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Fungal contaminants of stored wheat vary between Australian states

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Abstract

A survey was undertaken to determine the mycoflora associated with stored wheat in four states across Australia including Western Australia, South Australia, Victoria and New South Wales. A total of 482 fungal isolates from 15 genera were isolated. The most common genera isolated were *Alternaria* spp., *Aspergillus* sp., *Aureobasidium* sp., *Cladosporium* spp., *Drechslera* sp., *Fusarium* spp., *Mucor* sp., *Nigrospora* sp., *Penicillium* sp., *Rhizopus* sp., *Stemphylium* sp., *Eutiarosporella* spp., *Ulocladium* sp., *Epicoccum* sp., and an undescribed genus from the Hypocreales. Diversity profiling also identified fungi that were not isolated using traditional methods including *Paecilomyces* spp., *Dendryphiella* and *Cryptococcus*. The results indicate that the mycoflora of stored wheat varies between Australian grain growing regions, and that diversity profiling analysis identifies different fungal contaminants compared to traditional methods. This study also revealed that some pathogens of high risk to humans (e.g. *Cryptococcus*) may occur with a high frequency in wheat.

Keywords: Postharvest; Mould; Diversity analysis; *Cryptococcus*

Introduction

Wheat is the main cereal crop grown in Australia, and the major wheat producing states are Western Australia (WA), South Australia (SA), Victoria (Vic), New South Wales (NSW) and Queensland (Qld) (Murray and Brennan 2009). Australia is the fourth biggest wheat exporter in the world after the United States, Canada, and the European Union (Yang et al. 2014). WA is the largest grain producer in Australia, at 40 % of the nation's crop (Yang et al. 2014). As a high value export crop, it is important to understand the abiotic (e.g. temperature and moisture) and biotic (microorganisms and insect pests) factors that affect yield loss. Once harvested, grain is always influenced by how it is handled and stored, as well as the environmental conditions it is exposed to.

Many different fungi can infect wheat grains during or after harvest and during transfer of the grain into storage (Riba et al. 2008). Fungal contamination is a major concern because it can cause economic losses to stored grain as well as a potential decline in human and animal health if the contaminated grain is consumed (Marasas 1995). In Australia, there is limited recent information about the mycoflora associated with wheat grain and their distribution across the main production areas (Berghofer et al. 2003). A survey by Berghofer et al. (2003) determined the mycoflora associated with Australian wheat in flour milling fractions and end products, and the most common fungi found were *Aspergillus*, *Penicillium*, *Cladosporium* and *Eurotium* spp. An earlier survey by Blaney and Williams (1991) found that Australian-grown grains including maize, sorghum and wheat were contaminated with *Alternaria* and *Fusarium*.

The objective of this study was to isolate and identify the species of postharvest fungi present in stored wheat grain across four states of Australia. These regions have a wide range of climatic conditions; rainfall decreases from north to south, with the Southern Region (Victoria and South Australia) having a temperate climate and the Western Region (Western Australia) a Mediterranean climate, whereas the Northern Region (New South Wales) has a climate ranging from temperate to subtropical depending on the growing area. Soils in the Southern and Western Regions are poor (low fertility) with many subsoil constraints, while the Northern Region has relatively good soils. The

hypothesis of this study was that differences in storage fungi exist between the different states of Australia.

Material and methods

Sample collection

A total of 22 newly harvested (in February 2012/2013 or February 2013/2014) non-chemical treated Australian standard hard wheat samples were collected from four states (Vic, SA, WA and NSW) in Australia (Fig. 1). The wheat samples (1 kg) were taken from random farm bins (50–150 t capacity). The sampling ports were 1 and 2 m from the grain surface, and the two lots of sample were mixed and stored in 2 kg metal sample can. All samples were sent to Murdoch University, Western Australia within 2 days of collection and stored at −20 °C.

Before use, the grain was removed from storage and kept at 5 °C for 2 days to defrost. The defrosted wheat samples were moved to room temperature (25 °C) for 24 h and then placed into a sealed chamber (2 L) and allowed to equilibrate at 25 °C and 55 % relative humidity for one week and the moisture content (wet basis) determined with an electronic moisture meter (Graintec HE 50 electronic moisture meter, Graintec Pty Ltd., Toowoomba, Australia). Based on preliminary experiments (data not shown), the wheat samples were adjusted to a final moisture content of 15 % by adding a calculated volume of distilled water into a sealed flask (3 L) at 25 °C for 1 week.

Isolation of fungi from grain

Each farm sample was divided into four sub-samples after moisture adjustment to allow for four different isolation methods. In Method 1, grains were surface sterilised by immersion for five min in 2.5 % sodium hypochlorite followed by three washes in sterile distilled water, and dried on sterile filter paper for ten min in the laminar flow. In Method 2 grains were not surface sterilised. All grain from these two isolation methods was plated onto full strength potato-dextrose-agar (PDA; Becton Dickinson and Company Sparks, MD USA) with streptomycin (133 mg/l) and incubated at room

temperature (20–25 °C) in the dark. Method 3 consisted of surface sterilised grain (as described above) that was placed in humidity chambers on filter paper moistened with sterile distilled water. Method 4 grains were not surface sterilised and were placed in moist chambers. Methods 3 and 4 were also incubated at room temperature in the dark. From 24 h to 14 days after plating for Methods 1 and 2, plugs of 5 mm2 were taken from emerging colonies, transferred onto fresh PDA plus streptomycin and incubated at 20–25 °C in the dark. Whilst for Methods 3 and 4, small fragments of mycelium growing from the grains, were transferred by sterile needle onto fresh PDA plus streptomycin and incubated at 20–25 °C in the dark. There were three replicate plates of ten grains per plate for each of the four methods.

Mycological analysis

Fungal colonies were grouped based on culture morphology and identified using the morphological criteria of Barnett and Hunter (1972), Booth (1971), Ellis (1971), and Nelson et al. (1983). Selected isolates from each group were chosen on the basis of morphological and cultural similarities for further identification using molecular methods and all isolates were also lodged with the Department of Agriculture and Food Western Australia (DAFWA) herbarium.

DNA extraction, amplification and sequencing of fungal isolates

Mycelium from selected individual isolates was frozen in liquid nitrogen, and ground to a fine powder. Genomic DNA was extracted using a hexadecyl trimethyl ammonium bromide protocol but modified by the addition of 100 µg/ml Proteinase K and 100 µg/ml RNAse A to the extraction buffer as per Andjic et al. (2007). The internal transcribed spacer (ITS) region of the ribosomal DNA operon was amplified using the primers ITS-1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). PCR products were purified and sequenced as described by Sakalidis et al. (2011). Sequences were tentatively identified by using a BLAST search to match to known sequences in GenBank, and confirmed by phylogenetic analysis using Geneious.

Diversity analysis of wheat samples

In addition to ITS analysis, a bulked sample consisting of up to 10 g of grain was prepared by combining 2 g of grain from each farm (one bulk sample for each state). This was milled to a fine powder in a coffee grinder (Breville, Australia) and used for DNA extraction by the Australian Genome Research Facility (AGRF). The composition of species diversity in high-throughput amplicon sequencing data was carried out using the Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.6 (http://qiime.org; Caporaso et al. 2010).

Statistics

Differences in the fungal communities between states were assessed using community ecology statistics, in which each state was considered a community. Both the number of different isolates in each state and the frequency with which they occurred were assessed using the freeware Paleontological statistics software package (PAST) V 3.01 (Hammer et al. 2001, available for download from http://folk.uio.no/ohmmer/past/). The multiple farms in each state constituted replicates for the analysis. The Non-Metric Multidimensional scaling (nMDS) statistical technique, with the Bray-Curtis distance measure, was used to visualise patterns of fungal diversity across states, with stress values of less than 0.2 being interpreted as providing a useful figure (Clarke and Warwick 2001). One-way non-parametric multivariate analysis of variance (PERMANOVA), based on the Bray-Curtis measure, was used for statistical comparisons of the fungal communities from each state. Following significant results in PERMANOVA, similarity percentages (SIMPER) were used to determine which isolates made the greatest contribution to differences between states (Hammer and Harper 2006).

Farms within states could not be compared with the above approach because there was no replication within each farm. However, if each farm is regarded as an ‘ecological community,’ the ecological statistics species richness (the number of species from the farm), species diversity (Shannon diversity, $H' = -\Sigma pi(\ln pi$, where pi is the proportion of isolates from species i), and equitbility ($E = H'/H'max$, where $H'max$ = the highest value H' can assume for the data for a farm) (see

Krebs 1999). These statistics can be compared at a significance level of 0.05 using a permutation test for equality (Hammer and Harper 2006, p. 197). Given the multiple tests, the significance value of 0.05 was adjusted for each state using the Bonferroni correction.

Results

Fungi associated with stored grain in Australia

From the 22 farm samples from across Australia a total of 472 isolates from 15 genera were isolated (Table 1). These included *Alternaria* spp., *Aspergillus* sp., *Aureobasidium* sp., *Cladosporium* spp., *Drechslera* sp., *Fusarium* spp., *Mucor* sp., *Nigrospora* sp., *Penicillium*sp., *Rhizopus* sp., *Stemphylium* sp., *Eutiarosporella* spp., *Ulocladium* sp., *Epicoccum* sp., and an undescribed genus from the Hypocreales. The highest numbers of isolates were from WA wheat grain, followed by SA, Vic and NSW (Table 2).

Distribution of fungal species across states

Visual inspection of the nMDS plot indicated that NSW wheat grain had a distinct fungal community, whereas there was considerable overlap in the other states (Fig. 2). This was confirmed in PERMANOVA, where there was a significant (pseudo F = 2.042, $p = 0.01$) difference in the fungal communities between grain from the different states. Subsequent paired testing found significant ($p < 0.05$) differences between NSW and SA, NSW and Victoria, and NSW and WA.

Considering all states together, SIMPER showed that the pattern of occurrence of *Alternaria infectoria* explained 48 % of the variability across samples. *Alternaria infectoria* was also the major contributor to each significant paired comparison between states, with the scope of its contribution varying from 52 % between SA and Victoria to 45 % between NSW and WA. The next most common species was *Rhizopus* sp., which contributed 8.4 % to the variability across states (Supplementary Table 1). There was evidence of heterogeneity between farms in Western Australia only, with

significant differences in species richness, diversity and equitability of fungi recovered (Supplementary Table 2).

Molecular identification and phylogenetic analysis

Based on sequence data and phylogenetic analysis, seven of the *Alternaria* isolates appeared to be most closely related to *A. alternata*, two were related to *A. chatarum*, one belonged to *A. malorum* and ten to the *A. infectoria* clade (Table 2). Four isolates were closely related to *Cladosporium cladosporioides* and one isolate to *Cladosporium herbarum* (Table 2). In addition, two isolates of *Fusarium* were closely related to *Fusarium tricintum* and one each to *Fusarium pseudograminearium*, *Fusarium equiseti* and *Fusarium oxysporum* (Table 2). Two isolates of *Aureobasidium* aligned to *Aureobasidium pullulans* and two isolates appeared to be putative new *Aureobasidium* species (Table 2). The phylogenetic analyses for *Nigrospora*indicated one isolate was close to *Nigrospora oryzae* and another appeared to be an unidentified *Nigrospora* sp. (Table 2). One isolate each of *Penicillum cordubense* and *P. dipodomyicola* were confirmed, and one isolate appeared to be an undescribed *Stemphylium*sp. (Table 2).

Diversity analysis

Diversity profiling analysis of bulked farm samples resulted in the identification of additional species that were not identified by ITS analysis, but confirmed similar variation between growing regions to that detected using traditional isolations. Based on sequence reads, in Western Australia *Paecilomyces* was the dominant genus, with 78 % of all reads, followed by *Cryptococcus victoriae* at 10 % (Table 3). In South Australia, the most common species based on the number of sequence reads was *Dendryphiella arenaria* at 26 %. *Cryptococcus* was recorded in almost 40 % of the SA sequences, with *C. macerans, C. victoriae* and *C. oeirensis*all being present (Table 3). Victorian grain also had high numbers of sequence reads of *Cryptococcus macerans* (48 %) and *C. victoriae* (34 %) (Table 3). In NSW, *Aspergillus* species made up the highest number of reads at 42 % (Table 3). *Cryptococcus* species were all less than 1 % in NSW.

Discussion

This study provides an insight into fungi currently present in apparently healthy wheat grain stored in silos in four states of Australia. Although only a small snapshot in time, there were clear differences in fungal communities between the four states, with the highest number of fungi isolated from WA grain, followed by SA, Victoria and NSW.

The observations of different patterns of occurrence in fungal communities between states is interesting and may possibly be explained by differences in climatic conditions during the growing seasons of each state and the subsequent variation in the mycoflora at those locations. Some fungi such as *Aspergillus niger* are widespread in warmer climates, both in field crops and stored foods (Riba et al. 2008). Therefore it would be expected that some fungi would be common cross all Australian sites, and others more restricted in their distribution. The reasonable explanation for the results in the current study is fungi can be influenced by various environmental factors such as temperature, rainfall frequency and geographic distribution or extreme events (i.e., floods in New South Wales during the seasons collected).

The major storage pathogen of wheat grain was *Alternaria* spp., in the storage of wheat grain under Western Australia conditions. This is in agreement with Pitt and Hocking (2003) who reported *Alternaria* as the main problem in Australian wheat, including *Alternaria alternata*and *A. infectoria.* The high number of *Alternaria* isolations in the current study is similar to Patriarca et al. (2007) and Kulik et al. (2014) who found that *Alternaria* spp. were common spoilage fungi of crops in the field and caused postharvest decomposition of various grains including wheat and rye in Poland. *Alternaria* has a worldwide distribution, is ubiquitous and includes various pathogenic plant and saprophytic species that can spoil grain pre-harvest or postharvest, if conditions are suitable. In Australia, *Alternaria*, especially *A. alternata* and *A. infectoria*, have previously been reported on wheat, sorghum and barley (Pitt and Hocking 2006; Webley et al. 1997; Blaney and Williams 1991).

Contamination of wheat by *Alternaria* species is a concern as the genus has the ability to produce mycotoxins (Patriarca et al. 2007). Pitt and Hocking (2003) found that *Alternaria*produced a range of

toxins in wheat including tenuazonic acid, altenuene, alternariol and alternariol monomethyl ether. The results of the present study indicate that it is essential to further study the current toxigenic ability of *Alternaria* spp. in stored wheat across Australia due to its dominant presence. However, there should be a particular focus on wheat that is at higher risk of mould development, for example when storage conditions are breached allowing ingress of moisture, the presence of grain storage insect pests, or when the growing season has been particularly wet. As *Alternaria* also has the ability to produce mycotoxins, it will be critical to monitor stored grain for the presence of these toxins in the future, should on-going monitoring for the pathogen show a continued high level presence.

Other storage fungi found in high counts from almost all the wheat grain samples included *Aureobasidium*, *Rhizopus*, *Cladosporium* and *Penicillium*. These results concur with Berghofer et al. (2003) who detected *Aureobasidium*, *Alternaria* and *Fusarium* in Australian wheat. A similar study by Shipton and Chambers (1966) found *Cladosporium, Rhizopus* spp., *Penicillium* spp., *Alternaria* spp., *Stemplium* spp., and *Ulocladium* spp., associated with Western Australian wheat. In similar studies in Mediterranean environments *Cladosporium*and *Rhizopus* species were isolated from wheat in Argentinia (González et al. 1999), *Aspergillus* (mainly *A. flavus*, *A. niger* and *A. versicolour)* from wheat in Algeria (Riba et al. 2008) and *Cladosporium* spp., *Alternaria* spp., *Penicillium* spp., *Aspergillus* spp. and *Fusarium* from wheat in Iran (Mostafa et al. 2011).

The diversity profiling analysis identified a number of fungi potentially present on the grain that were not isolated using traditional methods. Again, although only a snapshot in time, this method may be useful for the detection and identification of pathogenic fungi without isolation of cultures for morphological determination. Of most concern was the presence of *Cryptococcus macerans* which has been described as a human pathogen and the cause of diseases such as Meningoencephalitis (Lindsberg et al. 1997). Additionally, *C. victoriae* was identified, although has previously only been reported from the Antarctic environment (Montes et al. 1999). *C. oeirensis* was detected in all states but is known to occur as a 'black head mould' or weak pathogen on ripening or ripe wheat heads (Nicolaisen et al. 2014). *Cryptococcus* species can spread by wind or human activities such as

shipping and handling to silos. The data obtained in this study indicate that the human fungal pathogen *C. macerans*can be detected on grain and therefore is of epidemiological significance and importance. The occurrence of the other *Cryptococcus* species in stored grain is unusual for Australia as to our knowledge these species have not recorded on wheat before. Ghannoum et al. (1992) reported that synthetic amino acid medium-fungal and yeast nitrogen based media are the best to support the growth of yeasts such as *Cryptococcus* species, these media might be useful in future for postharvest grain storage isolations to confirm if *Cryptococcus* is present and viable.

A recent study of the mycobiome of Danish wheat, using a similar diversity analysis method, reported different saprophytes and pathogens including *Alternaria*, *Cladosporium*, *Phaeosphaeria*, *Fusarium* and *Microdochium* and also *Cryptococcus tephrensis* and *Cryptococcus victoriae* (Nicolaisen et al. 2014). The present study is different to their study because our focus was on the fungi that are present in storage rather than at harvest. However, both studies indicate through diversity profiling analyses that more fungi are present on grain than what we previously knew. Such microbiome studies could assist in better management practices in the future by taking whole fungal populations into consideration when designing postharvest facilities

Other fungi detected using the diversity profiling technique were also unexpected. For example *Dendryphiella arenaria*, which is frequently found in marine environments, was putatively identified in all states but was particularly high in the South Australian grain.
Previously, *Dendryphiella arenaria* has only been isolated from plant debris in sea foam and from marine sediments (Michaelis et al. 1987). Similarly, sequences of *Sporobolomyces roseus* were also obtained from the South Australian grain. This species has also been found in seawater (Hernandez-Saavedra et al. 1992; Gadanho et al. 2003) and freshwater (Libkind et al. 2003). An explanation for the presence of these fungi is that the spores are present in the atmosphere and were spread to the storage facilities when the grain was harvested and transported to the silo.

Typical soil fungi such as *Aspergillus* spp. were also detected on wheat using diversity analysis, indicating that this group of fungi can colonise wheat pre- or immediately postharvest, which has been demonstrated to occur if the grain has moisture content in equilibrium with a relative humidity

between 70 and 90 % (Flannigan 1978). Isolates belonging to the genus *Paecilomyces* present on Western Australian grain have been isolated from *Taxus mairei* and *Torreya grandis* from south east China and are known to produce a wide range of bioactivity metabolites (Wang et al. 2002). Why such a high percentage of *Paecilomyces* spp., were detected in WA grain samples is not known. However, *Paecilomyces*are common environmental moulds (Domsch et al. 1980) and can grow under warm and dry conditions (Ying and Feng 2004), which are conditions typical to WA.

Conclusion

In conclusion, this study found that the mycoflora of stored wheat varies between Australian grain growing states. This study revealed that *Alternaria* occurs with a high frequency in comparison to other genera, and that some pathogens of high risk to humans, either directly (e.g. *Cryptococcus*) or indirectly (e.g. *Fusarium* which produces mycotoxins) may also be present. The presence of fungi with the ability to produce toxins could pose chronic adverse health effects to human and livestock fed on contaminated wheat or wheat products. Future investigations should include the toxigenic potential of Australian isolates of *Alternaria*species considering the frequency at which they were isolated from across Australia. Further work should also be conducted on the potential presence of *Cryptococcus* species, using both traditional and advanced technologies to determine if the genus is a common occurrence on stored grain or not. If common, it would be useful to determine how it is entering grain silos and under what conditions it might pose a threat to human and animal health.

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Fig. 1 The collection locations of wheat grain from farms across different states of Australia: Western Australia (WA); South Australia (SA), New South Wales (NSW), and Victoria (Vic)

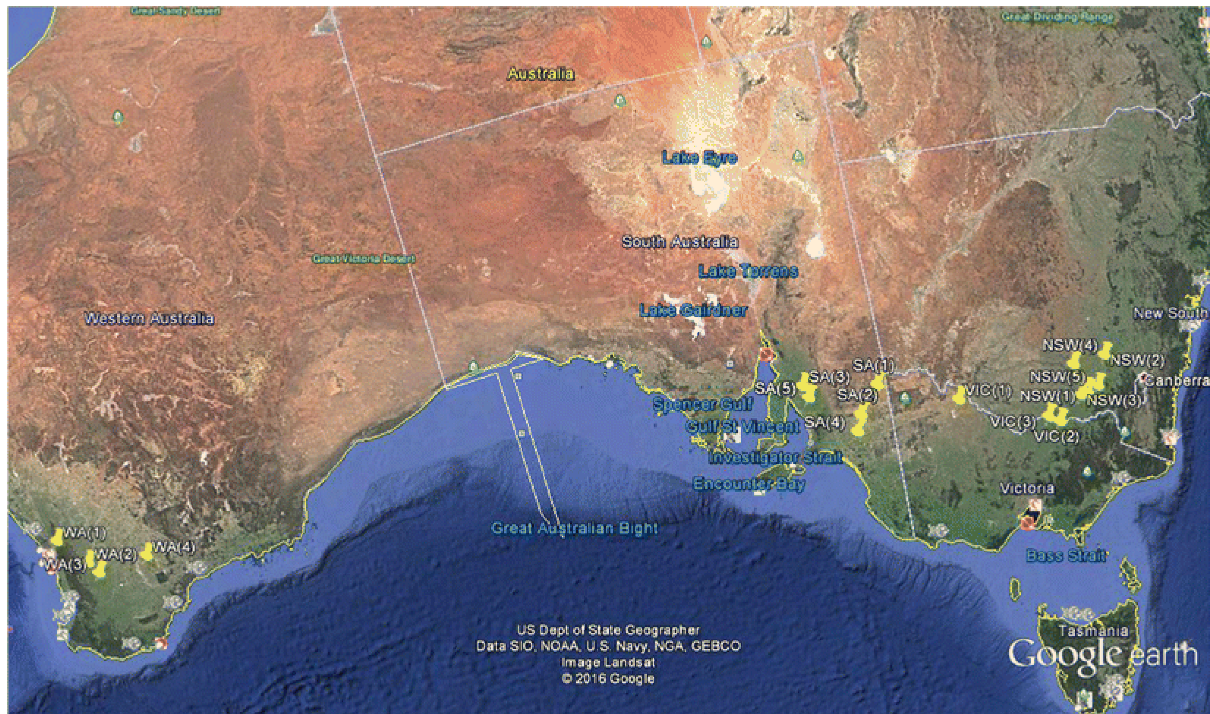

Fig. 2 Non-metric multidimensional scaling model (nMDS) plot comparing the fungal communities isolated from grain samples using traditional methods, collected from farms across Australia: New South Wales (NSW) Victoria (VIC), Western Australia (WA) and South Australia (SA). Minimum convex hulls (the smallest polygon embracing all points for a state) are shown. The stress was 0.13

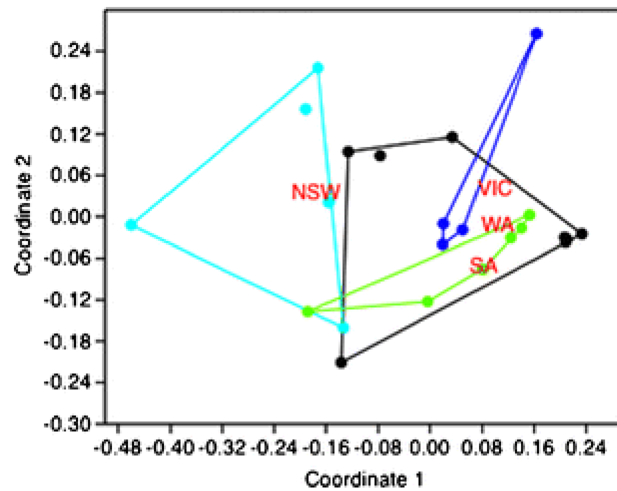

Table 1 Genera, species and numbers of fungal isolates identified (using traditional methods) from wheat samples collected from farms in Western Australia, South Australia, New South Wales and farms in Victoria. Number in brackets indicates number of farms sampled

Genus/Species	Number of Isolates			
	WA (8)	SA (6)	NSW (5)	VIC (3)
Alternaria alternata	18	3	2	6
Alternaria chartarum	0	1	0	1
Alternaria infectoria	167	94	1	35
Aspergillus spp.	4	5	1	1
Aureobasidium pullulans	8	3	14	5
Aureobasidium spp.	1	0	0	0
Cladosporium cladosporioides	3	1	2	1
Cladosporium herbarum	3	2	0	3
Drechslera spp.	3	0	0	0
Epicoccum nigrum	1	0	0	0
Eutiarosporella spp.	1	0	0	1
Fusarium tricinctum	2	0	0	0
Fusarium equiseti	0	1	0	0
Fusarium oxysporum	2	0	0	1
Fusarium pseudograminearum	0	1	0	0
Mucor circinelloides	0	0	0	4
Nigrospora spp.	1	0	0	0
Nigrospora oryzae	3	0	1	0
Penicillium cordubense	0	6	0	0
Penicillium dipodomyicola	0	0	23	0
Rhizopus spp.	10	16	7	9
Stemphylium spp.	2	0	0	0
Ulocladium spp.	1	0	0	0
Unidentified species from the Order Hypocreales	1	0	0	0
TOTAL	231	133	51	67

Table 2 Identity of isolates selected for ITS sequencing. Western Australian Culture Collection (WAC) numbers allocated by the Department of Agriculture and Food Western Australia Herbarium

Species.	**WAC Numbers**
Alternaria alternata	13,842, 13,843, 13,844, 13,845, 13,846, 13,847, 13,848
Alternaria chartarum	13,826, 13,827
Alternaria infectoria	13,849, 13,850, 13,851, 13,852, 13,853, 13,854, 13,855, 13,856, 13,857, 13,858
Alternaria malorum	13,818
Aureobasidium pullulans	13,820, 13,821
Aureobasidium sp.	13,824, 13,825
Cladosporium cladosporioides	13,838, 13,839, 13,840, 13,841
Cladosporium herbarum	13,833
Fusarium equiseti	13,829
Fusarium oxysporum	13,828
Fusarium pseudograminearum	13,837
Fusarium tricinctum	13,834, 13,835
Nigrospora oryzae	13,836
Nigrospora sp.	13,823
Penicillium cordubense	13,831
Penicillium dipodomyicola	13,832
Stemphylium sp.	13,859

Table 3 Common fungal species detected in wheat grain samples following diversity analysis of bulked grain samples from Western Australia, New South Wales, Victoria and South Australia.

Species	**Percentage of Sequencing reads per State**			
	WA	SA	NSW	VIC
Paecilomyces sp	78	0.5	11	0.1
Cryptococcus macerans	4	19	0.7	48
Cryptococcus victoriae	10	13	0.5	34
Aspergillus sp	0.4	0	42	0.3
Unidentified Ascomycota	0.1	21	15	4
Dendryphiella arenaria	3	26	1.3	4
Unidentified Eurotiales	0.2	0	17	0.1
Cryptococcus oeirensis	0.2	6	0.2	1
Sporobolomyces roseus	0.2	5.5	0.1	1.1