Received: 21 October 2015 | Revised: 6 March 2017 | Accepted: 21 March 2017

DOI: 10.1111/mec.14132

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

Organic farming increases richness of fungal taxa in the wheat phyllosphere

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Funding information
Svenska Forskningsrådet Formas, Grant/Award Number: 2010-1945; The SLU Centre for Biological Control (CBC).

Abstract

Organic farming is often advocated as an approach to mitigate biodiversity loss on agricultural land. The phyllosphere provides a habitat for diverse fungal communities that are important for plant health and productivity. However, it is still unknown how organic farming affects the diversity of phyllosphere fungi in major crops. We sampled wheat leaves from 22 organically and conventionally cultivated fields in Sweden, paired based on their geographical location and wheat cultivar. Fungal communities were described using amplicon sequencing and real-time PCR. Species richness was higher on wheat leaves from organically managed fields, with a mean of 54 operational taxonomic units (OTUs) compared with 40 OTUs for conventionally managed fields. The main components of the fungal community were similar throughout the 350-km-long sampling area, and seven OTUs were present in all fields: *Zymoseptoria*, *Dioszegia fristingensis*, *Cladosporium*, *Dioszegia hungarica*, *Cryptococcus*, *Ascochyta* and *Dioszegia*. Fungal abundance was highly variable between fields, 10^3–10^5 internal transcribed spacer copies per ng wheat DNA, but did not differ between cropping systems. Further analyses showed that weed biomass was the strongest explanatory variable for fungal community composition and OTU richness. These findings help provide a more comprehensive understanding of the effect of organic farming on the diversity of organism groups in different habitats within the agroecosystem.

KEYWORDS
diversity measures, farming systems, high-throughput sequencing, microbiome, microbiota, *Triticum aestivum*

1 | INTRODUCTION

Diverse microbial communities inhabit the above-ground parts of plants, that is the phyllosphere (Newton, Gravouil, & Fountaine, 2010). Phyllosphere microorganisms are important for plant health and productivity and include plant pathogens, saprotrophs and antagonists (Peñuelas & Terradas, 2014; Vorholt, 2012). Previous research on agricultural phyllosphere has mostly focused on bacteria, while relatively little is known about fungi (Rastogi, Coaker, & Leveau, 2013). The phyllosphere microbiota is influenced by both biotic and abiotic factors, including agricultural practices (Rastogi et al., 2013). With the exception of some fungal plant pathogens, it is not well understood how agricultural management affects phyllosphere fungi.

Diversity patterns of microbial communities are associated with a number of ecosystem services. For example, Wittebolle et al. (2009) showed that initial community evenness is important for maintaining

the denitrifying capacity of bacterial communities under salinity stress. Moreover, more diverse microbial communities have in theory a higher probability of containing antagonists to pathogens or higher antagonistic co-evolutionary potential (Kinkel, Bakker, & Schlatter, 2011).

Organic farming aims at limiting the negative environmental impacts of agriculture, such as eutrophication, pesticide accumulation in the environment and biodiversity loss. Numerous studies have examined the effect of organic farming on diversity patterns of a number of organism groups and often reported a positive effect on species richness (Gasser & Berg, 2011; Hole et al., 2005; Tuck et al., 2014). However, studies of phyllosphere microbiota have been rare compared to studies on the soil microbiota and mainly focused on fruit and vegetable crops (Granado et al., 2008; Jensen et al., 2013; Leff & Fierer, 2013; Ottesen, White, Skaltsas, Newell, & Walsh, 2009). Little is known about how organic farming affects the phyllosphere microbiota of wheat, although wheat is grown on a larger proportion of land worldwide than any other crop (Food and Agriculture Organization of the United Nations, 2013). In addition, in organic fruit production and viticulture, alternatives to synthetic fungicides such as copper preparations are often used. The amount of copper in grapes has been shown to be negatively correlated with population size, species richness and evenness of grape yeasts and yeast-like fungi (Martins et al., 2014). These alternative substances are rarely used in organic cereal production (European Commission, 2007). We therefore hypothesize that organic farming practices have different effects on the phyllosphere microbiome in cereal cultivation than in fruit production and viticulture.

The differing cultivation practices used in organic and conventional cropping systems can be expected to influence phyllosphere fungi in several ways. First, the use of fungicides in conventional production potentially has direct impact on fungal communities. Fungicides may also have indirect effects on the fungal community by delaying senescence of the plant (Bertelsen, de Neergaard, & Smedegaard-Petersen, 2001). In a recent study, we showed that fungicides affect fungal community composition in the wheat phyllosphere (Karlsson, Friberg, Steinberg, & Persson, 2014). Second, organic and conventional systems also differ in the amount and quality of fertilizer applied, which influences the nutrient status of the plant. For example, it is known that nitrogen fertilization can increase the severity of some fungal diseases (Thomas, Cook, & King, 1989). Third, organic farming is characterized by more diverse cropping sequences. A more diverse crop rotation has been shown to reduce the severity of some fungal foliar diseases (Bailey, Gossen, Lafond, Watson, & Derksen, 2001). Finally, the microclimate is likely to differ between organic and conventional fields due to a generally lower crop density and higher weed abundance in organic fields. Crop density is known to influence fungal disease development (Tompkins, Fowler, & Wright, 1993).

Interestingly, there are several reports of less fungal disease in organic production, but the mechanisms behind this are poorly understood (Bernhoft, Clasen, Kristoffersen, & Torp, 2010; Gosme, de Villemandy, Bazot, & Jeuffroy, 2012). In the case of Fusarium head blight, some studies have shown that the amount of *Fusarium* and mycotoxin contamination is lower (or equal to) in organic cereal production than in conventional production (Bernhoft et al., 2010; Birzele, Meier, Hindorf, Kramer, & Dehne, 2002; Edwards, 2009). It has been hypothesized that management factors such as cereal intense rotations and high nitrogen application in the conventional system could contribute to higher levels of disease (Bernhoft et al., 2010; Edwards, 2009). Indigenous antagonistic microorganisms may also contribute to the observed differences in fungal disease. Competition for nutrients between saprotrophs and pathogens has been suggested to act as a naturally occurring biocontrol process in the phyllosphere (Fokkema, 1993). An important step in exploring the potential for biocontrol in the phyllosphere is to characterize the microbiome in different agricultural production systems. The advent of high-throughput sequencing has provided an opportunity to do so in more detail compared with first generation sequencing or culturing.

Comparing biodiversity in organic and conventional production is difficult as there are many possible confounding factors. Pairing of organic and conventional farms or fields is often used to limit variation due to, for example, climate conditions or soil type (Granado et al., 2008; Hyvönen, Ketoja, Salonen, Jalli, & Tiainen, 2003; Yeates et al., 1997). However, pairing farms to limit variability could eliminate the very differences between the two systems (Hole et al., 2005). Therefore, we used an agricultural intensity index (Herzog et al., 2006) to get an objective measure of two of the main factors differing between organic and conventional production: pesticide applications and the amount of nitrogen applied.

In this study, we used 11 paired organically and conventionally cultivated fields in Sweden to characterize the fungal community on wheat leaves. The fields were paired to account for effects due to plant genotype (wheat cultivar) and geographical location. We used 454 high-throughput sequencing and real-time PCR of the internal transcribed spacer (ITS2) to describe fungal communities in the leaves. The aim of the study was to compare fungal richness, evenness and community composition in organic and conventional winter wheat production and to explore important environmental and agricultural factors, including agricultural intensity, for phyllosphere fungi.

2 | MATERIALS AND METHODS

2.1 | Sampling, data collection and plant material

Wheat leaf samples were collected during July 2012 from 11 pairs of organically and conventionally managed fields in Sweden. Fields were paired based on proximity and cultivar. Paired fields were adjacent or located between 0.5 and 9.5 km from each other and the winter wheat cultivar grown was either 'Olivin' or 'Stava' (Table 1; Figure 1).

At sampling, the leaf below the flag leaf was picked from 10 plants evenly distributed along a 10-m-long diagonal transect starting 5 m into the field. The 10 leaves collected from each field were pooled into one plastic bag and stored at 4°C during sampling and at −20°C in the laboratory until DNA extraction. Leaves were collected using gloves to prevent cross-contamination. The proportion of

TABLE 1 Characteristics of the wheat fields included in the study. In total, 22 organically and conventionally managed wheat fields in Sweden were included

	Organic (n = 11)			Conventional (n = 11)		
	No. of fields			No. of fields		
Wheat cultivar 'Olivin'	8			8		
Wheat cultivar 'Stava'	3			3		
Field ploughed	10			8		
Nonsynthetic fertilizer applied	8			3		
Mechanical weeding	7			2		
	Min	Mean	Max	Min	Mean	Max
Years in organic production	5	10.8	22	—	—	—
Developmental stage (DC)	65	69	70	67	69	70
Field size (ha)	3	9.1	17	3.5	14	31
Sowing (days since 1 September)	5	24	35	5	23	34
Crop biomass (g/m^2)	399	776	1259	447	1131	1693
Weed biomass (g/m^2)	15	80	142	5	30	132
Crop diversity[a]	1	2.7	4	1	1.6	3
Total nitrogen applied (kg/ha)	0	51	138	108	157	204
Glyphosate applications	—	—	—	0	0.5	1
Herbicide applications	—	—	—	0	0.8	1
Fungicide applications	—	—	—	0	0.9	1
Insecticide applications	—	—	—	0	0.2	1
Total pesticide applications	—	—	—	1	2.4	3
Yield (t/ha)	2.3	3.2	4.5	3.5	5.2	7
Agricultural intensity index[b]	0	13	34	43	78	100

[a]No. of crops grown during previous 3 years, ley counted as two crops.
[b]No. of pesticide applications and amount of nitrogen applied (kg/ha) were used to calculate the agricultural intensity index.

diseased leaf surface was visually estimated for each pool of ten leaves, which included symptoms of several pathogens. To capture both endophytic and epiphytic fungi, DNA was extracted from the whole leaf tissue using the DNeasy Plant Mini kit (QIAGEN, Germany) as described previously (Karlsson et al., 2014). From each pooled sample of 10 leaves, three extraction replicates were made.

A number of additional data were collected in each field (Table 1). Wheat developmental stage was estimated according to the Zadoks' scale (Zadoks, Chang, & Konzak, 1974). At the start and end of the transect, two 0.25 m^2 squares were laid out and wheat plants and weeds (all plants other than wheat) in each square were cut and separated, and the plant material was dried for 24 hr at 105°C to determine dry mass. We hypothesized that fields with dense crop or weeds would have a different microclimate. The farmers managing the sampled fields were interviewed about the preceding crops, soil tillage, pesticides, fertilization and other measures applied. These data were also used to calculate an agricultural intensification index for each field based on the index proposed by Herzog et al. (2006). The original index by Herzog et al. includes three indicators: livestock density, the amount of nitrogen applied and the number of pesticide applications. Only the two latter indicators were applicable in this study and used to calculate the agricultural intensity index.

Weather data from weather stations in the area of the sampled fields (6–43 km from the fields, Figure 1) were taken from the LANTMET database (http://www.ffe.slu.se/lm/) (Table 2).

2.2 | PCR amplification and 454 sequencing

The ITS2 region was amplified as described previously (Karlsson et al., 2014) using primers fITS7 (Ihrmark et al., 2012) and ITS4 (White, Bruns, & Taylor, 1990), which mainly amplify the basidiomycete and ascomycete phyla. Each extraction replicate was barcoded individually (Table S1) and run in three PCR replicates. The PCR replicates were pooled and purified using AMPure (BeckmanCoulter, CA, USA), and DNA concentrations were measured using capillary electrophoresis on a MultiNA (Shimadzu, Japan). The amplicon pool was sent to Eurofins MWG Gmbh (Ebersberg, Germany) for purification by gel extraction, adaptor ligation and sequencing on one eight of a plate of a GS Junior 454 sequencer (Roche, Switzerland).

Raw sequence data were quality filtered and clustered using the SCATA pipeline (Brandström Durling et al. [http://scata.mykopat.slu.se]). The quality filtering consisted in removing reads, which had mismatches to the barcodes or more than two mismatches to the primers, low-quality scores (mean quality score <20, base score <10) or were shorter than 200 bp.

The clustering method implemented in SCATA is single linkage clustering. In single linkage clustering, a sequence is included in the cluster if it has less than 1.5% dissimilarity to *any* other sequence in the cluster, meaning that the distance between two sequences within a cluster can be larger than the cut-off (Lindahl et al., 2013). Clustering into operational taxonomic units (OTUs) approximating fungal species was performed at 1.5% dissimilarity cut-off. The cut-off level was chosen where basidiomycete yeast species were separated into different OTUs. However, no cut-off will perfectly reflect biological species (Ryberg, 2015). To test the robustness of the results in relation to the chosen OTU cut-off, the statistical analyses were repeated on data clustered at 3% and 5% dissimilarity cut-off with similar results (Fig. S1).

Global and per-sample singletons were removed from the data set (Lindahl et al., 2013). The most abundant sequence in each OTU was screened against the NCBI nucleotide database (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm, and the output was checked for nonfungal and erroneous OTUs (hits with identity or coverage

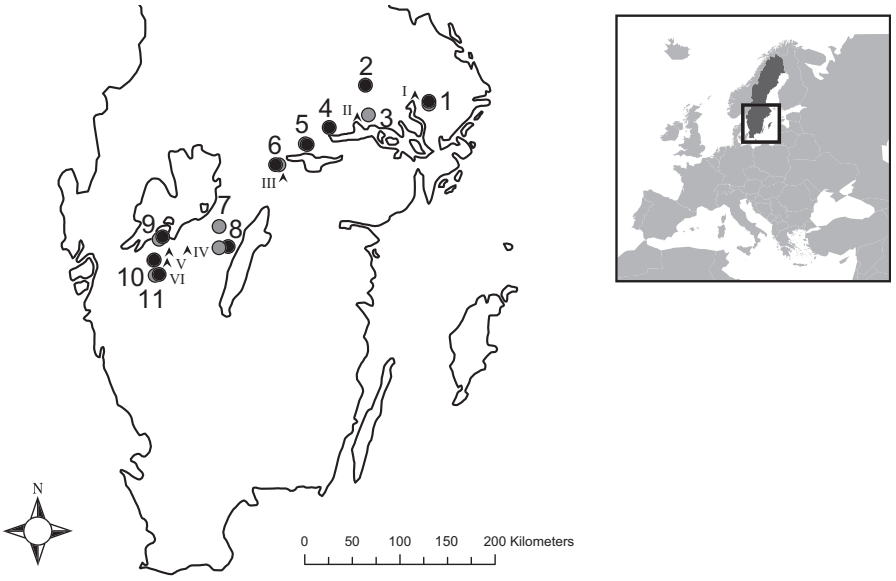

FIGURE 1 Sampling sites for the 11 pairs of wheat leaf samples in Sweden. Paired organically and conventionally managed fields were close to each other and grew the same wheat cultivar. Fields in organic production are represented by grey dots and fields in conventional production by black dots. Individual fields within a pair are not always visible when located close to each other. Weather data were taken from LANTMET stations (http://www.ffe.slu.se/lm/), which are indicated by Roman numerals (Table 2)

TABLE 2 Weather data from LANTMET[a] weather stations located close to the sampled fields. Location of weather stations I–VI is shown in Figure 1. Data are summarized from the 4 weeks preceding sampling in 2012. Degree days are the sum of days with mean daily temperature above 0°C during the period. Values in brackets are mean value for the corresponding period 2009–2011

No.	LANTMET[a] weather station	Degree days (°C)	Accumulated rainfall (mm)	Mean relative humidity (%)
I	Ultuna	456 (492)[b]	80 (44)[b]	77 (71)[b]
II	Brunnby	453 (459)	126 (59)	76 (70)
III	Säbylund	436 (447)	98 (63)	76 (72)
IV	Götdala	377 (393)[c]	185 (76)[c]	82 (77)
V	Lanna	384 (402)	136 (87)	86 (80)
VI	Bjertorp	387 (406)	99 (73)	81 (77)

[a]Data from http://www.ffe.slu.se/lm/.
[b]Data missing for 2009.
[c]Missing data, degree days calculated from mean of 15 days within the 4 weeks' period.

<70%). Species identification was performed with the RDP classifier of The Ribosomal Database Project (Wang, Garrity, Tiedje, & Cole, 2007) and complemented by including reference sequences in SCATA. The RDP classifier was run with the ITS data set from the UNITE fungal database (https://unite.ut.ee/) at a confidence level of 80%. The UNITE database is curated by experts (Kõljalg et al., 2013; Nilsson et al., 2014). Sequences of fungal origin that could not be classified to the phylum level was manually checked against the NCBI database. All samples including the extraction controls were treated in the same way.

2.3 | Real-time PCR

While 454 sequencing provide information on the relative abundances of OTUs in a community, we aimed for a more quantitative description by estimating the total fungal abundance in the samples. To this end, we quantified the number of fungal ITS copies per sample with real-time PCR using the same primers as for 454 sequencing. The PCR reactions contained 2.5 μl template, forward primer at 500 nM and reverse primer at 300 nM in Evagreen master mix (Biotium, CA, USA). The PCR conditions were 3 min at 98°C and 40 cycles of 15 s at 98°C, 20 s at 57°C and 45 s at 72°C. A dilution series of plasmid containing an ITS fragment from *Fusarium* sp. containing 1.5×10^2 to 1.5×10^6 copies per reaction was used to generate a standard curve.

The number of ITS copies per sample was normalized to the amount of wheat DNA quantified with plant-specific primers Hor1 and Hor2 (Nicolaisen et al., 2009). This is a common way to account for variation in the amount and water content of starting material and DNA extraction efficiency among samples (Schena et al., 2013). The PCR conditions were 2 min at 98°C, 40 cycles of 5 s at 98°C and 10 s at 60°C. The PCRs contained 2.5 μl template and primers at 400 nM in Evagreen master mix. A dilution series of DNA from healthy wheat leaves grown in a greenhouse containing a final concentration of 0.02–20 ng DNA per reaction was used to generate a standard curve.

Samples were diluted 1:100 to avoid PCR inhibition and run in triplicate 12.5-μl reactions on a CFX 96-well thermocycler (Bio-Rad, Carlsbad, CA, USA), followed by dissociation curve analysis at 65–95°C.

2.4 | Statistical analyses

The statistical analyses consisted of: (i) testing the effect of the cropping system (organic or conventional) and agricultural intensity index on fungal OTU richness, Pielou's evenness and fungal abundance using univariate statistics; (ii) testing the effect of cropping system/agricultural intensity index on community composition using multivariate generalized linear models (GLM); and (iii) evaluating the importance of different management practices on community composition using stepwise model selection.

The OTU table generated with 454 sequencing (proportional data) was multiplied with the total fungal abundance for each sample (real-

time PCR data). A mean OTU table based on three extraction replicates per field was calculated and used for further statistical analyses (Table S4). All statistical analyses were performed in R (R Core Team, 2014). Read count and qPCR data are available in Tables S5 and S6.

High-throughput sequencing typically results in a varying number of reads between samples, and there are several methods to account for this technical bias (Goodrich et al., 2014). We chose to incorporate the number of 454 reads per field (mean of three extraction replicates) as the first explanatory variable in the statistical models (Bálint et al., 2016).

The effect of cropping system and agricultural intensity index on OTU richness was tested using GLMs as the data were not normally distributed. A model including the number of 454 reads, field pair, number of ITS copies per ng of wheat DNA (to account for differences in fungal abundance) and cropping system was used to model OTU richness using the *glm.nb* function in the 'MASS' package (Venables & Ripley, 2002) with a negative binomial probability distribution. Thereafter, the *F*-values were adjusted by calculating a dispersion parameter (as in the quasi-Poisson function) to adjust for remaining overdispersion.

The effect of cropping system and agricultural intensity index on Pielou's evenness (Pielou, 1966) was tested using a linear model including the number of 454 reads, field pair and cropping system. Evenness was box-cox transformed to stabilize variance and improve normality.

The effect of cropping system on fungal abundance (number of ITS copies per ng wheat DNA) was tested using a paired *t*-test for unequal variance on log-transformed data to account for a non-normal distribution. The effect of agricultural intensity index on fungal abundance was tested using a GLM.

The effect of cropping system on diseased leaf area was tested with a paired *t*-test as above. The correlation between diseased leaf area and the most abundant pathogen (OTU 0 *Zymoseptoria*) was tested using Spearman rank order correlation.

The effect of cropping system and agricultural intensity index on community composition was tested using GLMs. GLMs are better in handling the mean–variance relationship typical of count data, than distance-based methods such as ordination or PERMANOVA (Warton, Wright, & Wang, 2012). 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 (Wang, Naumann, Wright, & Warton, 2012) with a negative binomial probability distribution. Significance was assessed using the *anova.manyglm* function, 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.

To visualize community composition among fields, we performed nonmetric multidimensional scaling (NMDS) 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 mitigate the effects of unequal variance between abundant and rare species. Default transformation was turned off. Subsequently, 95% confidence interval areas were fitted to the ordination using the *ordiellipse* function and vectors were fitted using the *envfit* function.

The effect of environmental and agricultural variables on fungal community composition and abundance was evaluated with redundancy analysis (RDA) using the *rda* function in the 'VEGAN' package. Model selection started from a model including the number of 454 reads and field pair as conditional variables. Correlated variables were excluded from the analysis, for example the amount of nitrogen applied, which is correlated with crop biomass. Crop diversity, type of fertilizer, crop biomass, weed biomass, number of fungicide applications and type of soil tillage were then included in the model selection. Crop diversity was calculated as the number of different crops grown on the fields in the last 3 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. To evaluate which factors were most important for OTU richness, the same variables were included in GLMs, with the number of 454 reads, field pair and fungal abundance as covariates. The GLMs were performed as the analyses of OTU richness described above. Interview data were missing for one of the fields (6O), and therefore, this field was excluded from these analyses.

3 | RESULTS

3.1 | Sequence data quality

Sequencing of ITS2 amplicons from 66 samples from a total of 220 wheat leaves from 11 pairs of fields in organic or conventional production yielded 150,180 reads, of which 75% passed SCATA quality filtering. In total, 284 fungal OTUs were revealed at 1.5% dissimilarity cut-off (Table S2). The number of 454 reads per field was 1043–2802, with a mean value of 1714 ± 531 (*SD*) reads per field. The eight DNA extraction controls only yielded a small number of reads in total (36).

3.2 | Fungal abundance and species composition

Fungal abundance, quantified by real-time PCR, was highly variable between fields, ranging from 1,081 to 95,181 ITS copies per ng wheat DNA (Figure 2a), indicating large variation in fungal biomass between fields.

The Ascomycota were represented by 72% of the OTUs and the Basidiomycota by 25%. The Basidiomycota were mainly represented by yeast species among the most frequent OTUs (Table S2). On evaluating the total number of ITS copies, the Ascomycota also dominated the data set, with 72% of the copies, compared with 28% for the Basidiomycota. Seven OTUs (0.2‰ of the ITS copies) could not be classified to phylum level by the RDP classifier. BLAST searches revealed these were probably of ascomycete origin. Seven OTUs were present across all fields: *Zymoseptoria* (OTU 0), *Dioszegia fristingensis* (OTU 2), *Cladosporium* (OTU 4), *Dioszegia hungarica* (OTU 1),

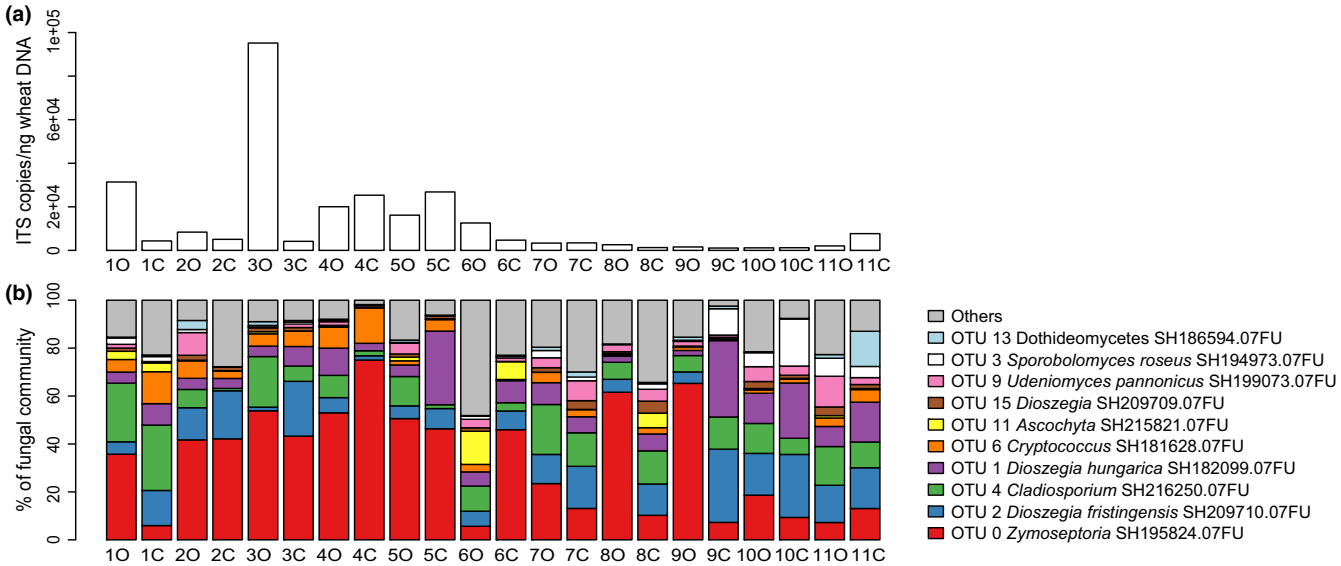

FIGURE 2 (a) Fungal abundance and (b) community composition in wheat leaves from different cropping systems. Eleven pairs of fields in organic (O) or conventional (C) production were included and numbered on a south-western gradient in Sweden starting from the north (Figure 1). In (b), the 10 operational taxonomic units (OTUs) with the highest incidence are shown, while less abundant OTUs are grouped into 'Others'. Species hypothesis accession codes in the UNITE database are indicated when available (http://unite.ut.ee/)

Cryptococcus (OTU 6), *Ascochyta* (OTU 11) and *Dioszegia* (OTU 15) (Figure 2b, Table S2).

The most abundant OTU in the data set was *Zymoseptoria* (OTU 0), contributing 46% of the total ITS copies and 6%–75% of the ITS copies per field. This OTU matched several *Zymoseptoria* species, including *Z. tritici* (*Mycosphaerella graminicola*), *Z. brevis* and *Z. pseudotritici*. *Zymoseptoria tritici* is a common pathogen on wheat leaves, causing septoria tritici blotch, while the other two are newly described pathogens on graminicolous hosts, but little is known about their distribution and host range (Quaedvlieg et al., 2011; Stukenbrock et al., 2012). However, symptoms of septoria tritici blotch were observed during sampling, indicating that *Z. tritici* was present on the leaves. A correlation between visual assessments of diseased leaf area (which included symptoms caused by several pathogens) and the amount of *Z. tritici* was observed (Spearman rank correlation coefficient = 0.86, $p < .001$). However, the diseased leaf area was not significantly different between cropping systems ($t = 0.8556$, $p = .412$). In addition to *Z. tritici*, other important wheat pathogens were identified, including *Parastagonospora nodorum* (*Phaeosphaeria nodorum*) (OTU 5), *Blumeria graminis* (OTU 12), *Monographella nivalis* (OTU 21) and *Pyrenophora tritici-repentis* (OTU 34) (Fig. S2).

3.3 | Effect of cropping system and agricultural intensity on fungal communities

We used species richness and Pielou's evenness to evaluate the effect of cropping system on fungal diversity in the wheat phyllosphere, as these measure two different dimensions of diversity (Magurran, 2004; Purvis & Hector, 2000). Species richness, estimated as the number of OTUs per 10 wheat leaves, was higher on wheat leaves from organic production, with a mean of 54 ± 8.8 (*SD*) OTUs compared with 40 ± 24.3 (*SD*) OTUs for conventional production (Table 3a; Figure 3a). However, for three pairs, OTU richness was higher in the conventional field (Figure 3a). Species richness was not affected by fungal abundance (Table 3a).

As regards Pielou's evenness, there was no significant difference between the two cropping systems (Table 3a; Figure 3b). However, evenness was affected by the number of 454 reads per sample (Table 3a), with a negative relationship to the number of reads.

The mean number of ITS copies was ~18,000 in organically managed fields and ~8,000 in conventionally managed fields (Figure 3c), but a paired *t*-test showed no significant difference between the two cropping systems ($t = 1.37$, $p = .200$).

The multivariate GLM showed a significant effect of cropping system on fungal community composition (Table 4a). After controlling for multiple testing, two OTUs were found to be affected by the cropping system: OTU 38 Ascomycota (GLM: deviance = 32.2, $p = .027$) and OTU 54 Ascomycota (GLM: deviance = 38.8, $p = .013$), and both were more abundant in the organic system (Fig. S2). The RDP classifier only classified these OTUs to the phylum level, but a BLAST search revealed that OTU 38 had 98% similarity to a sequence (KT203193) from a fungal endophyte isolated from the leaves of the grass *Elymus mollis* (David, Seabloom, & May, 2015). OTU 54 had 96% identity to *Penidiella strumelloidea* (EU019277), and 95% identity to the sequence of another fungal endophyte from the study of David et al. (KT203050). The field pair variable was significant in the analyses of community composition (Table 4), and the more south-western fields (8–11) tended to cluster towards the top right of the NMDS ordination (Figure 4).

The mean agricultural intensity index was lower for organically managed fields than for conventionally managed, and the variation was larger in the conventional group (Table 1; Figure 3d–f). The

TABLE 3 Effect of (a) cropping system and (b) agricultural intensity on the richness and evenness of wheat phyllosphere fungal communities. Species richness is estimated as the number of operational taxonomic units (OTUs) per 10 wheat leaves; Fungal abundance is estimated as internal transcribed spacer (ITS) copies per ng wheat DNA. Eleven field pairs in conventional and organic production were included. Agricultural intensity was measured as an index including the amount of nitrogen applied (kg/ha) and the number of pesticide applications; one field was excluded from this analysis due to missing data. Richness was analysed using generalized linear models (GLM) and evenness using linear models

	(1) Richness				
a) Cropping system	***df***	**Deviance**	**Resid. *df***	***F***	***p***
No. of 454 reads	1	0.02	20	0.01	.933
Fungal abundance	1	0.47	19	0.20	.667
Field pair	10	26.0	9	1.09	.460
Cropping system	1	18.2	8	7.64	**.025***
	(2) Pielou's evenness				
	df	**SS**	**MS**	***F***	***p***
No. of 454 reads	1	0.030	0.030	11.0	**.009****
Field pair	10	0.069	0.007	2.5	.088
Cropping system	1	0.003	0.003	1.1	.317
Residuals	9	0.024	0.003		
	(1) Richness				
b) Agricultural intensity	***df***	**Deviance**	**Resid. *df***	***F***	***p***
No. of 454 reads	1	0.02	19	0.01	.936
Fungal abundance	1	0.58	18	0.23	.649
Field pair	10	35.9	8	1.39	.339
Agricultural intensity index	1	22.2	7	8.59	**.022***
	(2) Pielou's evenness				
	df	**SS**	**MS**	***F***	***p***
No. of 454 reads	1	0.027	0.027	9.9	**.014***
Field pair	10	0.070	0.007	2.5	.102
Agricultural intensity index	1	0.003	0.003	1.3	.296
Residuals	8	0.022	0.003		

*$p < .05$, **$p < .01$.

effect of agricultural intensity index on fungal communities followed a similar pattern. Agricultural intensity index had a negative effect on species richness but no effect on evenness (Table 3b; Figure 3d–e). Fungal abundance was not affected by agricultural intensity index (GLM: deviance = 1.26, $df = 9$, $p = 0.29$, Figure 3f), but there was a significant effect of field pair (deviance = 7.08, $df = 10$, $p = .002$). Agricultural intensity index also had an effect on fungal community composition (Table 4b).

3.4 | Importance of agricultural and environmental factors for fungal communities

Organic and conventional cropping systems differ in a number of agricultural practices. Here we evaluated the agricultural and environmental factors that were most important for fungal community composition and OTU richness and could contribute to the difference observed between the two systems. The diversity of previous crops on the field, type of fertilizer, crop biomass, weed biomass, number of fungicide applications and type of soil tillage were evaluated. For community composition, weed biomass in the field was the only variable included by the model selection procedure. In the RDA with the selected model, 61% of inertia was attributed to the conditional variables (no. of 454 reads and field pair), 11% to constrained inertia (weed biomass) and 28% to unconstrained inertia. The permutation test for RDA showed a significant effect of weed biomass ($F = 3.19$, $p = .008$). For OTU richness, the model including weed biomass had the lowest Akaike's information criterion (AIC), indicating a better fit of the model, followed by fungicide use and these two factors had statistically significant effects (Table 5).

4 | DISCUSSION

This evaluation of the effect of organic farming on fungal communities in the wheat phyllosphere revealed higher richness of fungal OTUs in the organically managed fields compared with the conventionally managed. While little is known about the cereal phyllosphere, higher fungal species richness has previously been reported in the phyllosphere of organically produced fruit. For example, Granado et al. (2008) found higher taxon richness of both epiphytic and endophytic fungi in organic apple production compared with integrated production, while Martins et al. (2014) reported higher species richness of yeasts on organically produced grapes compared with conventionally produced. For many organism groups, including plants, birds, arthropods and soil microbes, organic farming has been shown to have a positive effect on species richness (Tuck et al., 2014). Our study of fungal communities in the wheat phyllosphere thus adds further knowledge on the effects of organic farming on biodiversity.

Some previous studies have reported higher numbers of fungal colony-forming units on organically produced fruit than on conventionally produced (Granado et al., 2008; Martins et al., 2014). Although comparison of biomass is an important factor to fully understand the ecology of fungal communities (Baldrian et al., 2013), this is not always included in amplicon sequencing studies of microbial communities. For leaf fungal communities, the total biomass of, for example, a pathogen compared to other fungi provides more information on the potential impact on the plant than its relative abundance in the community. We quantified the total abundance of fungal ITS copies on the leaves using real-time PCR to estimate fungal biomass. We found no indication of differing fungal abundance between the two production systems and abundance was not related to OTU richness. Fungal abundance was highly variable between fields, even within field pairs. This highlights the importance of complementing amplicon sequencing data with estimates of fungal biomass to get a more complete view of fungal communities.

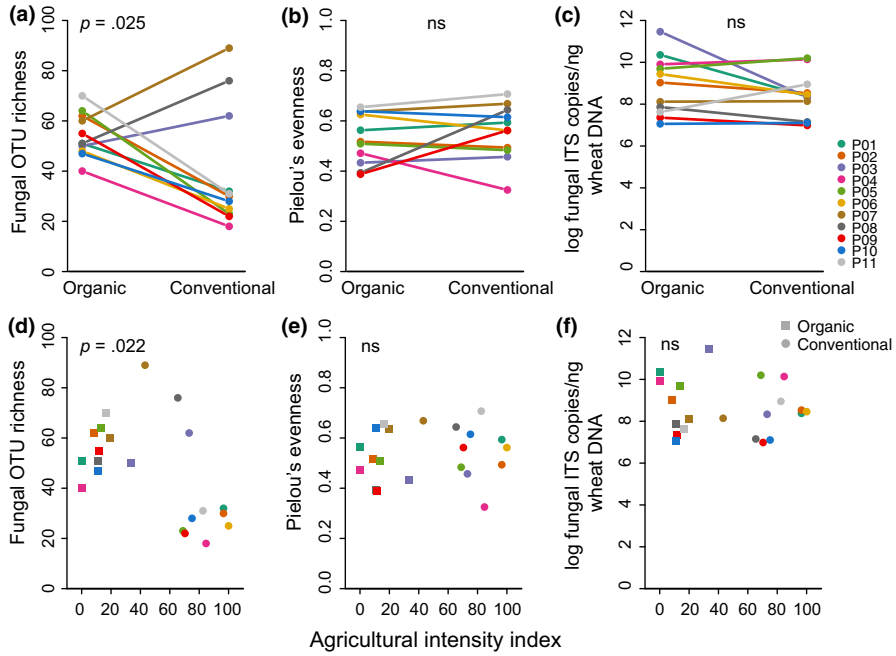

FIGURE 3 Richness, evenness and abundance of phyllosphere fungal communities in organically and conventionally managed wheat fields. Distribution of fields regarding (a) and (d) richness of operational taxonomic units (OTUs), (b) and (e) Pielou's evenness and (c) and (f) abundance (number of internal transcribed spacer (ITS) copies per ng wheat DNA). In panel a-c, data are grouped by cropping system and in panel d–f the agricultural intensity index is indicated on the *x*-axis. Field pairs of one conventionally and one organically managed field are colour-coded. Test results in Table 3. ns = nonsignificant

TABLE 4 Effect of (a) cropping system and (b) agricultural intensity on fungal community composition in the wheat phyllosphere. Eleven pairs of conventionally and organically managed wheat fields were sampled in Sweden. Agricultural intensity was measured as an index including the amount of nitrogen applied (kg/ha) and the number of pesticide applications, one field was excluded from this analysis due to missing data. Data were analysed with multivariate generalized linear models (using the *manyglm* function in the 'MVABUND' R package) with a negative binomial probability distribution

	Residual *df*	*df*	Deviance	*p*
(a)				
No. of 454 reads	20	1	567	.147
Field pair	10	10	3024	**.011***
Cropping system	9	1	650	**.003****
(b)				
No. of 454 reads	19	1	563	.16
Field pair	9	10	3029	**.003****
Agricultural intensity index	8	1	704	**.003****

*$p < .05$, **$p < .01$.

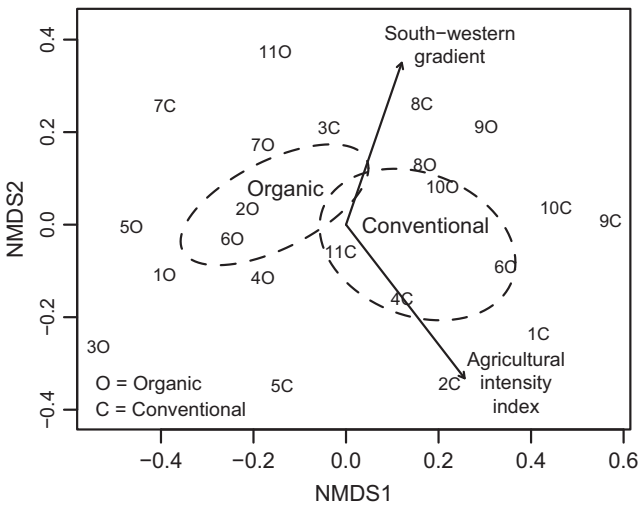

FIGURE 4 Nonmetric multidimensional scaling (NMDS) ordination of fungal phyllosphere communities. Eleven pairs of organically and conventionally managed wheat fields were included. Fields are shown with fitted environmental variables. Pairs are numbered on a south-western gradient starting from the north (Figure 1). Ellipses represent 95% confidence areas for organic and conventional groups, respectively. Stress: 0.14

However, it is worth noting that the number of ITS copies in the genome varies between fungal species and individuals (Liti et al., 2009; Rodland & Russell, 1982). This may influence comparisons of both community composition and abundance of fungal communities between samples based on ITS (Baldrian et al., 2013).

The sampling year, 2012, had the highest occurrence of leaf blotch diseases in the northern part of the sampling area since 1998 (Swedish Board of Agriculture, 2012). The sampled leaves had up to 70% of diseased leaf area (Table S3). This was also reflected in the molecular data, with up to 75% of the ITS copies per field represented by *Zymoseptoria* (OTU 0), including *Z. tritici*, one of the main pathogens responsible for leaf blotch diseases in wheat. However, in

TABLE 5 Importance of agricultural and environmental variables for fungal OTU richness in the wheat phyllosphere. Variables were evaluated using generalized linear models with a negative binomial probability distribution. All models included the number of 454 reads, field pair and fungal abundance as covariates. Eleven pairs of conventionally and organically managed wheat fields were sampled. One field was excluded due to missing data

	Akaike's information criterion (AIC)	Deviance	*p*
Weed biomass (g/m^2)	164.6	29.5	**.001*****
No. of fungicide applications	186	6.4	**.04***
Type of fertilizer (organic/inorganic/both)	187.5	1.9	.24
Crop biomass (g/m^2)	191.1	3.5	.10
Crop diversity (no. of crops during last 3 years)	192	3.1	.12
Type of soil tillage (ploughed/reduced)	193.2	2.5	.16

*$p < .05$, ***$p < .01$.

the fields with the highest estimated fungal biomass, 3O and 1O, *Zymoseptoria* accounted for 54% and 35% of the fungal community, respectively, so the presence of this pathogen could not fully explain the high amount of fungal DNA on leaves from these fields, as other fungi were also abundant (Figure 2). The whole sampling area had experienced lower temperatures and received more rainfall before the sampling in July 2012 than in the same period in previous years (Table 2), and it is known that rainfall favours infection by *Z. tritici* (Thomas et al., 1989).

The dominance of *Zymoseptoria* resulted in lower Pielou's evenness overall compared to our previous study (Karlsson et al., 2014). This might be one reason why no difference in evenness was observed between the two farming systems. The effect of organic farming on evenness has been less well studied than the effect on species richness. A meta-analysis suggests that these two measures of diversity may be independent of each other (Crowder, Northfield, Gomulkiewicz, & Snyder, 2012). Higher Pielou's evenness of fungal communities has previously been observed in the apple phyllosphere in organic production compared with conventional (Glenn, Bassett, & Dowd, 2015). In soil fungal communities, Hartmann, Frey, Mayer, Mäder, and Widmer (2014) observed the same effect of organic farming as in our study, with increased species richness and no effect on Smith Wilson evenness. However, the extent to which soil and phyllosphere fungal communities can be expected to respond similarly to agricultural management is unclear.

Organic farming also had an impact on the overall composition of the fungal community, and two OTUs were significantly affected. These OTUs were more abundant in the organic system and both had similarities to *Penidiella strumelloidea* and fungal endophytes isolated from grasses (David et al., 2015). *Penidiella strumelloidea* has been reported as a foliar pathogen on cucumber in Iran (Nosrati, Esmailzadeh-Hosseini, & Sarpeleh, 2010). In a previous study, the culturable fungal community of strawberries differed to only a small extent between organic and conventional systems (Jensen et al., 2013). Other studies have reported more significant differences between cropping systems. On grapes, the yeast *Sporidibolus* has been reported to dominate in conventional systems, while *Aureobasidium* dominates in organic systems (Martins et al., 2014; Schmid, Moser, Muller, & Berg, 2011).

A notable finding was that there was no difference between the two systems for any of the OTUs representing pathogenic species in the data set, although the use of fungicides could be expected to reduce the abundance of pathogens in the conventional system. Lower incidence of leaf blotch diseases in organically managed fields has also been reported, with one possible explanation being differences in nutritional status of the plant (Gosme et al., 2012). For example, nitrogen fertilization has been shown to increase the disease severity of septoria tritici blotch (Simón, Cordo, Perelló, & Struik, 2003). *Zymoseptoria* (OTU 0) was the most abundant OTU in the present study, but no significant difference in its abundance or in visual disease estimations (including symptoms of several pathogens) was observed between the two cropping systems. Similarly, the response was not consistent across pairs for other potential pathogens (Fig. S3). This could be due to variable environmental and weather conditions. In addition, fungicide resistance and timing of control measures applied could be important.

Fungicide use is one of many factors differing between organic and conventional systems and can be expected to have a direct effect on phyllosphere fungi. It is known that different fungicides have different effects on phyllosphere fungi (Bertelsen et al., 2001). The fields in our study received different fungicide treatments, but all fungicide-treated fields had received a strobilurin in combination with a triazole/triazolinthione, two chemical groups with different mode of action (Table S3). Fungicide use was associated with significantly lower OTU richness and could be one of the factors explaining the observed differences between the two systems. Fungicide use has been reported to lower the richness (Sapkota, Knorr, Jørgensen, O'Hanlon, & Nicolaisen, 2015) and evenness of fungal communities on wheat leaves (Karlsson et al., 2014). But in a study on wheat kernels, only minor effects on nontarget fungi was reported (Hertz et al., 2016).

Weed biomass in the fields emerged as the most important predictor for both community composition and OTU richness. Weeds are more difficult to control in organic production and weed biomass tended to be higher in the organically managed fields in our study (Table 1). The conventional fields had been treated with different herbicides, where synthetic auxins and ALS-inhibitors were the most common (Table S3). So far, we can only speculate about the cause of this observation. It could be because weeds contribute to a different microclimate in the field, favouring a different fungal community, or because high weed biomass correlates with higher weed species richness, with different weeds possessing distinct microbiota that can spread to the crop, the two hypotheses being compatible. Weed flora is also different between organic and conventional fields in Sweden, with more nitrophilous species in the conventional system (Rydberg & Milberg, 2000). The microbiota of common weed species

have not yet been described, but plant genotype has been associated with differences in fungal phyllosphere communities (Agler et al., 2016; Sapkota et al., 2015). However, weed biomass might be correlated with other factors that vary between fields, and therefore, more controlled experiments would be necessary to confirm the influence of weed biomass on fungal communities in the cereal phyllosphere.

Overall, the main components of the fungal community were similar regardless of cropping system and geographical location, as seven OTUs were present across all fields. In a previous study, we identified six OTUs that were present across all fields sampled in two regions in Sweden in 2011 (Karlsson et al., 2014). Four of the OTUs were shared between the 2 years, namely *Cladosporium* (OTU 4), two *Dioszegia* (OTUs 1 and 2) and one *Cryptococcus* (OTU 6) OTU. These genera have also been observed in other studies of wheat leaves (Blixt, Olson, Lindahl, Djurle, & Yuen, 2010; Sapkota et al., 2015) and on wheat kernels (Hertz et al., 2016; Nicolaisen, Justesen, Knorr, Wang, & Pinnschmidt, 2014). This indicates that many members of the phyllosphere mycobiome of wheat are cosmopolitan over large geographical distance. A similar observation has been made in plant communities, where the most abundant species were shared between intensively and extensively managed fields, indicating that intensification mainly removes the rarer species rather than completely reshapes the community (Kleijn, Rundlöf, Scheper, Smith, & Tscharntke, 2011).

Farmers practising organic production often grow different wheat cultivars than farmers in conventional production, as higher disease resistance and nutrient use efficiency are required (Lammerts van Bueren et al., 2011). In our study, we paired one field in organic production with one field in conventional production growing the same wheat cultivar, as plant genotype can affect the phyllosphere microbiota (Agler et al., 2016). Our study included two cultivars: 'Olivin' which is common in conventional production and 'Stava' which is targeted for organic production (http://www.lantmannenlantbruk.se/sv/produktkatalog/vaxtodling/hostutsade/hostvete/, 19/09/2016). For this reason, the pairing based on cultivar might have introduced bias while selecting farms growing a cultivar nontypical of the respective system. Furthermore, as organic production has to comply with certain regulations, it can be expected that the cropping practices varies less within the organic group than within the conventional group. This was reflected in our data set as not all the conventional farmers had applied fungicides or fertilizer. We used an agricultural intensity index to directly evaluate the effect of the main cropping practices differing between the two systems. The index includes the number of pesticide applications and the amount of nitrogen applied. As expected, the mean agricultural intensity index was lower for organically managed fields and lower agricultural intensity was associated with higher species richness. Interestingly, the three conventional fields where there was a higher richness than in the organic field in the pair, all tended towards the lower range of the agricultural intensity index of the conventional group. Thus, agricultural intensity index and cropping system were correlated in our study with similar effects on fungal communities.

The pairing was also intended to account for geographical variation and field pair was included as a variable in all analyses. Field pair had a significant effect only in some of the analyses, including fungal abundance and community composition. This indicates that different aspects of phyllosphere fungal communities may have different drivers. Fields within each pair were located within less than 10 km of each other, but it is possible that some aspects of fungal communities varies on a smaller spatial scale. Dispersal of fungal spores could be dependent on landscape characteristics and can be limited by for example patches of large trees. Fields located more to the south-west of the sampling area clustered to the top right of the NMDS plot (Figure 4), indicating regional differences. Weather data collected from stations throughout the sampling area indicated lower temperatures and lower humidity in north-eastern parts of the area than in south-western parts in the month preceding sampling (Table 2). Fungal taxa have previously been found to overlap between air and leaves, and average daily temperature has been shown to be correlated with the amount of fungal spores in the air (Levetin & Dorsey, 2006). Differences in weather throughout the sampling area may thus have influenced fungal communities.

High-throughput sequencing is often associated with variable read numbers, which is a technical bias, not representing the variation in abundance of the organism between samples (Goodrich et al., 2014). To overcome this problem, we included read numbers as a factor in the statistical models. This approach was chosen over the common practice of rarefying count data, which has been criticized for omitting valid data (McMurdie & Holmes, 2014). Read numbers may vary by several orders of magnitude between samples, but in our data set, the variation was limited to about twofold. This may explain why read numbers did not affect species richness or community composition. However, evenness was affected by the number of reads, with high read numbers associated with lower evenness.

Another common feature of high-throughput sequencing data (and other count data) is a positive mean–variance relationship. Using distance-based methods such as PERMANOVA on this type of data may confound location and dispersion effects (Warton et al., 2012). We noted a clear mean–variance relationship in our data indicating that GLMs were suitable in this case.

In conclusion, we found a positive effect of organic farming on species richness of wheat phyllosphere fungi, which could be expected but which has never yet been demonstrated. Our findings contribute to a more comprehensive understanding of the effect of organic farming on the diversity of different organisms and in different habitats within the agroecosystem. Further studies are necessary to determine which management factors contributed to the observed difference between cropping systems. Our results indicate that the weed flora in the field can be an important factor to study. Variable disease pressure between years is another factor to take into account when evaluating the effect of organic farming on phyllosphere fungal diversity. Furthermore, it would be of interest to place the diversity of phyllosphere fungi within the context of the agricultural landscape. High-throughput amplicon sequencing is a helpful tool which provides further insights into the ecology of phyllosphere microorganisms and their

response to agricultural management but it is important to combine with biomass estimations. We believe that this knowledge can be used to develop biocontrol strategies, which promote antagonistic indigenous microbial communities in the phyllosphere. In the light of increasing fungicide resistance and the general aim of decreasing the environmental impact of agriculture, such strategies are likely to become more important in the future.

ACKNOWLEDGEMENTS

We would like to thank the farmers for their participation in the study, Maria Kedmark and Rickard Almers for assistance in the field, Tina Astor for critical review of the manuscript and Johannes Forkman for advice on statistical analyses. This work was supported by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (http://www.formas.se/en/), [grant number 2010-1945] and the SLU Centre for Biological Control (http://www.slu.se/cbc).

AUTHOR CONTRIBUTIONS

I.K., H.F., C.S. and P.P. designed the study; I.K. performed research; I.K., H.F., A.-K.K., C.S. and P.P. analysed and interpreted data; I.K., H.F., A.-K.K., C.S. and P.P. wrote the manuscript.

DATA ACCESSIBILITY

Raw sequence data have been deposited in the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under the Accession no. SRP057731. Representative sequences of the most common OTUs have been uploaded to GenBank (KY006587–KY006643). Supplementary tables including OTU abundance data, explanatory variables and the R code used to analyse the data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.01f10.

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How to cite this article: Karlsson I, Friberg H, Kolseth A-K, Steinberg C, Persson P. Organic farming increases richness of fungal taxa in the wheat phyllosphere. *Mol Ecol*. 2017;26:3424–3436. https://doi.org/10.1111/mec.14132