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Agricultural factors affecting *Fusarium* communities in wheat kernels

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Abstract

Fusarium head blight (FHB) is a devastating disease of cereals caused by *Fusarium* fungi. The disease is of great economic importance especially owing to reduced grain quality due to contamination by a range of mycotoxins produced by *Fusarium*. Disease control and prediction is difficult because of the many *Fusarium* species associated with FHB. Different species may respond differently to control methods and can have both competitive and synergistic interactions. Therefore, it is important to understand how agricultural practices affect *Fusarium* at the community level.

Lower levels of *Fusarium* mycotoxin contamination of organically produced cereals compared with conventionally produced have been reported, but the causes of these differences are not well understood. The aim of our study was to investigate the effect of agricultural factors on *Fusarium* abundance and community composition in different cropping systems. Winter wheat kernels were collected from 18 organically and conventionally cultivated fields in Sweden, paired based on their geographical distance and the wheat cultivar grown. We characterised the *Fusarium* community in harvested wheat kernels using 454 sequencing of translation elongation factor 1-α amplicons. In addition, we quantified *Fusarium* spp. using real-time PCR to reveal differences in biomass between fields.

We identified 12 *Fusarium* operational taxonomic units (OTUs) with a median of 4.5 OTUs per field. *Fusarium graminearum* was the most abundant species, while *F. avenaceum* had the highest occurrence. The abundance of *Fusarium* spp. ranged two orders of magnitude between fields. Two pairs of *Fusarium* species co-occurred between fields: *F. poae* with *F. tricinctum* and *F. culmorum* with *F. sporotrichoides*. We could not detect any difference in *Fusarium* communities between the organic and conventional systems. However, agricultural intensity, measured as the number of pesticide applications and the amount of nitrogen fertiliser applied, had an impact on *Fusarium* communities, specifically increasing the abundance of *F. tricinctum*. There were geographical differences in the *Fusarium* community composition where *F. graminearum* was more abundant in the western part of Sweden. The application of amplicon sequencing provided a comprehensive view of the *Fusarium* community in cereals. This gives us better opportunities to understand the ecology of *Fusarium* spp., which is important in order to limit FHB and mycotoxin contamination in cereals.

Keywords: *Triticum aestivum*; grain; *Gibberella*; high-throughput sequencing; pathogen ecology

1. Introduction

Fusarium head blight (FHB) is an economically important disease in cereal production world-wide caused by a range of *Fusarium* species. The disease can cause important yield losses, but the most problematic aspect of FHB is the associated contamination by harmful mycotoxins produced by many *Fusarium* species (Parry et al., 1995). Important *Fusarium* toxins include deoxynivalenol (DON), nivalenol (NIV), HT-2 and T-2, zearalenone (ZEA) and fumonisins, that have negative effects on human and animal health (D'Mello et al., 1999; Reddy et al., 2010). Furthermore, the possible health effects of several more recently discovered *Fusarium* toxins are not well understood (Jestoi, 2008). In the EU, there are legislative limits for DON, ZEA and fumonisins in cereals designated for human consumption and recommended limits for animal feed (European Commission, 2006a, 2006b). It is important to reduce human and animal exposure to *Fusarium* mycotoxins as well as the economic consequences for the farmer. These can be considerable since contaminated grain cannot be sold for food or feed. *Fusarium* fungi can produce significant amounts of mycotoxins already when the crop is growing in the field. Therefore, it is crucial to develop control measures that can reduce *Fusarium* infection and mycotoxin contamination in the field.

Fusarium head blight is caused by several *Fusarium* species of which *F. graminearum*, *F. culmorum* and *F. avenaceum* predominate, but up to 17 species have been associated with the disease (Parry et al., 1995). The many species involved in FHB makes the disease difficult to control. Different *Fusarium* species have different life cycles, host range, mycotoxin profiles, climatic preferences and respond differently to control methods (Xu and Nicholson, 2009). A better understanding of how environmental and agronomic factors affect the *Fusarium* community is important in order to make predictions and develop disease control strategies.

The *Fusarium* species involved in FHB generally produce asexual conidia, but some have sexual stages producing ascospores, e.g. *F. graminearum* (teleomorph *Gibberella zeae*) and *F. avenaceum* (teleomorph *G. avenacea*). Others, *e.g. F. culmorum* produce thick-walled chlamydospores that can persist for years in soil (Sitton and Cook, 1981). *Fusarium* survives saprotrophically on crop residues in the field and can infect the following crop (Champeil et al., 2004; Parry et al., 1995). Dispersal of conidia from crop residues by rain splashing has been proposed as a major infection route, but wind dispersal can be important, especially for species producing ascospores (Champeil et al., 2004; Keller et al., 2014).

Several agricultural practices and environmental factors are known to increase the risk of FHB (Champeil et al., 2004; Edwards, 2004). The crop is most susceptible at flowering and warm and wet weather conditions during this period favour *F. graminearum* infection and subsequent DON contamination (Obst et al., 1997). High nitrogen application and minimum tillage appears to be risk factors of *Fusarium* infection (Martin, 1991; Obst et al., 1997). The preceding crop is also important, where maize has been associated with the highest risk (Dill-Macky and Jones, 2000; Obst et al., 1997).

Fungicides have often shown limited and inconsistent efficacy against *Fusarium* infection and mycotoxin contamination (Parry et al., 1995). For example, in field trials inoculated with *F. culmorum* or *F. graminearum* FHB disease severity was reduced by 25-77% depending on cultivar, location and type of fungicide (Haidukowski et al., 2005). Fungicide application at flowering appears to be the most efficient in reducing *Fusarium* incidence and DON contamination (Chala et al., 2003). In contrast, fungicide application at

the beginning of heading to target leaf diseases may even increase *Fusarium* infection (Henriksen and Elen, 2005).

Interestingly, several studies have shown lower or equal levels of *Fusarium* and mycotoxin contamination in organic cereal production than in conventional production (Bernhoft et al., 2010; Birzele et al., 2002; Edwards, 2009; Gottschalk et al., 2009; Meister, 2009). In the UK, Edwards (2009) observed lower levels of HT-2 and T-2 in organically produced wheat, but no difference in DON contamination. Other studies identified lower DON levels in organically produced wheat than in conventionally produced (Bernhoft et al., 2010; Birzele et al., 2002). The cause of the lower mycotoxin contamination in organic production is poorly understood, but certain farming practices differing between the two systems have been proposed as explanations (Bernhoft et al., 2010; Edwards, 2009).

Characterising *Fusarium* communities using culturing is time-consuming and risk overestimating fast-growing species. Real-time PCR detection with species-specific primers requires many assays to cover all species involved in FHB. High-throughput amplicon sequencing (HTS) has enabled larger and more detailed studies of fungal communities (Meiser et al., 2014). However, the internal transcribed spacer (ITS), which is commonly used as a barcode for fungi (Schoch et al., 2012), lack species-level resolution for *Fusarium* (O'Donnell and Cigelnik, 1997). Recently, new *Fusarium*-specific primers targeting the translation elongation factor 1-α (TEF1) gene has been developed and employed to characterise *Fusarium* communities using HTS (Edel-Hermann et al., 2015; Karlsson et al., 2016). This new method enables the study of *Fusarium* in cereals as a community.

Pairing of organic and conventional farms or fields is often used to control for confounding factors such as climate or cultivar (Bernhoft et al., 2010; Granado et al., 2008). However, pairing could select for non-representative farms (Hole et al., 2005). Therefore, we used an agricultural intensity index to directly estimate two of the most important factors differing between conventional and organic systems: pesticide application and amount of nitrogen fertilisation.

In the present study, harvested wheat kernels were collected from pairs of organically and conventionally managed wheat fields in Sweden. Fields were paired to account for variability due to wheat cultivar and geographic location. The *Fusarium* community on the kernels was characterised using amplicon sequencing and real-time PCR. The aims of the study were: 1) to characterise the *Fusarium* community composition and abundance in harvested kernels in Sweden; and 2) to investigate the impact of agricultural factors on the *Fusarium* community in organic and conventional production.

2. Materials and methods

2.1 Sampling and data collection

Twelve pairs of organically and conventionally managed fields in central Sweden were included in the study (Fig. 1). Fields were paired to limit variation due to other factors than cropping system. Two conditions were used to pair the fields: 1) they had to lie close to each other; and 2) they had to contain the same winter wheat cultivar. Paired fields were adjacent or located between 0.5-9.5 km from each other and grew either winter wheat varieties 'Olivin' or 'Stava'.

At harvest 2012, farmers were asked to send a sample of harvested kernels from the fields. The farmers were asked to combine several subsamples into a representative sample of the field. Earlier in the season, in July 2012, wheat leaves were collected from the same fields and a number of additional data were collected at the same time (Karlsson et al.,

2017). At the start and end of the transect used for leaf sampling, two 0.25 m^2 squares were laid out and weeds and crop plants in each square were cut and the biomass was separated, dried for 24 h at 105°C and weighed to determine dry mass. The farmers managing the different fields were interviewed about the preceding crops, soil tillage, pesticides, fertilisation and other measures applied in the sampled fields (Table S1).

These data were also used to calculate the agricultural intensification index proposed by Herzog et al., (2006) for each field. The original index includes three indicators: livestock density, amount of nitrogen applied (kg/ha) and number of pesticide applications. Only the two latter were applicable to our study.

2.2 DNA extraction, PCR and 454 sequencing

In total, 18 farmers out of 24 provided a sample of harvested wheat kernels from the fields sampled in July. From each sample of about 1 kg wheat kernels, 100 g were milled with a Grindomix GM 200 (Retsch GmbH, Germany). DNA was extracted from two 100 mg subsamples for each milled sample using the DNeasy Plant Mini kit (Qiagen, Germany) according to the manufacturer's instructions. Kernel material, 300 µl 1 mm glass beads and 530 µl lysis buffer was added to a tube and the samples were run at 30 000 min-1 for 40 s on a Retsch MM 400 oscillatory mill. DNA extraction was completed in a QiaCube (Qiagen). Two water controls were included during the DNA extraction. DNA from the two extraction replicates was pooled before PCR.

A portion of the TEF1 gene was amplified using *Fusarium*-specific primers Fa (TCGTCATCGGCCACGTCGACTCT) and Ra (CAATGACGGTGACATAGTAGCG) (Edel-Hermann et al., 2015). PCR was run in 25 µl reactions containing 0.8 ng/µl template, 190 µM of each nucleotide, 2.75 mM $MgCl_2$, primers at 200 nM and 0.12 U/µl polymerase (DreamTaq Green, Thermo Scientific, MA, USA) in PCR buffer. The PCR conditions on a 2720 Thermal Cycler (Life Technologies, Carlsbad, CA, USA) were 3 min at 94°C, 20-33 cycles of [1 min at 94°C, 1 min at 67°C, 1 min at 72°C] and 10 min at 72°C. Size and concentration of amplicons was evaluated on 1% agarose gels. The Fa primer was tagged with a 9 bp sample identifying tag (Karlsson et al., 2016, supplemental file 1). In order to limit the risk of errors associated with many PCR cycles, the number of cycles was adapted for each sample to give weak to moderately strong bands on the gel.

PCR products were purified using AMPure beads (Beckman Coulter, CA, USA). DNA concentration was measured using capillary electrophoresis on a MultiNA (Shimadzu, Japan) and equal amount of DNA was pooled from each sample. The sample pool was sent to Eurofins MWG Gmbh (Germany) for adaptor ligation and sequencing on 1/4 of a plate using a GS FLX++ 454 sequencer (Roche, Switzerland). Before sequencing the amplicon pool was purified by gel extraction. Raw sequence data were deposited in the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession number SRP061762.

2.3 Bioinformatics and species identification

Raw 454 sequence data were quality filtered and clustered using the SCATA pipeline (Brandström Durling *et al*., [http://scata.mykopat.slu.se]) as described previously (Karlsson et al., 2016). Briefly, sequences were screened for matches to the primer sequences (allowing 1 mismatch) and the sample identifying tags. Sequences shorter than 500 bp and those with low quality scores were discarded. Sequences passing the quality control step were clustered into operational taxonomic units (OTUs) at 1.5% dissimilarity cut-off in SCATA. Mock communities of 10 *Fusarium* species were included as positive controls as described in a previous publication (Karlsson et al., 2016). The mock communities were used to determine the cut-off which best reflected *Fusarium* species and to set a limit for low-abundance OTUs as these may be the result of sequencing and PCR errors (Tedersoo et al., 2010). Consequently, OTUs with less than eight reads per OTU were removed as these

occurred as contaminants in the mock communities. In addition, singletons were removed on a per-sample-basis.

A first screening for *Fusarium* OTUs was done by subjecting the most abundant sequence in each OTU to a BLAST search against sequences in GenBank. OTUs that did not yield any hit with identity and coverage >70% were considered chimeric or artificial and removed. The remaining OTUs were screened for hits to *Fusarium* species or their teleomorph names, which were included in further analyses. *Fusarium* species identification was performed by including reference *Fusarium* TEF1 sequences in the clustering in SCATA. For OTUs related to *F. avenaceum*, BLAST analysis was performed with sequences in GenBank. The OTUs were identified to the species level with 99.4% similarity or more. Only sequences associated with a publication and a description of the corresponding strain were considered.

2.4 Real-time PCR quantification

The amount of *Fusarium* DNA was quantified with real-time PCR using the Fa/Ra primers as above at 500 nM (Edel-Hermann et al., 2015). The PCR conditions were 98°C for 2 min and 40 cycles of 98°C for 15 s, 67°C for 15 s and 72°C for 1 min. Wheat samples were diluted 1:10 before PCR. A dilution series of a mix of DNA from ten *Fusarium* species containing 0.003-3 ng per reaction was used to generate a standard curve.

Wheat DNA was quantified to account for variation in moisture content and DNA extraction efficiency between samples (Schena et al., 2013). A portion of the TEF1 gene was amplified with plant-specific primers Hor1 and Hor2 at 400 nM (Nicolaisen et al., 2009). The PCR conditions were 2 min at 98°C, 40 cycles of [5 s at 98°C, 10 s at 60°C]. A 10-fold dilution series of DNA from wheat kernels containing 0.005-50 ng per reaction was used to generate a standard curve.

All samples were run in triplicate 12.5 µl reactions containing 2.5 µl template in Evagreen master mix (Biotium, CA, USA). PCR was run on a CFX Thermal cycler (Biorad, Carlsbad, CA, USA), followed by dissociation curve analysis at 65-95°C. *Fusarium* abundance was estimated as pg *Fusarium* DNA per 100 ng of wheat DNA.

2.5 Statistics

The OTU table generated from 454 sequencing data was multiplied with the amount of *Fusarium* present estimated with real-time PCR and was used as input for the statistical analyses. All statistical analyses were performed in R (R Core Team, 2014).

Pairwise co-occurrence of *Fusarium* OTUs was tested using the probabilistic model implemented in the 'cooccur' package version 1.2 (Griffith et al., 2016). OTU pairs with only one co-occurrence were excluded from the analysis, which left 28 pairs tested.

In order to visualise *Fusarium* community composition between fields, non-metric multidimensional scaling (NMDS) was applied using the function *metaMDS* in the 'Vegan' package (Oksanen et al., 2013). The NMDS was performed on Bray-Curtis dissimilarities calculated from log-transformed data to account for unequal variance between abundant and rare species. Subsequently, continuous explanatory variables were fitted to the ordination using the *envfit* function.

The effect of cropping system and agricultural intensity on *Fusarium* community composition was tested using multivariate generalised linear models (GLMs). A model including the number of 454 reads, field pair and cropping system was fitted to each OTU using the *manyglm* function in the 'mvabund' package with a negative binomial probability distribution package (Wang et al., 2012). Significance was assessed using the *anova* function in 'mvabund', which provides a multivariate test for the community composition and univariate tests for each OTU. The likelihood-ratio test was used and p-values were

adjusted for multiple testing. Interview data from one of the fields (12C) was incomplete and was therefore excluded from the analysis of agricultural intensity.

Finally, the effect of agricultural variables on *Fusarium* communities was evaluated with redundancy analysis (RDA) using the *rda* function in the 'Vegan' package. Correlated variables were avoided, *e.g.* the amount of nitrogen applied which is correlated with crop biomass. Model selection started from a model including the number of 454 reads and field pair as conditional variables. Crop diversity, type of fertiliser, crop biomass, weed biomass, number of fungicide applications and type of soil tillage were included in the model selection. Crop diversity was calculated as the number of different crops grown on the fields in the last three years. Leys were counted as two crops regardless of the duration of the ley. Forward model selection was performed with the *ordistep* function and data were log-transformed prior to analysis. Statistical significance of the final model was assessed using the *anova.cca* function performing an ANOVA-like permutation test for RDA.

3. Results

Fusarium TEF1 sequences were obtained from harvested kernels from all fields by high-throughput 454 sequencing. The number of 454 reads per sample was 497-3681 with a median of 1982. In total, 12 *Fusarium* operational taxonomic units (OTUs) were identified from 18 samples of winter wheat kernels (Fig. 2a). Reads were distributed between four different OTUs related to *F. avenaceum*. The number of *Fusarium* OTUs per field ranged from two to eight with a median of 4.5. *Fusarium avenaceum* was the most frequent species recovered and was present in 94% of fields, followed by *F. poae* (83%), *F. graminearum* (78%) and *F. tricinctum* (72%) (Table S2). The amount of *Fusarium* DNA in the samples was 20-1667 pg per 100 ng wheat DNA (Fig. 2b). In terms of abundance, *F. graminearum* dominated the dataset with 84% of total DNA, followed by *F. avenaceum* (11%), *F. culmorum* (2.4%) and *F. tricinctum* (1.6%) (Table S2). Two pairs of *Fusarium* species co-occurred between fields. *F. poae* and *F. tricinctum* (p=0.012) and *F. culmorum* and *F. sporotrichoides* (p=0.022).

The GLM analysis revealed no significant difference in *Fusarium* community composition between organically and conventionally grown wheat kernels (Table 1a). However, agricultural intensity had an impact on *Fusarium* community composition (Table 1b). *F. tricinctum* was associated with higher agricultural intensity (Table 2, Fig. 3b). The mean agricultural intensity index was 80 and 11 for conventionally and organically managed fields, respectively.

Pairs of organically and conventionally managed fields were distributed on a gradient from the north-east to the south-west of Sweden. The variable field pair was included in the analyses and the GLM analysis revealed a significant difference between field pairs (Table 1). There was a significant difference in the abundance of *Fusarium graminearum* between field pairs and this species was more common towards the south-west of the sampling area (Table 2 and Fig. 3b).

Model selection including crop diversity, type of fertiliser, crop biomass, weed biomass, number of fungicide applications and type of soil tillage was performed to find the most important factors affecting *Fusarium* community composition. Soil tillage – ploughing or reduced tillage - was the only significant variable. However, only two fields were subject to reduced tillage (Fig. 3a). In the RDA with the selected model 83% of inertia were attributed to the conditional variables (field pair and 454 reads), 8% to constrained (soil tillage) and 9% to unconstrained inertia. The permutation test for RDA showed a significant effect of soil tillage (F=4.02, p=0.033).

4. Discussion

Several *Fusarium* OTUs were present in variable amounts in wheat kernels from all fields investigated in the present study. Surveys of individual fusaria in cereals in the Nordic countries often report high incidence of several species (Eskola et al., 2001; Fredlund et al., 2013; Kosiak et al., 2003; Lindblad et al., 2013; Nielsen et al., 2011). *Fusarium* spp. are also common in relation to other fungi present on wheat kernels. In Argentina, *F. graminearum* was the second most abundant fungus isolated from wheat kernels after *Alternaria alternata* (González et al., 2008). Using HTS of the ITS region, *Alternaria* and an OTU including *F. graminearum* and closely related species were the two most abundant fungal OTUs recovered from wheat kernels in Denmark (Nicolaisen et al., 2014). It has also been reported that *Fusarium* fungi only constitute a minor part of the fungal community of wheat heads (Hertz et al., 2016).

The TEF1 marker gene provided species-level resolution for the *Fusarium* community in our samples, and within-species variation was detected for *F. avenaceum*. *Fusarium avenaceum* is known to harbor high intra-specific diversity (Nalim et al., 2009). One of the *F. avenaceum* OTUs dominated with 24% of the reads followed by three minor ones (<2% of the reads). No pattern was identified regarding the distribution of the different *F. avenaceum* OTUs, but correlation between intra-specific variation and geographic distribution has been revealed in other fungi using amplicon sequencing (Millberg et al., 2015).

All samples in our study contained at least two but up to eight different *Fusarium* OTUs. The use of HTS of amplicon markers is clearly advantageous for detecting many *Fusarium* species simultaneously. Amplicon sequencing does not require beforehand selection of which species to survey, as opposed to real-time PCR with species-specific primers. Therefore, amplicon sequencing is especially useful for detecting *Fusarium* species colonizing new areas. Amplicon sequencing provides the relative abundance of each species in a community but not the total abundance. Therefore, we complemented the HTS data with real-time PCR quantification of *Fusarium spp*. using the same primers. *Fusarium* DNA was estimated to vary 100-fold between samples. The TEF1 is single-copy in *Fusarium* (Geiser et al., 2004), which allows for quantitative comparisons of both total and relative abundances between species. In our previous study, the relative abundance of *Fusarium* species in mock communities varied ±8% between *F. graminearum*, *F. avenaceum*, *F. langsethiae* and *F. tricinctum*, indicating comparable amplification efficiencies (Karlsson et al., 2016). We conclude that the combination of these two methods provided a detailed description of the *Fusarium* community present on winter wheat kernels.

Fusarium graminearum was the most abundant species, while *F. avenaceum* had the highest incidence in our study. *Fusarium* species present on wheat has been analysed in earlier studies in Sweden using PCR (Djurle et al., 2010; Lindblad et al., 2013). In the study by Lindblad et al., (2013), *F. avenaceum* and *F. poae* were present in 94-100% of all winter wheat samples in 2009 and 2011, *F. culmorum* in 90% and *F. graminearum* in 75-90% of samples. *F. tricinctum* and *F. langsethiae* had more variable presence and were recovered with 61-100% and 58-94% frequency, respectively. Djurle et al., (2010) reported that *F. poae, F. avenaceum* and *F. langsethiae* were the most common species in wheat in 2008 and 2009, while *F. culmorum* and *F. graminearum* were detected less often. Thus, *F. poae* and *F. avenaceum* had high incidence in all three studies, while for example *F. graminearum* and *F. langsethiae* had more variable presence that could be due to yearly variations in climate conditions.

We found that *F. graminearum* was more prevalent in the south-western part of the sampling area. This area has been particularly affected by high DON levels during 2011 and 2012 (http://www.lantbruk.com/node/104902, retrieved 06-01-2017), which was also observed by Lindblad et al., (2013) who reported high levels of *F. graminearum*. The cause of the high DON levels in Western Sweden is not well understood but the climate conditions were favourable for *Fusarium* during 2011. DON can be produced by both *F. graminearum* and *F. culmorum*. However, Lindblad et al., (2013) found the strongest correlation between *F. graminearum* and DON.

Previously, *F. culmorum* was considered more common in Northern Europe and *F. graminearum* more common in Southern Europe (Bottalico and Perrone, 2002). A shift in dominant species from *F. culmorum* to *F. graminearum* has been described in Northern Europe in recent years (Jennings et al., 2004; Nielsen et al., 2011; Stępień et al., 2008; Waalwijk et al., 2003). In North America, a shift in the population structure of *F. graminearum* towards more toxigenic populations has been observed (Ward et al., 2008). Climate factors, increased practice of reduced tillage and maize cultivation have been proposed as potential causes of this shift (Nielsen et al., 2011). Higher temperatures and more rainfall in a future climate can be expected to cause higher incidence of FHB, and more favourable conditions for ascospore formation by *F. graminearum* to increase the spread of this species (Parikka et al., 2012).

In addition to the DON-producers, other toxigenic species were also detected. *F. sporotrichoides* and *F. langsethiae* produce the highly toxic HT-2 and T-2 toxins (Thrane et al., 2004). *Fusarium poae* is known to produce nivalenol and beauvericin (Thrane et al., 2004), *F. avenaceum* to produce moniliformin and enniatins (Morrison et al., 2002) and *F. tricinctum* to produce enniatins (Cuomo et al., 2013). We did not measure mycotoxin content, but the range of *Fusarium* species present indicate a potential for mycotoxin formation in the sampled fields.

The co-occurrence analysis showed that two pairs of *Fusarium* species co-occurred between fields: *F. poae* with *F. tricinctum* and *F. culmorum* with *F. sporotrichoides*, but no negative correlations were observed. *Fusarium* species may have both synergistic and competitive interactions. In a controlled experiment by Xu et al., (2007), co-inoculation of several *Fusarium* species resulted in mostly competitive interactions where *F. graminearum* was the most competitive species. Furthermore, competition resulted in decreased amount of fungal biomass while mycotoxin production increased (Xu et al., 2007). Co-occurrence patterns may also depend on indirect associations where two species prefer the same environmental conditions rather than direct interactions.

Other studies of *Fusarium* species associations in cereals have identified variable patterns. In Norway, *F. culmorum* and *F. sporotrichoides* were found to be positively correlated, as in our study, as well as *F. culmorum* and *F. avenaceum* (Bernhoft et al., 2010). In the same study, several negative correlations between *F. graminearum* and other species were found. In a study including Hungary, Ireland, Italy and the UK, *F. culmorum* was positively correlated to *F. avenaceum* and negatively correlated to *F. poae* at harvest, but for some two-species interactions the results varied between countries (Xu et al., 2005). In a study by Infantino et al., (2012) significant associations were found between *F. graminearum* and *F. avenaceum* in bread wheat over three years as well as between *F. culmorum* and *F. equiseti*. The various statistical methods used to assess species co-occurrence may contribute to the variable patterns reported. Species co-occurrence patterns may also depend on the scale studied, if at the level of individual plants, or at field- or plot-scale where many individuals are mixed as in our study. Exhaustive sampling of all

Fusarium species present in a sample using HTS will facilitate the detection of co-occurrence patterns of *Fusarium* in cereals.

In our study, we could not detect any difference in *Fusarium* community composition and abundance between the organic and conventional systems. The was a large variation in both species composition and amount of *Fusarium* DNA between the fields. This heterogeneity could be the result of local environmental conditions or dispersal limitations and calls for selecting a larger number of fields for comparing *Fusarium* communities over different regions. Here we did not account for within-field variation which can be important (Oerke et al., 2010), and could have contributed to the observed variation.

Mycotoxin contamination of organically and conventionally produced grain was compared in a recent review, it could not be concluded that either of the systems increases the risk of mycotoxin contamination (Brodal et al., 2016). However, several studies have reported lower amounts of *Fusarium* and mycotoxin contamination in organic cereal production than in conventional production (Bernhoft et al., 2010; Edwards, 2009; Gottschalk et al., 2009; Meister, 2009). Pesticide use, high nitrogen application and cereal intense rotations in the conventional system have been hypothesised to cause these differences (Bernhoft et al., 2010; Edwards, 2009). Perhaps, there is an interaction between cropping system, environmental conditions and *Fusarium* epidemiology so that differences between the two systems only occur under certain conditions.

Pairing based on wheat cultivar may have selected for non-representative farms as different cultivars are often grown in the two systems. Organic agriculture is governed by specific criteria, while variation in cropping practices could potentially be large within the conventional system. We used an agricultural intensity index, including the number of pesticide applications and the amount of nitrogen applied, reflecting some of the important differences between the organic and conventional systems. We found that agricultural intensity affected the *Fusarium* community present in wheat kernels. Specifically, *F. tricinctum* was positively correlated with higher agricultural intensity. Previously, Bernhoft et al., (2010) reported lower infestation of wheat kernels by *F. tricinctum*, but also *F. culmorum* and *F. langsethiae* in organically produced wheat.

Of the different agricultural variables evaluated, soil tillage was found to be important for *Fusarium* community composition. But only two fields had been subjected to reduced tillage (2C and 3C), making it difficult to draw a definite conclusion. Higher incidence and severity of FHB has previously been reported in low- and no-tillage treatments (Dill-Macky and Jones, 2000). In our study, the two fields subjected to reduced tillage had rather low *Fusarium* abundance. The effect of reduced tillage may be driven by differences in community composition rather than abundance. Figure 2a indicates that *F. langsethiae* was present in these two fields, but only occurred in two more fields and then constituted less than 1% of the community.

In addition to environmental and agricultural factors, we would like to introduce the possible role of naturally occurring microorganisms for *Fusarium* contamination of cereals. In a greenhouse experiment, Liggitt et al., (1997) found that inoculation of wheat ears with saprotrophic fungi before *F. culmorum* inoculation reduced FHB severity. In the field, the phyllosphere of cereals harbour a diverse fungal community that is affected by cultural practices (Karlsson et al., 2014; Sapkota et al., 2015). Interactions between *Fusarium* and other fungi are likely to occur in the phyllosphere. Gradual upward movement of *Fusarium* infections towards the head throughout the season has been reported (Zinkernagel et al., 1997) and several *Fusarium* species have been identified on green leaves (Köhl et al., 2007; Vujanovic et al., 2012). Therefore, the interaction between the phyllosphere community and

Fusarium is a factor to consider when trying to understand *Fusarium* occurrence in different cropping systems in further studies.

In conclusion, we show that the structure of *Fusarium* communities in wheat is complex and dependent on both geographic location and agricultural practices. A combination of molecular methods targeting both community composition and abundance with multivariate statistics can be used to better understand the multiple agricultural and environmental factors affecting *Fusarium* communities. This knowledge will be important for developing strategies to predict and control FHB.

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Tables

Table 1 Effect of cropping system (a) (organic or conventional) and agricultural intensity index (b) on *Fusarium* communities on wheat kernels. The agricultural intensity index included the amount of nitrogen applied (kg/ha) and the number of pesticide applications. Data were analysed with multivariate generalised linear models. One field was excluded from the analysis of agricultural intensity due to missing data. ** P < 0.01, * P < 0.05

	Residual d.f.	d.f.	Deviance	P
a)				
No. of 454 reads	16	1	36.74	0.009**
Field pair	6	10	218.2	0.006**
Cropping system	5	1	23.13	0.11
b)				
No. of 454 reads	15	1	33.21	0.032*
Field pair	6	9	190.86	0.005**
Agricultural intensity	5	1	31.4	0.038*

Table 2 Effect of agricultural intensity index on *Fusarium* operational taxonomic units (OTUs) on wheat kernels. The agricultural intensity index included the amount of nitrogen applied (kg/ha) and the number of pesticide applications. Twelve *Fusarium* OTUs were included in the analysis but only significantly affected OTUs are shown. 17 fields were included and data were analysed with multivariate generalised linear models. * $P < 0.05$

	OTU 0 *F. graminearum*		OTU 8 *F. tricinctum*	
	Deviance	P	Deviance	P
No. of 454 reads	0.163	0.997	0.061	0.997
Field pair	51.545	0.012*	14.878	0.874
Agricultural intensity	0.091	0.912	22.136	0.034*

Figures

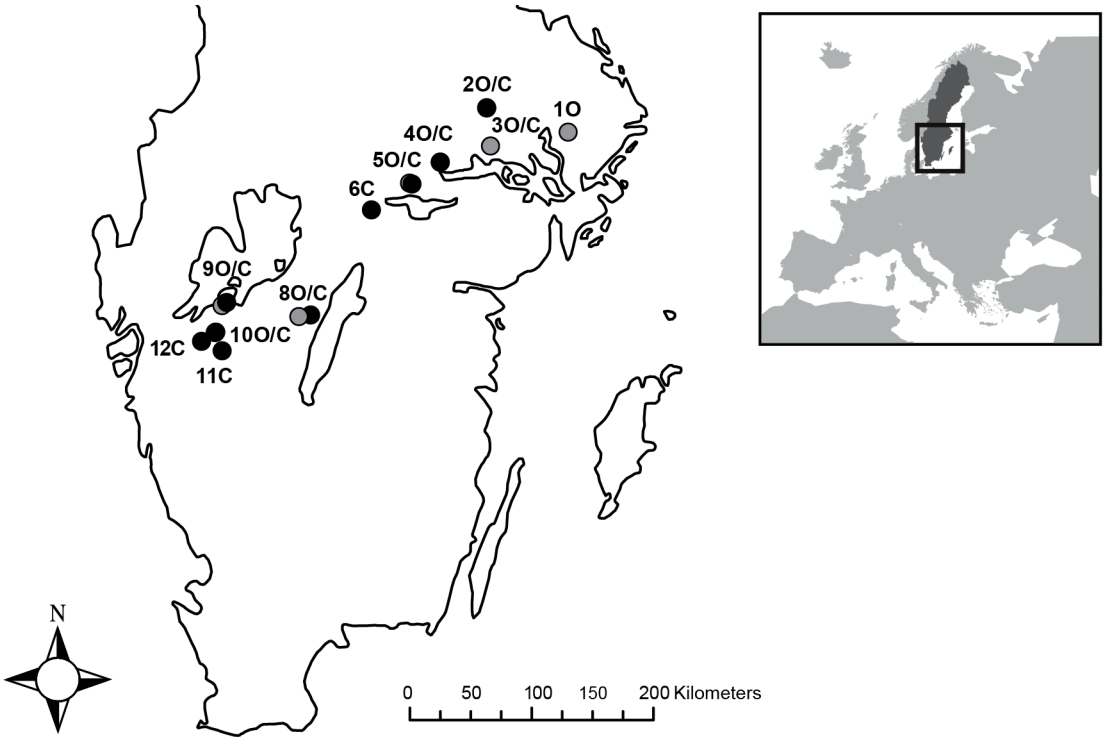

Fig. 1 Map of sampled wheat fields.
Wheat kernels were collected from pairs of organically and conventionally managed fields in Sweden. Fields are paired based on location and wheat cultivar. Some pairs are incomplete since kernel samples were not available. Samples from 18 fields were included. Organically managed fields are represented by grey dots and conventionally managed by black dots. Individual fields within a pair are not always visible when located close to each other. O = organically managed field, C = conventionally managed field.

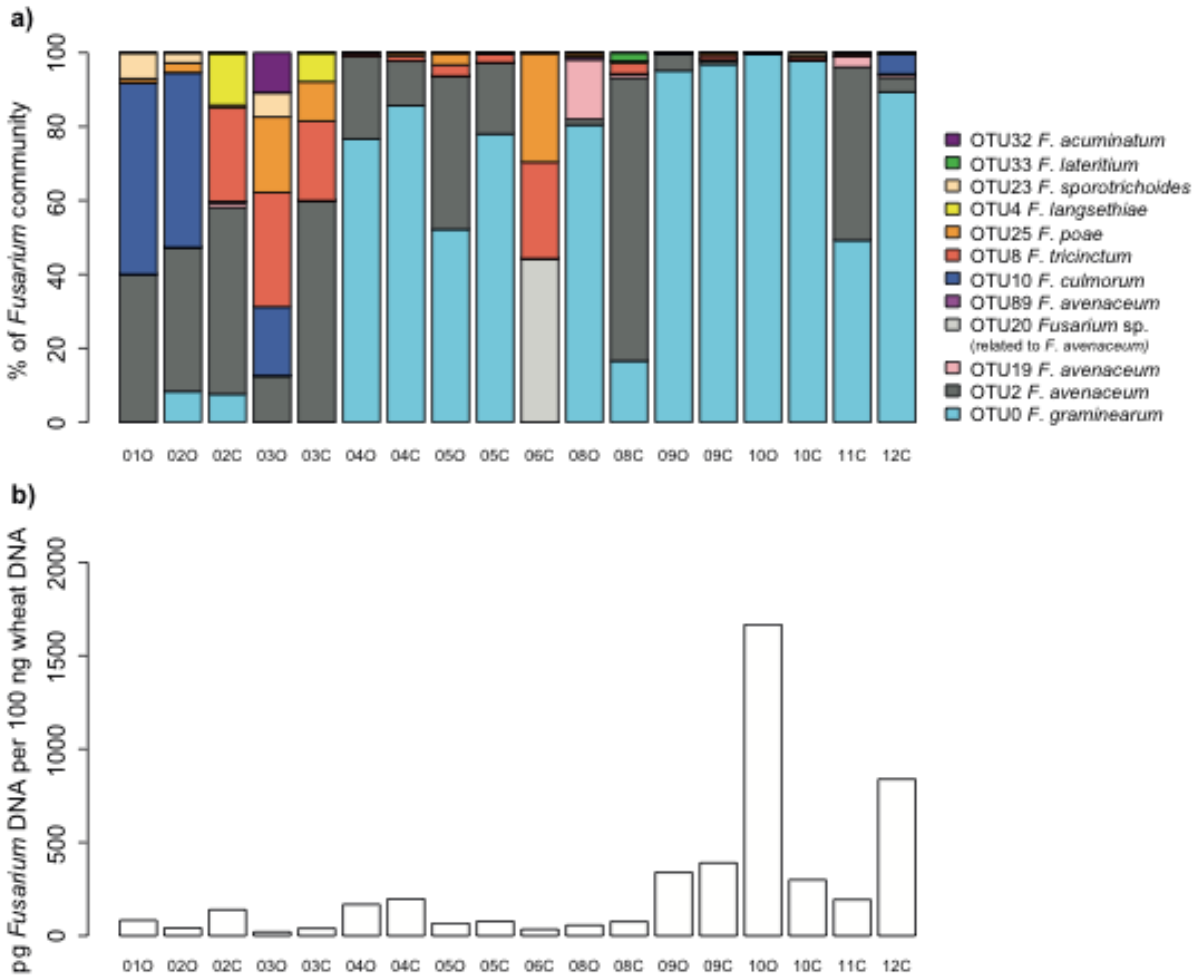

Fig. 2 *Fusarium* community composition and abundance in winter wheat kernels. a) *Fusarium* species composition analysed with 454 sequencing of TEF1 amplicons. b) Amount of *Fusarium* DNA estimated with real-time PCR with *Fusarium*-specific primers targeting the TEF1. Harvested kernels were collected from 18 paired fields in organic (O) or conventional (C) production. Field pairs are numbered on a south-western gradient over Sweden starting from the north (Fig. 1).

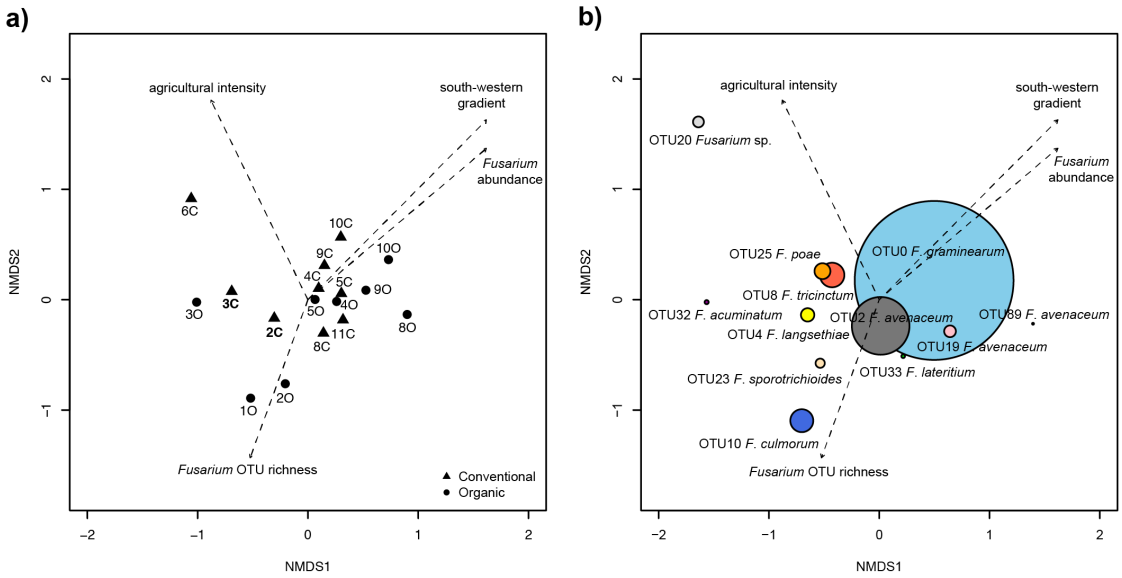

Fig. 3 *Fusarium* community composition in harvested wheat kernels from organic and conventional production.
Non-metric multidimensional scaling (NMDS) ordination of *Fusarium* communities showing a) fields and b) *Fusarium* operational taxonomic units (OTUs) with fitted explanatory variables. Fields are paired and numbered on a south-western gradient starting from the north (Fig. 1). Two fields managed with reduced tillage are marked in bold in a). In b), the abundances of *Fusarium* OTUs are represented by the area of the circles and color-coded as in Fig. 2a. 17 fields were included in the analysis.