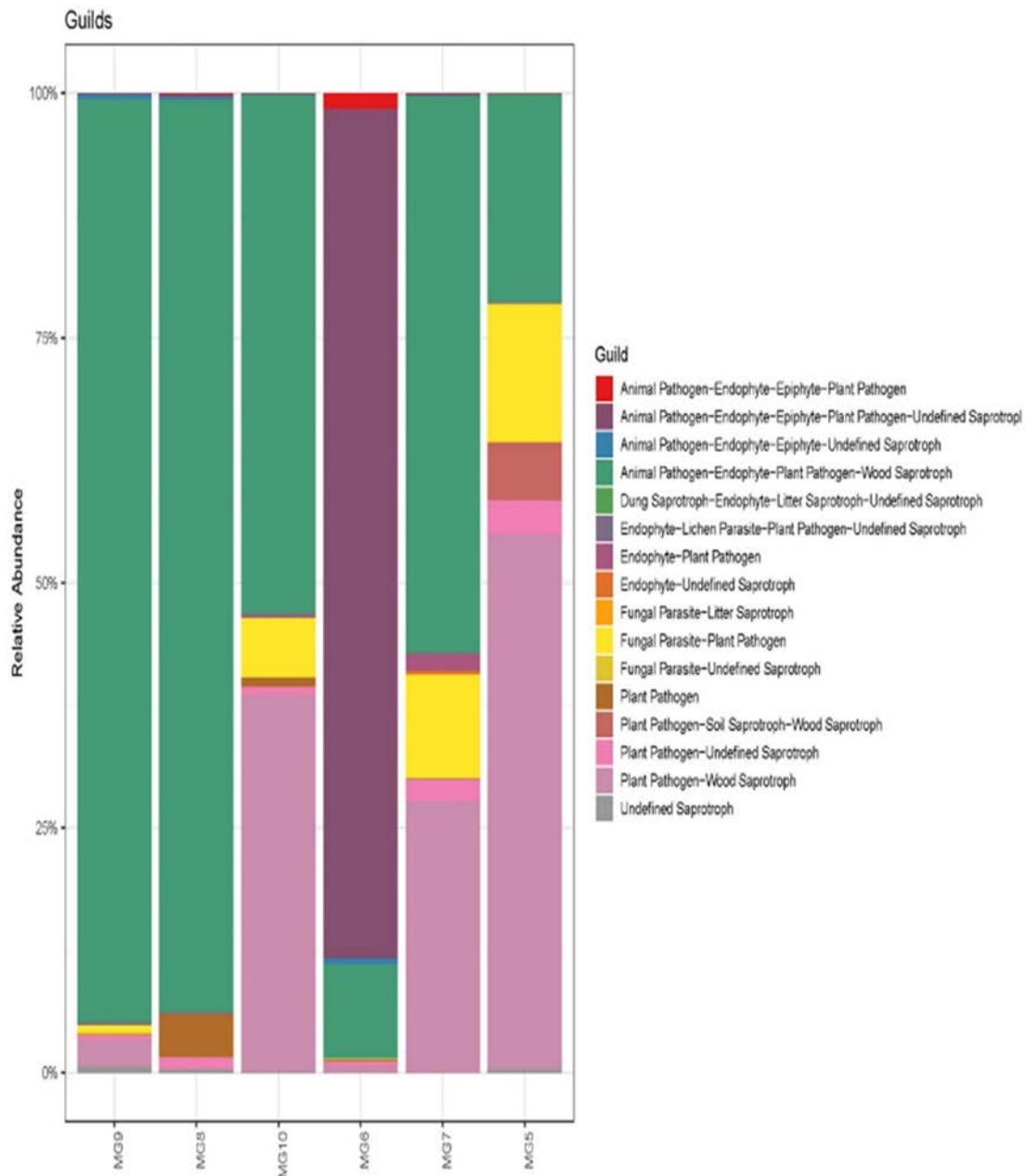
Figure 4 – Ecological guilds assigned with FUNGuild to prominent MOTUs that could be identified to genus level.

Three groups of *Aureobasidium* sequences formed, including *A. namibae* (bootstrap support 92%), three different sequences grouping with *A. melanogenum*, and a possible unnamed species (Fig. 5d). Species sequences from GenBank showed (Fig. 6a) that MOTUs grouped with *Cryptococcus luteolus* (MG10 7046, bootstrap support 100%), MG9 25372 grouped close to *C.*

terrestris (bootstrap support 99%), and MG7 5975 grouped in a clade consisting of *C. flavescens* and *C. laurentii* (bootstrap support 80%). Three reads of *Aspergillus* grouped close to *Aspergillus reticularus*, *Aspergillus penicillioides* (bootstrap support 94%) and *Aspergillus restrictus*, respectively (Fig. 6b). Three reads (MG33980, MG617262 and MG107179) identified as *Erysiphe* (Fig. 6c) grouped close to *Erisyphe multappendicis* (Bootstrap support 92%) and *E. berberidicola*.

Unfortunately, the partial ITS region, and even the ITS region alone, had inadequate resolutions to distinguish between current genera in the Cladosporiaceae and Dydimellaceae, and MOTUs could thus not be assigned to an appropriate genus. More detailed multi-gene phylogenetic analyses will be required for these taxa. It is thus unclear if reads assigned as "*Phoma*" and "*Epicoccum*", or "*Cladosporium*" and "*Toxicladosporium*" truly belong to these genera. Although the particular dataset used for the *Cochliobolus/Bipolaris* group showed a majority of *Bipolaris* taxa grouping close to the read, the generic status of these genera were weakly supported and needs to be validated further. It is thus difficult to assign a proper genus to the sequences. However, the reads did group with sequences of *Bipolaris victoriae*, *B. zeicola* and *B. sorokiniana*, and close to sequences labelled as *Cochliobolus lunatus* and *Curvularia lunata* (Fig. 6d).

Discussion

We characterized the seed mycobiome of six cultivars of sorghum using Illumina sequencing of the ITS2 region. Rarefaction curves showed that more samples should be analysed for a better reflection of the true endophytic diversity. Besides this limitation, all seeds were found to contain fungi with complex ecological roles belonging to 41 MOTUs (99% similarity cutoff). The most prominent family was the Pleosporaceae, represented mostly by the MOTUs assigned to the phytopathogen genus *Alternaria*. Other potentially detrimental fungi were also detected in the seed after phylogenetic analyses was conducted. This is in agreement with previous observations (Tesfaendrias et al. 2011, Beukes et al. 2017), suggesting that seeds are often infected by phytopathogenic fungi.

Alternaria and *Aureobasidium* species and those in the *Didymellaceae* (assigned by the pipeline to "*Phoma*" and "*Epicoccum*") dominated the fungal communities. However, *Aureobasidium* was only dominant in one cultivar. Taxa assigned to *Fusarium*, *Colletotrichum*, *Aspergillus*, *Cryptococcus* and *Erisyphe*, were also found but they were not as dominant and ubiquitous. Other important pathogens or problem groups, namely taxa linked to *Cochliobolus* and Cladosporiaceae, were also detected but in low numbers. These, and MOTUs of possible importance in the *Didymellaceae* and some in the *Pleosporaceae*, could not be satisfactorily assigned to an appropriate genera without the use of additional gene regions.

Additional phylogenetic analyses assisted greatly in more precise identification of MOTUs and yielded more information on the possible roles of the fungi in the seeds. Even if precise species identifications were not possible with ITS data in taxonomically extensive fungal groups, the MOTU identities could be narrowed down to some candidate species, or placed in sections or species complexes, as is the case for *Alternaria* and *Fusarium*. It also elucidated the occurrence of more than one MOTU within fungal genera or families.

Presence, absence or prominence of important groups or species can be revealed, which influence control measures or quarantine. In the case of *Aspergillus*, the three MOTUs could be closely assigned to species not really known to be important as mycotoxin producers (Sklenář et al. 2017), while important species such as *A. fumigatus* and *A. flavus* were not detected. *Colletotrichum sublineolum* that causes anthracnose of sorghum and represents one of the main constraints in sorghum production throughout the world (Basavaraju et al. 2009), was not detected. Instead the MOTU was identified as *C. truncatum*/*C. aciculare*/*C. jasminigenum*, none of which are important on sorghum, with *C. truncatum* an important pathogen on various other crops such as peppers (Damm et al. 2009). However, in some cases results can be misleading since the ITS sequence for certain species has not been produced. For instance, there is no ITS sequence in GenBank for *Curvularia penisetti*, which is known to be associated with disease symptoms of sorghum (Patil et al. 2008).

A large number of taxa that could not be assigned to genus level were detected. This suggests that endophytic fungi are substantially understudied or underrepresented in the UNITE database, especially those from South Africa. Initiatives such as the Top50 search function in UNITE will be useful to refine the identities of these unknown sequences (Nilsson et al. 2016).
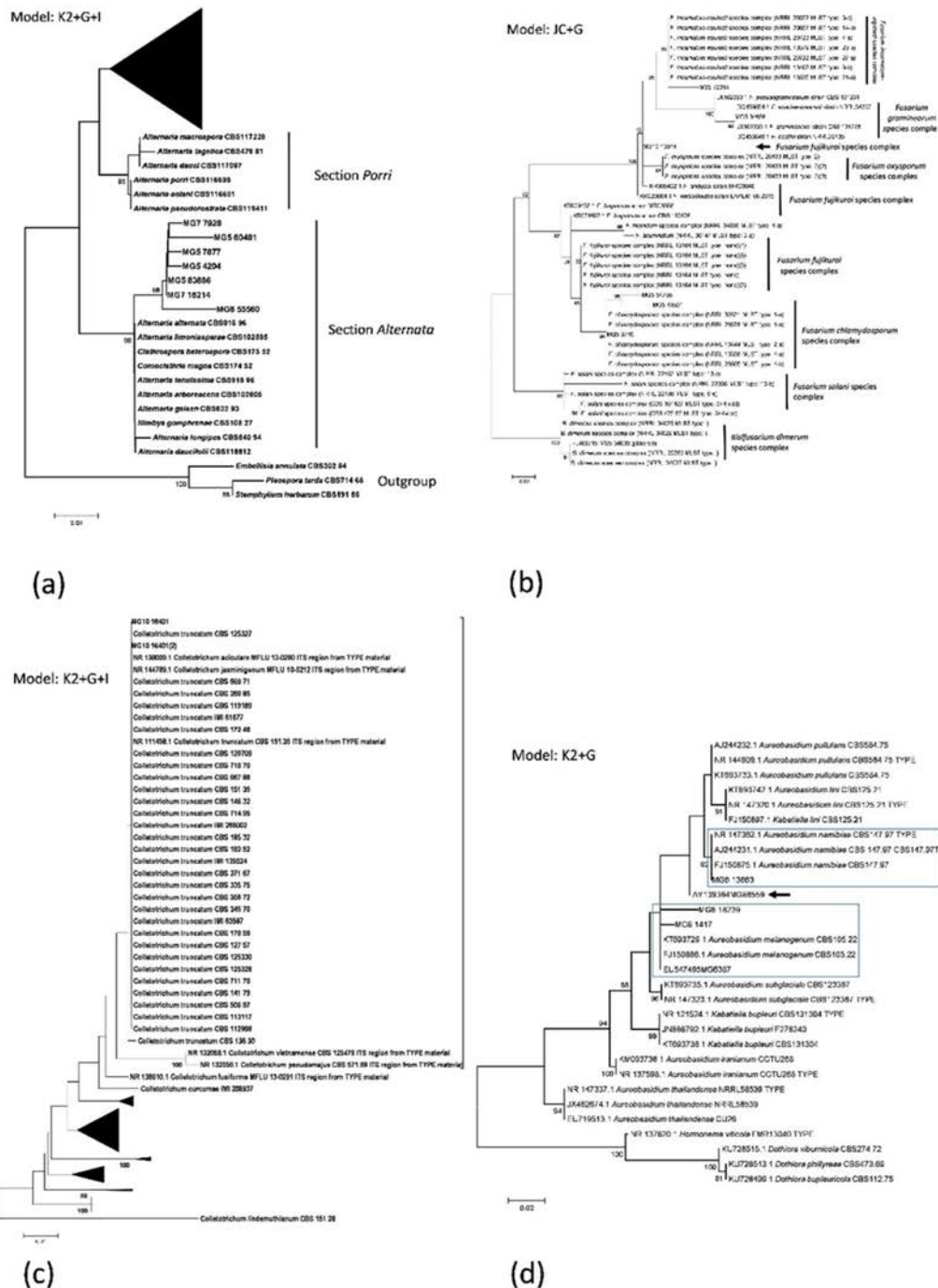
Figure 5 – Phylograms obtained from Maximum Likelihood analyses of the Internal Transcribed Spacer 2 region evolutionary model used indicated in the figure. Confidence levels ≥ 80% (1000 replicate bootstrap analysis) are indicated on the branches. Sequence reads obtained in this study are indicated with MG codes (a) *Alternaria*. (b) *Fusarium*. (c) *Colletotrichum*. (d) *Aureobasidium*.
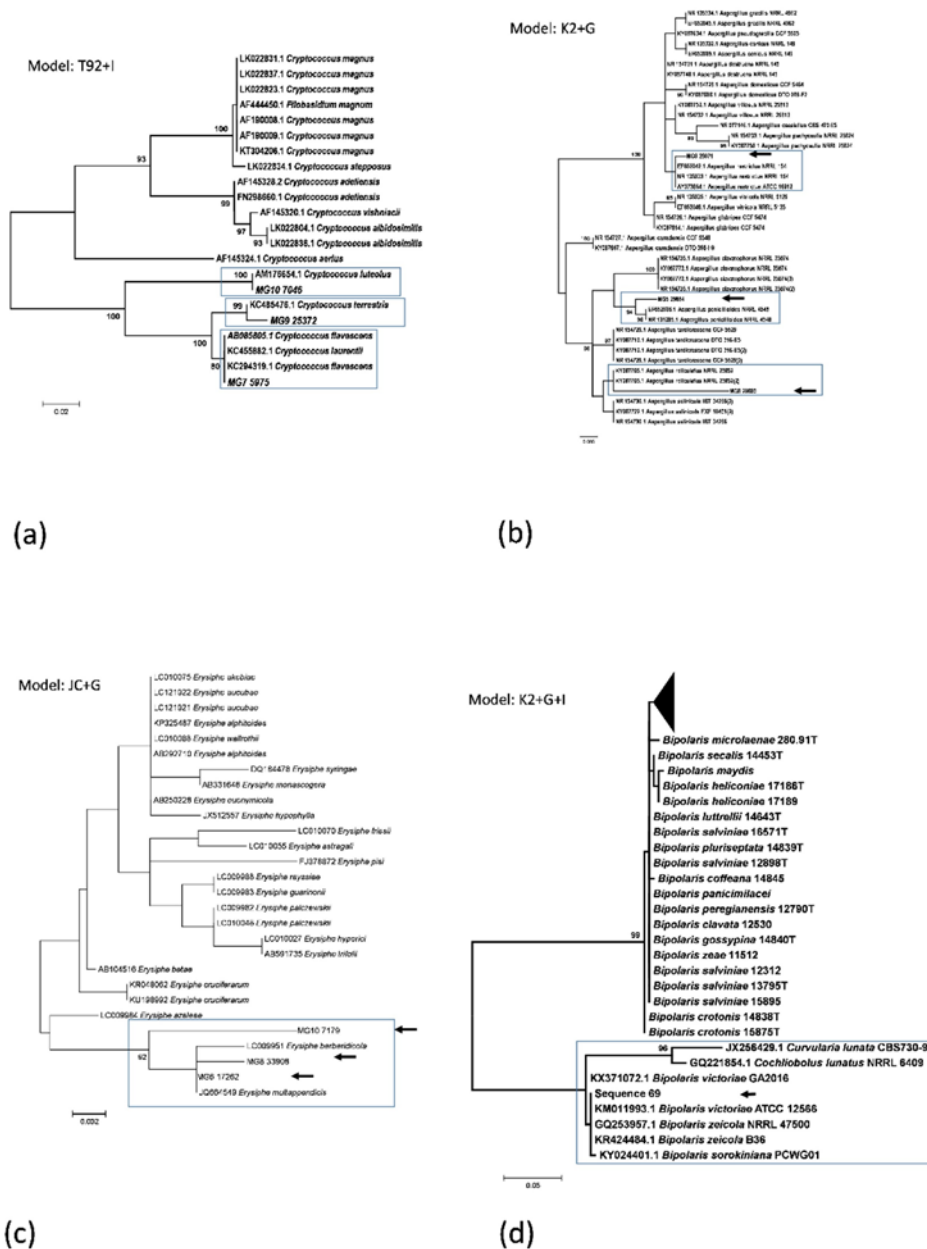
Figure 6 – Phylograms obtained from Maximum Likelihood analyses of the Internal Transcribed Spacer 2 region evolutionary model used indicated in the figure. Confidence levels ≥ 80% (1000 replicate bootstrap analysis) are indicated on the branches. Sequence reads obtained in this study are indicated with MG codes (a) *Cryptococcus*. (b) *Aspergillus*. (c) *Erysiphe*. (d) *Cochliobolus*.

In this study we have shown that with careful consideration and additional analyses more information on possible genus and even species identities can be obtained. This may yield meaningful ecological data and aids the detection of problematic fungi. Trends shown on order or family level can also be better explained. For instance, the Dothideales were mostly represented only by one genus, namely *Aureobasidium*, and the prominence of the Pleosporaceae across all cultivars were mostly due to the prominence of one genus, namely *Alternaria*. However, results showed that care should be taken when assigning generic and species names to MOTUs and should

only be done in careful consideration to existing, often complex, taxonomies and responsible name assignments (Nilsson et al. 2011, Jeewon et al. 2018, Hongsanang et al 2018).

A major limitation to sorghum production is grain mold disease (Sharma et al. 2010), which is caused by species of *Fusarium*, *Alternaria*, and the *Cochliobolus/Curvularia/Bipolaris* complexes (Tarekegn et al. 2006, Tesfaendrias et al. 2011). Commonly isolated species are *Fusarium thapsinum*, *Alternaria alternata* and *Curvularia lunata* (Tarekegn et al. 2006, Tesfaendrias et al. 2011). Infection can significantly reduce grain yield and quality or contribute to deterioration during storage (Stack & Pedersen 2003). Members of these fungi have been detected in the tested seeds, with especially *Alternaria* being omnipresent and in high proportions. In contrast, *Fusarium* and the *Cochliobolus* complex were detected sporadically across the tested cultivars and in low proportions. Although it is not known whether these fungi are viable in the seed, as amplicon sequencing can detect DNA from both viable and non-viable organisms (Louws et al. 1999, Tringe & Ruben 2005), it nonetheless indicates the degree to which seeds have been infected, most likely while being in the field (Stack & Pedersen 2003).

PCoA analyses showed that the fungal assemblages of some seedlots were similar in composition. However, the origin of the seeds used for isolations is unknown, and they could originate from different localities in South Africa. Significant variations in sorghum fungi have been found across locations, and among sorghum lines at a particular location (Thakur et al. 2003). Without such knowledge it will thus be difficult to ascertain if this similarly reflects geographic origin, if it could be a cultivar effect or an artefact of sampling.

A relatively low number of MOTUs were shared between the seeds from all six cultivars. These include a number of unassigned taxa, but especially *Alternaria* MOTUs. Species in this pathogenic and mycotoxin-producing genus is known to have a cosmopolitan occurrence and wide host range, including seed substrates (Bottalico & Logrieco 1998, Woudenberg et al. 2013). The fact that *Alternaria* is so prominent in samples from these six cultivars, which include seeds with unknown origins, indicates that this genus can readily infect sorghum seeds.

Seed-borne fungal pathogens and mycotoxin producers play a significant, yield-limiting role in sorghum production world-wide, and could cause severe health problems in humans and live-stock after consumption. Despite the limitations in species level identification, the use of NGS and phylogenies applied in the present study has provided a more precise dissection of the diversity and composition of the seed transmitted mycobiome in sorghum. A further advantage of the approach is that it can be done for a high number of samples, which greatly reduces cost (Adey et al. 2010). Previously extensive and exhaustive culturing would have been needed to reveal the diversity from the same sample size. Although care should be taken that DNA of killed organisms on the seed surface could maybe influence results, such information can contribute to better understanding of fungal communities in seeds of sorghum cultivars. It may also lead to better management practices, and aid studies investigating possible differences in the seed mycobiome of cultivars, areas and even other crops.

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