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Fungal communities in wheat grain show significant co-existence patterns among species

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ABSTRACT

The wheat grain mycobiome is only scarcely investigated and focus has been on seed-transmitted wheat pathogens of agricultural importance. In this study, we used next generation sequencing to study the mycobiome of Danish wheat grain samples at harvest. In total 228,421 sequences were obtained from 90 samples that were taken from locations across Denmark during three years. These sequences could be grouped into 173 non-singleton operational taxonomic units (OTUs) of which 21 OTUs, identified as belonging to genera such as *Fusarium*, *Alternaria*, *Cladosporium*. *Phaeosphaeria* and *Microdochium*, were identified as 'core' OTUs as they were found in all or almost all samples and accounted for almost 99 % of all sequences. The remaining OTUs were only sporadically found and only in small amounts. Cluster and factor analyses showed patterns of co-existence among the core species. Cluster analysis grouped the 21 core OTUs into three clusters: cluster 1 consisting of saprotrophs, cluster 2 consisting mainly of yeasts and saprotrophs and cluster 3 consisting of wheat pathogens. Principal component extraction showed that the *Fusarium graminearum* group was inversely related to OTUs of clusters 1 and 2.

Introduction

Winter wheat is the most important cereal crop in Europe, but grain yield and quality is often restricted by disease epidemics, which may be managed through deployment of resistant varieties, fungicide applications or agricultural practices in general. Average losses from diseases in Danish winter wheat are about 10 %, but they vary considerably across years, depending on the earliness of disease onset and actual disease severity levels in seasons (Jørgensen et al., 2000). Some of the most important diseases in wheat are caused by *Mycosphaerella graminicola*, *Bipolaris sorokiniana*, *Blumeria graminis*, *Puccinia striiformis*, *Pyrenophora tritici-repentis*, *Tilletia caries*, *Phaeosphaeria nodorum*, *Microdochium* spp. and several *Fusarium* spp. Of these pathogens, many can be found associated with seeds, such as *M. graminicola*, *P. tritici-repentis*,

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T. caries, *P. nodorum* and *Fusarium* spp. (Bockus et al., 2010) together with a range of other less pathogenic or non-pathogenic fungi such as *Alternaria* spp., *Cladosporium* spp., *Epicoccum nigrum* and *Phoma* spp. (e.g. González et al., 2008). After storage, *Penicillium* spp. and *Aspergillus* spp. can be found in high amounts if grain is not dried (Filtenborg et al., 1996). Wheat grain, thus, represents an ecological niche that is inhabited by a large variety of fungi. The importance of many of these in plant growth and health is not very well known, and interactions among species are largely unknown, as well. Most studies of the wheat grain mycobiome have, for obvious reasons, focused on the fungal pathogens, whereas very few studies have looked at entire seed-associated fungal communities and the co-existence patterns of species within these communities, although such analyses may reveal patterns of, for example, possible antagonism by competition for space or by antibiosis, different growth optima or similarities in response to agronomic practice. Previous studies identified a limited number of fungal species and provided only limited quantitative data on these, mainly because of technological limitations and because of the focus on pathogens (González et al., 2008; Rehman et al., 2011; Supronienė et al., 2011; Levic et al., 2012).

Next generation sequencing technologies (NGS) have enabled studies of microbial communities at a much higher resolution than was previously possible using classical culturing methods or cloning of PCR products followed by Sanger sequencing (e.g. Xu et al., 2012). In this study, we used 454 pyrosequencing (Margulies et al., 2005) of amplicons from the fungal internal transcribed sequence 1 (ITS1) to analyze the composition of fungal communities associated with wheat grain at harvest collected from Danish wheat fields in the years 2003, 2004 and 2005 (Nielsen et al., 2011). We hypothesized that NGS would reveal a significantly higher biodiversity than has been observed previously in culturing studies, and that significant co-existence patterns of fungal species exist depending on their lifestyles. This is, to our knowledge, the first published study where NGS has been used to explore fungal communities in cereal grain.

Materials and methods

Field samples

Field samples of cereal grain were collected at harvest from 2003 to 2005 (Supplementary Table 1) and consisted of harvested seeds from fields evenly distributed throughout Denmark as part of a stratified monitoring program in Danish cereal crops from conventionally grown wheat (Nielsen et al., 2011)

A sample of 100 g was taken out of an original 1 kg grain sample harvested from farmers' fields. In total, 90 samples from 2003 to 2005 (30 samples each year) were selected. The samples represent a broad variation in Danish farming practices with respect to wheat cultivar, soil type, fertilization, and fungicide treatment (Nielsen et al., 2011). After harvest, the grain samples were dried to a water content of 15–16 % and the samples were stored at −20 °C before DNA extraction.

Extraction of DNA

Wheat grain samples (100 g) were initially homogenized in a blender and approximately 5 g were further ground in liquid N_2 with eight steel balls using a Geno/Grinder 2000 (OPS Diagnostics, Bridgewater, NJ, USA), and then a subsample of this was used for DNA extraction, as described in Nicolaisen et al. (2009). One hundred mg ground material was mixed with 300 µl water and then 700 µl CTAB buffer (20 g l^{-1} CTAB, 1.4 M NaCl, 20 mM Na_2EDTA, 0.1 M Tris–HCl, pH 8.0) was added together with 10 µl RNase solution (10 mg ml^{-1}) before incubation at 65 °C for 30 min. Thereafter, 10 µl proteinase K solution (20 mg ml^{-1}) was added before an additional incubation at 65 °C for 30 min. Samples were centrifuged at 12,000 ×*g* for 10 min and the supernatant was transferred to a tube with 500 µl chloroform, vortexed and centrifuged at 12 000 ×*g* for 15 min. This step was repeated once. The upper phase was transferred to a new tube and 2 volumes of CTAB precipitation solution (5 g l^{-1} CTAB, 0.04 M NaCl, pH 8.0) were added and the samples were incubated at RT for 60 min before centrifugation at 12 000 ×*g* for 5 min. The pellet was dissolved in 350 µl 1.2 M NaCl, then 350 µl chloroform was added before vortexing and centrifugation at 12 000 ×*g* for 10 min. The upper phase was precipitated with 0.6 volumes of isopropanol and incubated at RT for 20 min and centrifuged at 12 000 ×*g* for 10 min. The pellet was washed in 70 % ethanol, air dried and resuspended in 100 µl TE buffer. The DNA samples were further purified using a DNeasy kit (QIAGEN Gmbh., Hilden, Germany) according to the manufacturer's instructions except that the lysis and QIAshredder steps were omitted.

Estimation of fungal biomass using quantitative real-time PCR (Q-PCR)

To estimate the amount of fungal biomass in each sample, Q-PCR was carried out in a total of 12.5 µl consisting of 6.25 µl 2 × SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA), 300 nM of each primer (ITS1F (Gardes and Bruns, 1993) and ITS2 (White et al., 1990)), 0.5 µg $µl^{-1}$ bovine serum albumin (BSA) and 2.5 µl template DNA. Reactions were performed in duplicate on all samples. Genomic DNA from grain samples was diluted 1:10 before PCR on a 7900HT Sequence Detection System (Applied Biosystems) using the following cycling protocol: 2 min at 50 °C; 95 °C 10 min; 40 cycles of 95 °C for 15 s, 55 °C for 1 min, and 60 °C for 1 min followed by dissociation curve analysis from 60 to 95 °C. Standard curves were generated using five-fold dilution series of DNA from *F. graminearum* strain 1955 from the IBT culture collection at the Technical University of Denmark. The amount of fungal DNA in samples was calculated from cycle threshold (Ct) values using this standard curve.

PCR amplification and pyrosequencing

To generate ITS1 amplicons for 454 pyrosequencing, primers ITS1-F and ITS2 were used. The two primers were tag encoded using the forward primer 5′-CGTATCGCCTCCCTCGCGCCATCAG-MID-ITS1F-3′ and the reverse primer 5′-CTATGCGCCTTGCCAGCCCGCTCAG-ITS2-3′. Thirty 10-nucleotide MID primer tags for sample identification after pooling were selected from