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Are communities of microbial symbionts more diverse than communities of macrobial hosts?

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ABSTRACT

In this study, fungal viruses (mycoviruses) of plant-associated fungi were used to test the general assertion that communities of parasitic and mutualistic symbionts may be more species-diverse than communities of their hosts. Mycoviruses are poorly studied in general, but can affect the fitness and ecology of the fungi and plants with which they associate. To date, mycovirus incidence and diversity in natural communities remain largely unaddressed. Here, we compared the incidence and diversity of fungi associated with tallgrass prairie plants to the diversity and incidence of mycoviruses within those fungi. Specifically, we sampled viruses from fungi associated with a parasitic plant (*Cuscuta cuspidata*) and its most frequent host plant (*Ambrosia psilostachya*) in a tallgrass prairie habitat in Oklahoma. For each plant sample we cultured fungal endophytes from surface-sterilized aboveground tissues. From the cultured fungi we extracted DNA to identify fungi, and extracted double-stranded RNA (dsRNA) to detect mycoviruses. Mycoviruses were further characterized using reverse transcription-PCR and sequence analyses. We found at least 25 fungal taxa associated with the two plants, and 10 % of these fungi contained readily detectable viruses. Several mycovirus types were shared among fungal taxa, indicating that mycoviruses may be less specialized than originally thought. Although the virus community was not as diverse as the fungal endophyte community (16 taxa), species accumulation rates of mycoviruses (inferred from rescaled rarefaction curves) may be higher than those of their associated fungal hosts. Thus, mycoviruses represent a further layer of undocumented biodiversity in ecological communities.

Introduction

Symbiotic relationships among organisms are ubiquitous in nature, yet we are only beginning to understand patterns of symbiont diversity. Communities of microbial symbionts living in larger organisms often are more diverse than the communities of the larger host organisms. For example, plant-associated fungal communities may be 3.5–33 times as diverse as the plant communities with which they associate (Hawksworth 2001), leading to claims that some communities of fungal endophytes in particular are hyperdiverse (Arnold *et al.* 2000). A greater ratio of symbiont to host species

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also is found among human bacterial associates (e.g., Paster *et al.* 2001; Eckburg *et al.* 2005), viruses associated with aquatic bacterial communities (Breitbart *et al.* 2002; Desnues *et al.* 2008), and viruses associated with plant monocultures (e.g., Al Rwahnih *et al.* 2009; Coetzee *et al.* 2010).

Endophytic fungi are ubiquitous fungi inhabiting plant tissues without causing apparent symptoms of disease (Hyde & Soytong 2008). Endophytes are extremely diverse (Arnold *et al.* 2000), and have broad-ranging effects on their host plants and varied ecological roles (reviewed in Rodriguez *et al.* 2009). These fungi are often generalists, such that the same species of fungal endophytes are shared between parasitic plants and their host plants (Suryanarayanan *et al.* 2000), or between different plant species within the same community (Higgins *et al.* 2011). Mycoviruses (viruses that infect fungi) commonly infect fungal endophytes (Herrero *et al.* 2009).

Mycoviruses were discovered in cultivated mushrooms in 1960s (reviewed in Ghabrial & Suzuki 2009). Since then, mycoviruses have been discovered in a diverse range of fungi from *Basidiomycota* (e.g., Yu *et al.* 2003; Ikeda 2006) to *Ascomycota* (e.g., Nuss & Koltin 1990; Tuomivirta & Hantula 2005), and in fungi with diverse lifestyles from plant pathogens (Osaki *et al.* 2006) to saprotrophs (Yu *et al.* 2003) and mutualists (Márquez *et al.* 2007; Romo *et al.* 2007). Closely related viruses have been discovered in plants (Fukuhara *et al.* 2006; Roossinck 2010), oomycetes (Hacker *et al.* 2005), and protists (Scheffter *et al.* 1994). Most of these viruses comprise double-stranded RNA (dsRNA) genomes and are typically classified into the four families: *Totiviridae*, *Partitiviridae*, *Mycoreoviridae*, and *Chrysoviridae* (Sasaki *et al.* 2007). Other mycoviruses may have single-stranded RNA genomes, including mycoviruses in the *Hypoviridae* (Koonin *et al.* 1991), the *Endornaviridae*, and other groups (Preisig *et al.* 2000; Yu *et al.* 2003). In addition, Yu *et al.* (2010) discovered a geminivirus-related DNA mycovirus in the plant-pathogenic fungus *Sclerotinia sclerotiorum*. Representatives of several other virus families also have been identified and characterized, some of which were not closely related to any previously known viruses (Preisig *et al.* 2000; Yu *et al.* 2003; Márquez *et al.* 2007). In spite of the discovery of many mycoviruses associated with pathogenic and nonpathogenic fungi, little is known about their function, biodiversity or ecology. However, mycoviruses have the potential to alter characteristics of their host fungi, either increasing virulence (Ahn & Lee 2001) or decreasing virulence (Nuss & Koltin 1990; Preisig *et al.* 2000; Yu *et al.* 2010) of plant-pathogenic fungi, or even increasing the tolerance of a plant-fungal mutualism to abiotic stress (Márquez *et al.* 2007). By altering the fitness of their hosts, these mycoviruses could affect species interactions, species prevalence in communities, and thus community composition of host fungi or even associated plants.

Some virologists have claimed that mycoviruses are transmitted only through spores (vertically) and through anastomoses (hyphal connections) between vegetatively compatible fungal strains (e.g., Ghabrial 1994). However, there is some evidence for transmission of mycoviruses among related fungal species (Buck *et al.* 2003; Liu *et al.* 2003), or even between plants, oomycetes, and fungi (Roossinck 2010; Roossinck *et al.* 2011). Generally, most of what is known to date indicates that mycoviruses are relatively specialized to a fungal species. Typically, viruses representing several of the major families of mycoviruses have been found in each fungal species that has been examined extensively (Tuomivirta & Hantula 2005; Ikeda 2006). This pattern indicates that the ratio of mycoviruses to fungal hosts may be much higher than one—i.e., the diversity of mycoviruses may be extremely high.

Although a few surveys of viruses in economically important fungi have been conducted (Nuss & Koltin 1990; Buck *et al.* 2003; Ikeda 2006; van Diepeningen *et al.* 2006), very little is understood about the prevalence of plant or mycoviruses in ecological communities that are not agroecosystems (but see Herrero *et al.* 2009; Roossinck *et al.* 2010). Furthermore, focussing on one or a few economically important species in agroecosystems or other human-generated environments in which relatively few species dominate may bias estimates of the prevalence and diversity of mycoviruses. One recent survey (Herrero *et al.* 2009) showed that diverse unidentified dsRNA elements commonly occurred within fungi associated with several grass species from nonagricultural settings.

Here we show results from a 2-y biodiversity study of mycoviruses and their host fungal endophytes associated with a parasitic plant (*Cuscuta cuspidata*, or prairie dodder), and above-ground parts of its main host plant (*Ambrosia psilostachya*, or western ragweed) in a diverse tallgrass prairie habitat in northeastern Oklahoma (the Tallgrass Prairie Preserve, Pawhuska, OK, USA; Palmer 2007). With this information, we addressed the following questions: (1) How prevalent and diverse are fungal endophytes from two plant species on the Tallgrass Prairie Preserve? and (2) How prevalent and diverse are mycoviruses from this fungal endophyte community? Our data suggest that communities of both endophytic fungi and mycoviruses might be more diverse than the communities of their respective hosts.

Materials and methods

Field sites

The Tallgrass Prairie Preserve in Pawhuska, Oklahoma (USA) is a 15 837-hectare property managed by the Nature-Conservancy. Habitats include prairies interspersed with oak-hickory woodlands (crosstimbers). Plant material was sampled from four sites in the Tallgrass Prairie Preserve (three in each of 2 y) dominated by prairie vegetation including large populations of *Ambrosia psilostachya*, in which plants parasitized by *Cuscuta cuspidata* occurred. Plants were sampled from site 1 (36°48′13″N, 96°25′20″W), site 2 (36°45′15″N, 96°22′37″W), and site 3 (36°50′23″N, 96°27′29″W) in 2006 and from site 1, site 3, and site 4 (36°45′20″N, 96°22′43″W) in 2007. Within each year, each site was at least 1 km away from the others.

Plant materials

Cuscuta cuspidata Engelm. is an annual, obligate parasite in the *Convolvulaceae* (Stefanović *et al.* 2002), ranging through most of the central United States, from North Dakota and Wisconsin to Texas, east to Kentucky and west to Utah (USDA 2006). Members of the genus *Cuscuta* have reduced leaves, very little chlorophyll, and no true roots, instead forming haustorial

connections with above-ground parts of host plants, drawing food, and nutrients from host vascular tissues (Diggs *et al.* 1999). On the Tallgrass Prairie Preserve, this species primarily parasitizes (i.e., forms haustoria on) plants in the *Asteraceae*, including *Solidago*, *Helianthus*, and *Ambrosia*, but also parasitizes plants in other families, including *Fabaceae* and *Poaceae* (T. S. Feldman, pers. obser.). Plants emerge by late May or early June, flower in late summer, and set seed by midlate October. We chose to use a *Cuscuta*-host system for this study because *Cuscuta* species are well known as vectors of plant viruses (Hosford 1967), and species of endophytic fungi are shared between *Cuscuta* plants and their hosts (Suryanarayanan *et al.* 2000).

Ambrosia psilostachya DC., or western ragweed, is a common, perennial weed of roadsides, pastures, and prairies, ranging from Quebec and British Colombia, through most of the United States and south (USDA 2006). *Ambrosia psilostachya* plants emerge in April, and produce wind-pollinated flowers between August and November (Diggs *et al.* 1999), after which the above-ground portions of the plant senesce.

Plant sampling and identification of Cuscuta *species*

We sampled tissues from all *Cuscuta* plants that we could clearly distinguish as separate individuals at each site. In total, we collected tissues from 38 *Cuscuta* plants and their *Ambrosia psilostachya* hosts in 2006 and from 26 *Cuscuta*–*Ambrosia* pairs in 2007, along with additional unparasitized material from *A. psilostachya* in 11 of these host–parasite pairs and three completely unparasitized *A. psilostachya* plants in 2007. Tissues from 28 and 26 of these plant pairs were used for fungal sampling in 2006 and 2007, respectively. We sampled two additional *Cuscuta*-host plant pairs associated with another host plant (*Symphyotrichum ericoides*) in 2006, and two additional pairs in 2007 (*Cuscuta* on *Helianthus* and *Solanaceae*).

Most of the *Cuscuta* tissues were collected when plants had not yet produced flowers, so we used molecular tools to confirm the identities of the *Cuscuta* species. DNA was extracted from *Cuscuta* samples using Plant DNAzol® (Invitrogen), and amplified using universal primers for regions of the nuclear internal transcribed spacer (ITS) rDNA and chloroplast transfer RNA gene *trnL-trnF* (Stefanović *et al.* 2002, 2007). We used the following program on the Idaho Technologies Rapid Cycler 2: 94 °C for 1 min, 30 cycles of 94 °C for 0 s, 45 °C for 0 s, 72 °C for 30 s, followed by 72 °C for 5 min, and 37 °C for 5 min. The Rapid Cycler uses glass capillaries heated and cooled by bursts of hot and cool air around the capillaries, which reduces the sample temperature lag to less than a second. The sequences of the amplicons were obtained using an ABI 3700 sequencer, and were subsequently placed into the context of an existing phylogeny of *Cuscuta* (Stefanović *et al.* 2007) to determine probable species identities of all sequences. Because all sequences for each gene from the *Cuscuta* plants used in our analyses were identical, we aligned our consensus sequence with sequences from the previous phylogeny using Clustal (Higgins & Sharp 1988), and manually corrected the alignments in MacClade (Maddison & Maddison 2000). Plant sequence data obtained in this study have been submitted to the European Molecular Biology Laboratory's European Bioinformatics Institute (EBI) Nucleotide Sequence Database under accession numbers HE579168–HE579207 (ITS) and HE579450–HE579516 (*trnL-trnF*). To determine possible species identities of all plants, we used neighbour-joining and maximum parsimony bootstraps (100 bootstrap replicates, default settings, using PAUP; Swofford 2000), and used the consensus trees to determine the species with which our samples grouped in a well-supported clade.

Fungal isolation

Small branches of *Ambrosia psilostachya* parasitized with *Cuscuta* were surface-sterilized together using a 2 % sodium hypochlorite and 70–95 % ethanol solutions followed by several rinses with autoclaved water according to Schultz *et al.* (1993). In 2006, we used the imprint method to test our sterilization techniques on seven host–parasite pairs (as in Schultz *et al.* 1998), and results indicated that the method was effective. After surface sterilization about 0.5–1 cm pieces of *A. psilostachya* leaves and stem, and *Cuscuta* stem and haustoria were cut using sterilized forceps and scissors. Ten to 12 pieces of each plant tissue were plated onto 0.1× potato dextrose agar (0.1× PDA) with ampicillin, streptomycin, and tetracycline (all at 50 $\mu g\ ml^{-1}$), and were monitored for fungal growth. From *Cuscuta* plants, we plated a total of 336 and 312 tissue fragments in 2006 and 2007, respectively (216 from branches and 432 from haustoria). From *A. psilostachya* plants, we plated a total of 336 and 445 *A. psilostachya* tissue fragments in 2006 and 2007, respectively (262 from stems and 524 from leaves), including tissue from unparasitized stems.

As fungi emerged, isolated colonies were transferred to fresh plates with 0.1× PDA and antibiotics (e.g., Arnold *et al.* 2000). One morphologically similar fungus from each sample of each plant species was isolated to minimize the chances of collecting multiple samples of one fungal individual. To measure infection rates, we counted the number of fragments from which fungi emerged, and to measure diversity, we counted the number of morphologically (and genetically) distinct fungal taxa that emerged from each individual plant's tissues. We counted each individual plant, whether parasitized or not, as a replicate (rather than counting individual pieces of plant tissue as replicates).

Fungal identification

0DNA was extracted from 260 fungal isolates using a freeze/thaw, sarkosyl-polyethylene glycol (PEG) extraction protocol (Rodriguez & Yoder 1991). We then amplified the ITS regions of all fungal isolates using primers ITS1F and ITS4 (White *et al.* 1990; Gardes & Bruns 1993), and analyzed as described above for *Cuscuta* genes. All identified ITS sequences ($n = 253$; 133 in 2006 and 120 in 2007) were searched against the GenBank database using BLASTn searches (Altschul *et al.* 1997). In addition, we amplified the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) region of samples matching the genus *Stemphylium*, because this region may be more effective than ITS for resolving taxonomic differences at the species level (Câmara *et al.* 2002; Pryor & Bigelow 2003). Fungal sequence data obtained in this study have been submitted to the EBI Nucleotide Sequence Database under accession numbers HE579208–HE579449 (ITS), HE649304–HE649309 (ITS),

HE653988–HE653992 (ITS), and HE579517–HE579540 (GAPDH). Sequences matching *Alternaria* and *Stemphylium* in GenBank were subsequently placed into the context of existing phylogenies (Chou & Wu 2002 for *Alternaria*; Câmara *et al.* 2002 for *Stemphylium*) with sequences used in these phylogenies and sequences from additional related taxa from GenBank if available. We aligned sequences as described above for plant analyses, and determined possible species identities of all fungi with neighbour-joining and maximum parsimony bootstraps as described above, using the consensus trees to determine the species with which our samples grouped in a well-supported clade.

Measuring mycovirus biodiversity

dsRNA extraction, cloning and sequencing

Mycoviruses are usually composed of RNA genomes, rarely DNA, and all RNA mycoviruses produce dsRNA during infections (Pearson *et al.* 2009). Thus, extracting dsRNA from fungi enabled us to detect and acquire sequence information from most mycovirus groups in large numbers of fungal samples. Assessing diversity using sequences also allowed us to detect finer-scale taxonomic differences between viruses than other available methods.

To obtain partial sequences of mycoviruses, we extracted dsRNA from 252 fungal isolates (131 in 2006 and 121 in 2007; including seven unidentified fungi from 2007) and used agarose gels to screen these samples for the presence of viruses. For 33 of our isolates, dsRNA banding patterns indicated the possible presence of viruses. We performed random-primed reverse transcription-PCR (RT-PCR) on these samples according to procedures described previously (Márquez *et al.* 2007), with the exception of using random decamer primers for RT-PCR in 2007. This method allows amplification of cDNA without significant DNA contamination, even without using DNase (Roossinck *et al.* 2010). The amplification was performed using a Rapid Cycler 2 (Idaho Technologies Inc.) as described above. PCR products were cloned into pGemT-easy® vectors (Promega) and then transformed into DH5α *Escherichia coli* competent cells. Individual colonies were picked, plasmids were isolated and sequenced, and reactions were purified as described in Srivastava *et al.* (2010) with the exception of using the following primers for sequencing: 5′-CGA ATT GGG CCC GAC GTC GCA TGC TCC-3′ and 5′-GCG TTG GGA GCT CTC CCA TAT GGT CG-3′.

Additional sequences were obtained by designing specific primers from partial sequences that were similar to virus sequences based upon BLAST searches against the GenBank, and conducting RT-PCR. For smaller fragments (<1000 base pairs), the RT reaction was the same as described above. However, for larger fragments (>1000 base pairs), amplification was done on an MJ Research machine (BioRad) using x-Taq polymerase (Takara) with the following program: 94 °C for 1 min; 30 cycles of 94 °C for 30 s, 45 °C for 30 s, 72 °C for 5 min or less; 72 °C for 5 min; 37 °C for 5 min. Smaller fragments were sequenced from PCR products. Larger DNA products were extracted from agarose gels using gel extraction and purification kits from Qiagen, followed by cloning (as above). Colony PCR was performed on DNA when we expected inserts to be less than 2 kb in size.

After vector and low quality sequences were removed, the sequences were assembled into contigs using TIGR Gene Indices Clustering Tools (TGICL; Pertea *et al.* 2003). After examining the sequences and contigs in DNAstar (DNASTAR Inc.), BLASTn, BLASTx, and tBLASTx were used to search against GenBank databases. If sequences or contigs matched regions of viral sequences with *e*-values less than e^{-02}, they were classified as viral. If sequences or contigs did not match any other sequences, they were classified as 'no matches'.

454 sequencing

We ran RT-PCR followed by 454 massively parallel pyrosequencing on 33 fungal samples suspected of harbouring virus infections (based on dsRNA banding patterns) using a procedure described in Roossinck *et al.* (2010). Results for each fungal sample were sorted according to individual sequence tags and resultant sequences for each sample were assembled into contiguous sequences. Contiguous sequences for each sample were then submitted to BLASTn, BLASTx, and tBLASTx searches on GenBank, and matches with known viruses or unknowns were sorted. Virus sequence data obtained in this study have been submitted to the EBI Nucleotide Sequence Database under accession numbers HE579541–HE579731.

Statistical analyses

To test for effects of plant species, site, and year on the fraction of tissue segments from which fungi emerged, we transformed these proportional data using the arcsine square-root transformation, and used the general linear model function in R (R Development Core Team). To test whether the number of fungal taxa per plant differed among plant species, sites, and years, we conducted general linear models in R. For each of these two data sets, we tested for effects of tissue type, site, year, and tissue type-by-year interactions within each plant species.

Fungal communities were compared between *Cuscuta* and *Ambrosia psilostachya* plants using Shannon diversity indices. To calculate 95 % confidence intervals, we resampled from the data with replacement to produce 1000 replicate data sets for each community, from which the Shannon diversity index was calculated. To estimate how similar fungal communities were between plant hosts, we calculated two common indices of community similarity: Jaccard and Sørensen's indices. We calculated abundance-based measures for these indices (similar to Chao *et al.* 2005) from the data, and used a similar bootstrap method (as described earlier) to calculate bias-corrected 95 % confidence intervals as in Dixon (2001).

To estimate the expected number of species in the fungal and viral communities as a function of the number of individual plants and fungi sampled, respectively, we calculated species richness indices and rarefaction curves using EstimateS version 8.0 (Gotelli & Colwell 2001; Colwell 2005). For conservative estimates of the fungal community, we lumped all taxa into genera unless we could distinguish among species by phylogenetic analyses, and included only one representative of each taxon from each individual plant's tissues ($n = 225$; 114 from 2006 and 111 from 2007). For a conservative estimate of the viral community, we discarded from our diversity analyses all potential viruses from which only one sequence from RT-PCR matched a known viral sequence, and that we could

not confirm with further RT-PCR followed by cloning, 454 sequencing, or northern blot analyses. Also, we discarded potential viruses determined only from 454 sequences with less than five reads. To make the estimate of viruses as conservative as possible, viruses were lumped together if nucleotide sequences greater than 150 bases were more than 90 % similar. While plant or fungal sequences that are only 90 % similar are almost certainly from different species, viruses exhibit greater nucleotide diversity within species, such that sequences representing the same virus may be only 80–90 % similar (Bao *et al.* 2008). We compared patterns of species richness between fungal and viral communities by rescaling the *x*-axis of rarefaction curves for each community to reflect numbers of individual fungi or viruses, respectively, and plotting the two curves together (Colwell *et al.* 2004). By rescaling the rarefaction curves, we compare the species accumulation with additional samples of each respective taxonomic group (i.e., diversity), rather than species accumulation with additional samples of host taxa (i.e., a combination of frequency and biodiversity).

Results

Cuscuta *species identification*

Phylogenetic analyses using sequences from both ITS and *trnL-trnF* genes produced topologies well supported by high bootstrap scores (not shown), indicated that all of the *Cuscuta* plants used in our analyses were *Cuscuta cuspidata*. Two additional plants not used in our analyses clustered in a well-supported clade with *Cuscuta pentagona*. These plants parasitized *Symphyotrichum ericoides*.

Fungal community

Fungi emerged from 49 of 54 *Cuscuta cuspidata* plants sampled (91 %), and from 51 of 57 *Ambrosia psilostachya* plants sampled (89 %). More fungi emerged from individuals of both plant species in 2006 than in 2007 (Table 1; Fig 1). Specifically, fungi emerged from 359 of 672 tissue fragments (53 %) in 2006, and from 172 of 762 fragments (23 %) plated in 2007. More fungi emerged from haustoria of *C. cuspidata* plants in 2007 than from branch tissue (Table 1; Fig 1B), and more fungi emerged from *A. psilostachya* leaves in 2006 and stems in 2007 (Table 1; Fig 1B). Although more fungi emerged from these tissues, often fungi emerged from only some of the 10–12 tissue pieces plated for each plant, indicating that these endophytes may represent localized infections. No fungi grew from plant impressions made from seven surface-sterilized plants, while fungi grew from some of the plant tissues embedded in companion Petri plates. This indicates that our sterilization technique was effective.

More than one fungal taxon emerged per individual plant, on average (Fig 1C). The number of fungal taxa per plant was unaffected by plant species, site, or year (Table 1). More fungal taxa emerged per plant from *C. cuspidata* haustoria tissue than branch tissue in both years, but fewer taxa emerged in 2007 than 2006 (Table 1; Fig 1D). In addition, more fungal taxa emerged from *A. psilostachya* leaves in 2006 and stems in 2007 (Table 1; Fig 1D). Sometimes, more than one morphologically distinct fungus emerged from a single tissue fragment.

The fungal community comprised 14 taxa in 2006 and 23 in 2007, with an overall richness of 25 taxa (Fig 2A and B). Eighteen total taxa emerged from *C. cuspidata*, and 19 from *A. psilostachya*. Most of the emergent fungi belonged to the

Table 1 – Effects of species, site, year, and tissue type on fungal emergence and the number of fungal taxa per plant.

Response variable	Category	Effects	Df[a]	P-value[b]
The fraction of tissue fragments with fungi	**Both plant species**	Species	1	0.42
		Site	3	0.08
		Year	1	~0*
	***C. cuspidata* (*n* = 54 plants)**	Tissue type	1	0.015*
		Site	3	0.48
		Year	1	0.006*
		Tissue type × year	1	0.001*
	***A. psilostachya* (*n* = 57 plants: 54 parasitized and three unparasitized)**	Tissue type	1	0.21
		Site	3	0.13
		Year	1	0.008*
		Tissue type × year	1	~0*
The number of fungal taxa per plant	**Both plant species**	Species	1	0.61
		Site	3	0.60
		Year	1	0.95
	***C. cuspidata* (*n* = 54 plants)**	Tissue type	1	~0*
		Site	3	0.58
		Year	1	0.89
		Tissue type × year	1	0.005*
	***A. psilostachya* (*n* = 55 plants: 54 parasitized and one unparasitized)**	Tissue type	1	0.62
		Site	3	0.55
		Year	1	0.45
		Tissue type × year	1	~0*

a We report the test degrees of freedom—the difference in the degrees of freedom between the full model and a model reduced by one factor.
b The asterisks indicate that the effect is significant, accounting for Bonferroni corrected significance levels ($\alpha = 0.017$).

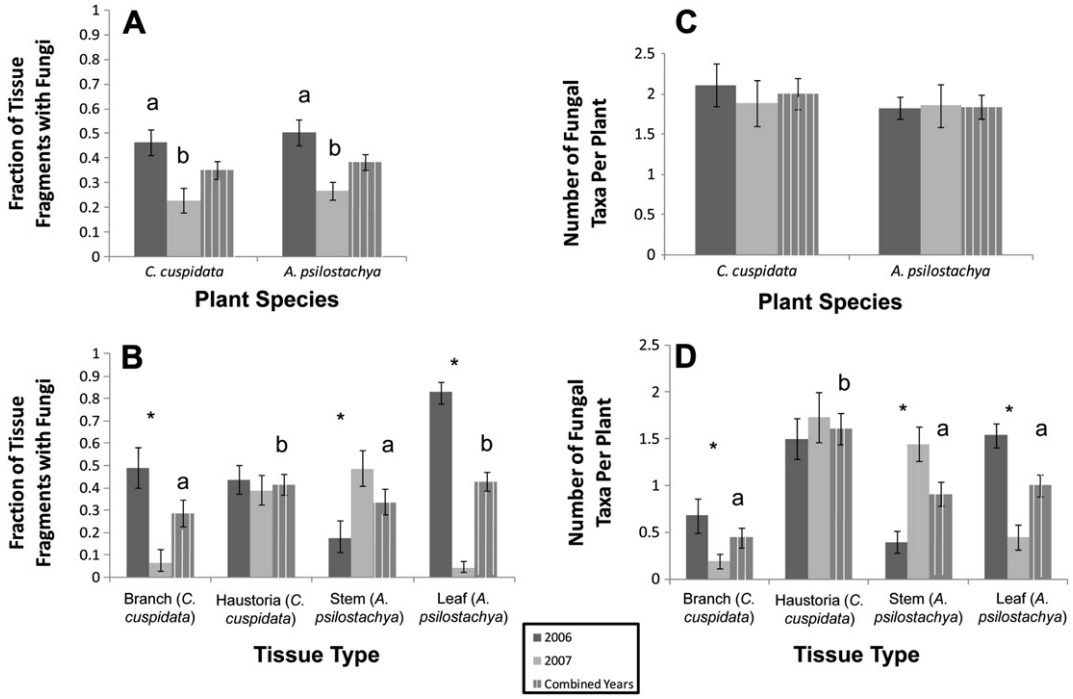

Fig 1 – The frequency and diversity of fungal infections on *C. cuspidata* and *A. psilostachya*. (A) The fraction of tissue fragments from which fungi emerged for each of the two plant species in each year and (B) the fraction of tissue fragments from which fungi emerged for each tissue type within each plant species for each year. Error bars indicate 95 % binomial confidence limits. (C) The number of fungal taxa per plant from each of the two plant species and (D) from each tissue type within each plant species for each year (mean ± standard error). Dark grey columns denote means for 2006, light grey columns denote means for 2007, and striped columns denote means for both years combined. Significant differences among years or among tissue types are indicated with letters, and significant year × tissue-type interactions are indicated with asterisks.

Ascomycota orders *Pleosporales* and *Capnodiales*, and included 20 genera e.g., *Alternaria*, *Cercospora*, *Cladosporium*, *Curvularia*, *Phoma*, and *Stemphylium* (Fig 2A and B). In *Alternaria*, we determined through phylogenetic analyses that samples were from two species: all samples but one were *Alternaria alternata*, and one sample (162H) was *Alternaria triticina* or *Alternaria infectoria*. All *Stemphylium* samples belonged to one species, *Stemphylium solani* (supported by phylogenetic analyses with GAPDH sequences). For fungi matching to genera such as *Cercospora*, *Phoma*, and *Curvularia*, multiple morphologies were associated with each genus, and GenBank matches were different among some of the samples within these genera. However, we could not determine species identities in these genera with the sequence data available in GenBank.

With two of the most abundant genera (*Alternaria* and *Stemphylium*), we were able to identify fungi to species by fitting the sequencing data into existing phylogenies (Câmara *et al.* 2002; Chou & Wu 2002) using neighbour-joining and maximum parsimony bootstrap analyses (data not shown). We considered fungal samples to be from a species if our sequences grouped together with known species in well-supported clades, and if our samples did not form clades separate from other species included in the phylogeny. However, sequences from 27 fungal samples that matched to *Alternaria helianthi* or *Leptosphaeria biglobosa* in the GenBank were difficult to place phylogenetically within *Alternaria* or *Leptosphaeria* (data not shown), thus, we called them *Pleosporales* sp. A and B.

The rarefaction curve produced in EstimateS for the overall fungal community did not reach an asymptote, indicating that additional samples, if collected, could include additional taxa (Fig 3A). When estimating fungal diversity (incorporating both richness and abundance), we counted each distinct taxon only once per plant. The Shannon diversity index for the fungal community was 1.72 (1.61, 1.78) in 2006 and 2.59 (2.48, 2.62) in 2007 (mean and 95 % confidence interval). Fungal communities overlapped considerably between *C. cuspidata* and *A. psilostachya*. The Sørensen index of community similarity between fungal communities on the two plant species was 0.61; (0.50, 0.73; mean and 95 % confidence interval), and the Jaccard index was 0.44 (0.33, 0.58), indicating that the fungal communities are similar.

Virus community

The dsRNA banding pattern on agarose gels is a rapid and reliable method for preliminary identification of the presence or absence of dsRNA mycoviruses, as well as ssRNA viruses that are not so common in fungi. However, DNA viruses (rare in fungi) and very low titre RNA viruses are not detected by this method. The dsRNA banding patterns consistent with viral infections were detected on agarose gels for 15 (12 %) and eight (7 %) of the isolated fungi in 2006 and 2007, respectively (examples shown in Fig 4). An additional three fugal isolates in 2006 and seven isolates in 2007 showed less clear evidence of

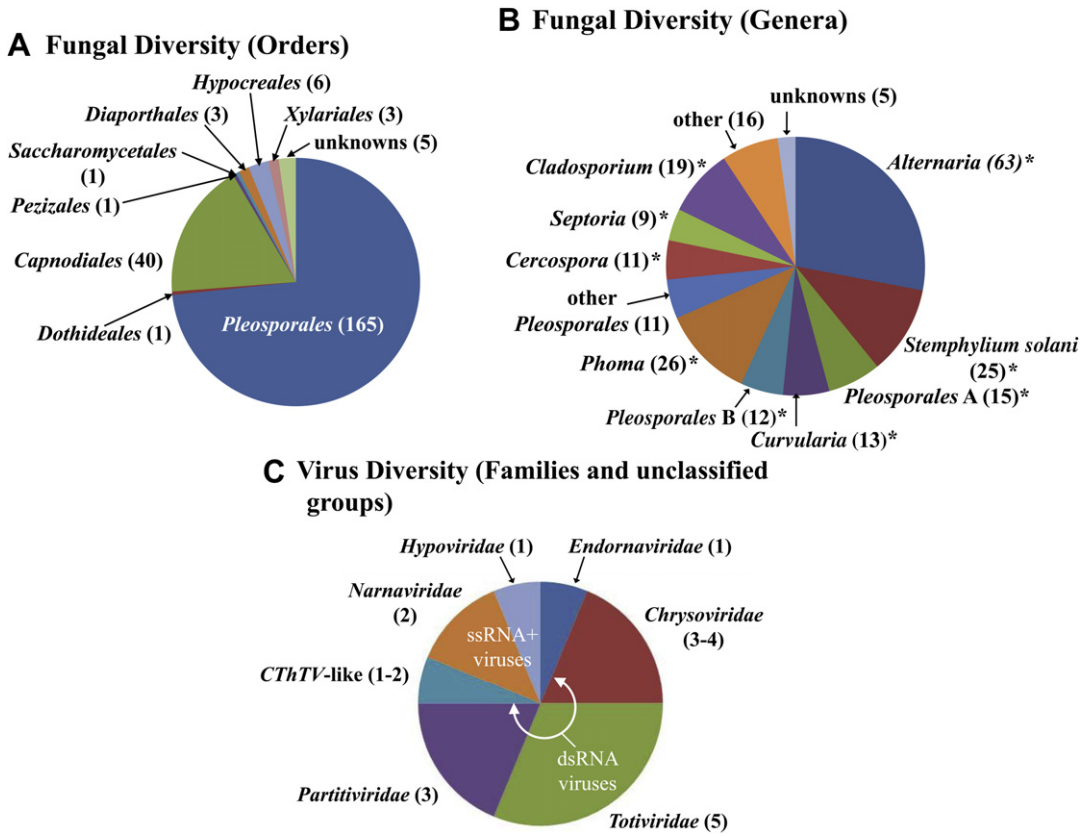

Fig 2 – Fungal and mycovirus diversity identified in this study (from 225 fungi and 16 mycoviruses used in diversity analyses). (A) Diversity of fungal orders (B) diversity of fungal genera or species (when we could identify them) found in plant samples. In (A) and (B), the numbers in parentheses represent the number of isolates sampled in each category. Asterisks indicate fungal types found in both the parasitic *C. cuspidata* and the host *A. psilostachya*. (C) Diversity of viral families and genera found in fungal samples. In (C), the numbers in parentheses represent the numbers of putative taxa represented by each section. The number of genome types of the different viruses is indicated on the figure.

possible virus infection, but no viruses were detected by sequence analysis of these samples. Out of 225 fungal samples analyzed, a total of 10 % contained detectable viruses. Low titre dsRNA viruses, ssRNA viruses, or DNA viruses would not be detected by these methods. Viruses were detected in most of the major fungal taxa sampled in this study, except the *Pleosporales* species A and B (Fig 5). In these taxa, large amounts of DNA copurified with the dsRNA, making definitive dsRNA pattern detection very difficult. No viruses were detected in these samples by sequence analysis. These samples were included in analyses, thus biasing our analysis in favour of increased fungal diversity relative to virus diversity.

Using information from sequence analyses, we confirmed the presence of 25 sets of viral sequences from 20 fungal isolates. Based upon our conservative criteria for lumping viruses (at least 90 % similarity over at least 150 bases), we grouped these viruses into 16 distinct taxa with sufficient sequence data to use in diversity analyses (Table 2; Fig 2C). Thirteen additional sets of unconfirmed sequences belonging to putative viruses were obtained from 11 fungal samples, four of which were also sources of confirmed virus sequences mentioned above. These sequences include nine lumped with confirmed virus taxa from this study, and three additional virus taxa with few associated sequences or that were not confirmed by pyrosequencing (Table 2; Fig 2C). A few sequences from fungal samples did not match anything in the GenBank, indicating that these sequences might belong to unreported viruses.

Single fungal species, and even individual fungal isolates, were associated with multiple virus taxa. For example, *Alternaria alternata* and *Stemphylium solani* were associated with seven and six viruses (respectively), and, in each case, four of which were detected in only that species (Table 2). Also, the single isolate from the second *Alternaria* species contained two viruses.

The nucleotide sequences of mycoviruses were similar to known fungal virus groups, including viruses in the *Chrysoviridae*, *Hypoviridae*, *Narnaviridae* (*Mitovirus*), *Partitiviridae*, *Totiviridae*, *Endornaviridae* families and those related to *Curvularia* thermal tolerance virus (CThTV) (Fig 2C). None of the nucleotide sequences of the confirmed viruses were more than 90 % similar to those of previously described viruses. Although mycoviruses have been found in *A. alternata* (Aoki *et al.* 2009), the seven viruses we detected from this species appear to be distinct from those found previously. The sequences of the remaining putative mycoviruses matched poorly with any known viruses, or matched no known sequences at all.

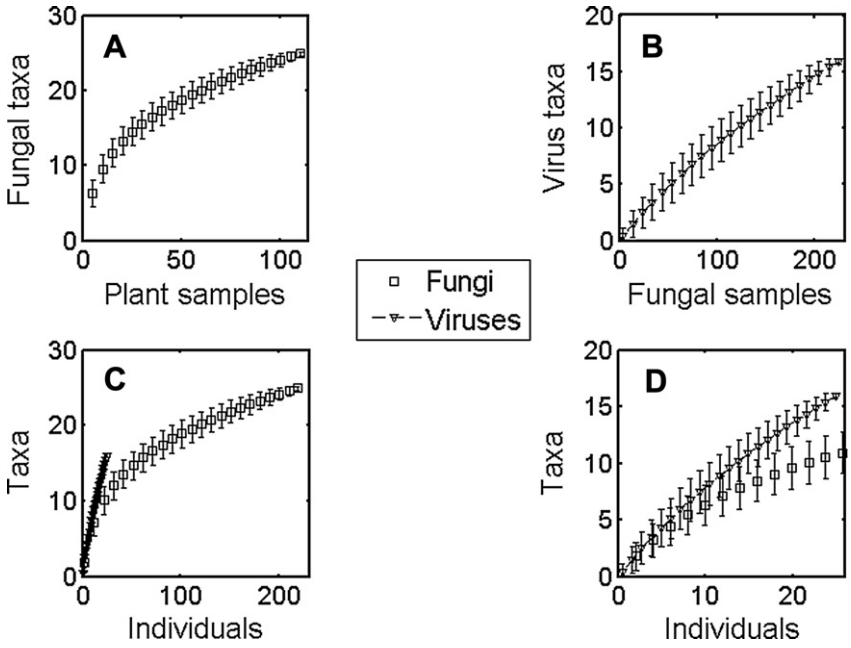

Fig 3 – Rarefaction curves for (A) fungi as a function of plant samples, (B) viruses as a function of fungal samples. In (B), the analysis includes only confirmed viruses. Because the pattern was the same when unconfirmed viruses were included (and when fungal species were split wherever possible given matches to known fungi in GenBank), we used the more conservative values in (C) and (D). (C) shows the rarefaction curves for both taxa, rescaled as a function of 'individual' (meaning 'individual fungus' for fungal endophytes, and 'individual virus' for mycoviruses). (D) shows the same curves as in (C), yet depicts only the region over which the two curves overlap. All rarefaction curves were run with 50 bootstrap samples.

Viruses from different fungal samples of the same species could represent single virus taxa in several cases: one of the *A. alternata* totiviruses (in samples 47P and 559P), and the endornavirus (in samples 63H, 71P, 122H, 139P, 325H, 401P, and 551P, which may be a separate strain from that in 32.1P). These and other virus taxa in the data set occurred in more than one fungal genus. The same endornavirus taxon, for example, occurred in several fungal genera including *Alternaria*, *Cercospora*, *Phoma*, and *Curvularia*. In addition, three of the partitiviruses detected may be the same virus taxon. The partitivirus in 545P may be the same as one in 87P (Table 2), as overlapping nucleotide sequences from each virus were at least 96 % similar. In addition, individual fungal samples sometimes contained multiple confirmed viruses. For example, sample 117.2H contained both a mitovirus and a partitivirus, and sample 46.1H contained both a totivirus and a chrysovirus (Table 2).

Like the rarefaction curve for the fungal community, the rarefaction curve for the virus community did not reach an asymptote (Fig 3B). Rescaled rarefaction curves comparing fungal and viral communities are shown in Fig 3C and D. Although the curve for mycoviruses contained fewer total species, the rate of species accumulation of mycoviruses with additional virus samples was higher than the rate of accumulation of fungi with additional fungal samples. This result was essentially the same even under the most extreme circumstances in which fungi were split into as many species as possible based solely upon matches to known fungi in the GenBank (analyses not shown), and when the virus data set excluded all of the samples with poor representation in the sequence data (all of those found only by 454 sequencing listed in Table 2).

Discussion

In this study, each plant species, and even individual plants, were associated with multiple fungal taxa. The same pattern seems to be true of mycoviruses—fungal species, and even individual fungal isolates, were associated with multiple viruses. For instance, the best-sampled fungal species, *Alternaria alternata* and *Stemphylium solani*, were associated with multiple virus taxa, several of which were detected in only the one fungal species (Table 2). In addition, the rate of virus taxa accumulation was greater than the rate of fungal taxa accumulation, as indicated by the rescaled rarefaction curves (Fig 3C and D). The rarefaction curve for mycoviruses may be further from reaching an asymptote. Thus, the community of mycoviruses may be more diverse than the community of plant-associated fungi. In the following paragraphs, we discuss factors affecting our main results, and implications of these results.

In several respects, our results are similar to those of previous studies. Higgins *et al.* (2011) suggested that endophytes are generalists among plants occurring at the same sites. Many of the fungal taxa we found occurred in both of the plant species we sampled and many of the same taxa were shared among sites and years. Also, several fungi, all *Ascomycetes*,

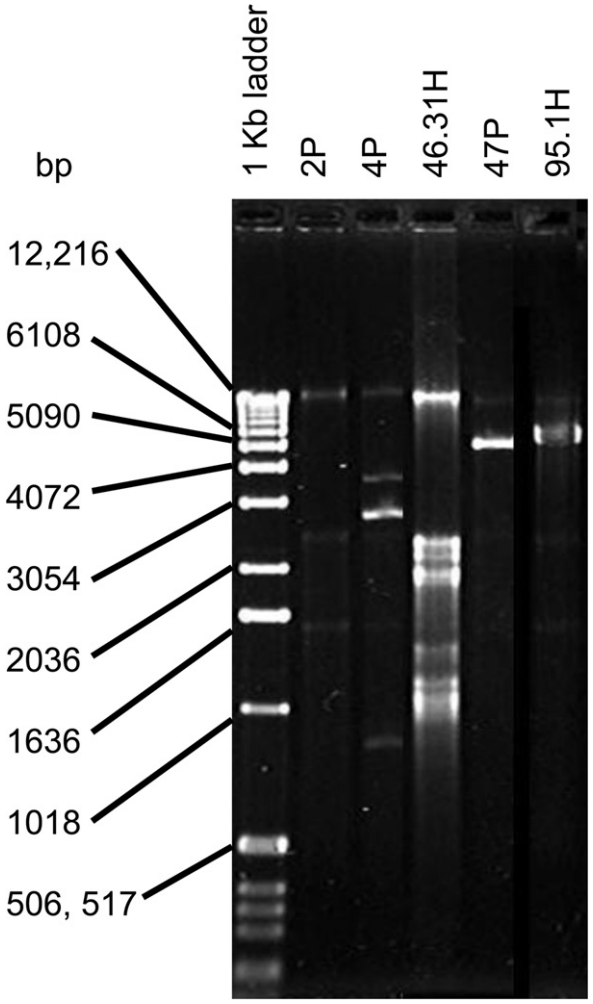

Fig 4 – Electrophoresis of samples of dsRNA isolated from various fungi. The gel was stained with ethidium bromide and bands were visualized under UV light. 1 kb ladder, MW standard from Invitrogen; 2P, *Alternaria* with no virus; 4P, *Alternaria* with chrysovirus; 46.31H, *Stemphylium* with hypovirus; 47P, *Alternaria* with totivirus; 95.1H, *Alternaria* with totivirus.

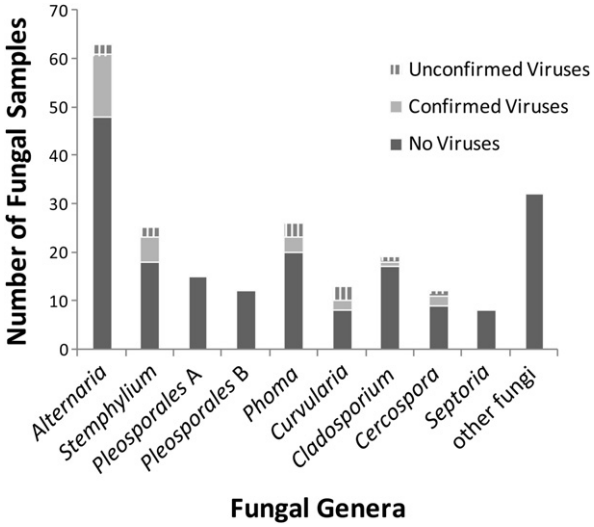

Fig 5 – Frequency of virus occurrence in different fungal genera. The darker regions below indicate fungal samples without virus, the lighter central region indicates samples with confirmed viruses, and the upper dark regions indicate samples with unconfirmed viruses.

cooccurred within tissues of each plant species, and fungal taxon richness did not reach an asymptote, similar to results of previous studies of class three fungal endophytes (e.g., Arnold *et al.* 2000; Higgins *et al.* 2011). We found more endophytes in 2006, which might reflect increased plant stress due to reduced rainfall in 2006 relative to 2007 (data not shown, from Oklahoma Mesonet site for Foraker, OK). Other studies have shown that endophytes are often associated with plants that are under stress (Márquez *et al.* 2007; Rodriguez *et al.* 2009). We also found that these endophytes were infrequently infected by mycoviruses, similar to previous surveys (van Diepeningen *et al.* 2006; Herrero *et al.* 2009). Our study shows that the mycoviruses of endophytic fungi are potentially very diverse, and many are related to, but distinct from, previously described taxonomic groups of viruses.

Matching sequences from several virus types may indicate that single virus types occur in different fungal genera. Although virus transmission between different host organisms has occurred several times during the evolutionary history of many mycovirus families (Liu *et al.* 2003; Roossinck 2010; Roossinck *et al.* 2011), we cannot claim that matching virus sequences from different fungal samples in our study belong to the same virus species without analyzing complete sequences from these viruses. Because our goal in this study was to assess biodiversity of mycoviruses relative to their fungal hosts, we lumped these virus taxa as a way to conservatively estimate virus diversity. If matching virus sequences from different fungal samples actually represent separate virus species, then this would further support our hypothesis that symbiont diversity is greater than host diversity. Also, none of the samples used in our analyses contained more than one virus from a given family, so it is unlikely that we biased our results by designating different segments of single segmented viruses as different virus types.

Our results depended upon our ability to distinguish among taxa. If we had been able to discern differences in fungal taxa at the species level (or the strain level), then we might have obtained a much higher estimate of fungal diversity. Uncovering cryptic fungal taxa might also change our results about community overlap between *Cuscuta cuspidata* and *Ambrosia psilostachya*. Although fungal genera and even fungal species may occur in multiple plant hosts, fungal strains within a plant species may be host specific (Arnold 2007 and references therein). That said, the potential diversity of mycoviruses is very high, and the same caveat described for fungi applies to viruses as well. In our estimates of fungal virus diversity, we attempted to be conservative, lumping virus types together when in fact these types may represent separate viruses or strains.

By comparing rarefaction curves of fungi and viruses, we assumed that our definition of operational taxonomic units was comparable between the fungal and viral communities,

Table 2 – A summary of viruses found in this study, including information about taxonomy, source fungi, and sequences from 454 and cloning.

Virus[a]	Fungal host genera/species	Sample ID numbers[b]	# of contigs[c]	Contig length[d]	# of reads[e]	Virus taxon[f]
Endornavirus 1	*Alternaria alternata*	**32.1P**	4 (6)	7141	1090	*Endornaviridae*
		63H	1 (2)	9565	1169	
		71P	3 (2)	5637	567	
		139P	2 (1)	4873	754	
		122H	1 (0)	241	2	
	Curvularia	**551P**	11 (0)	683	53	
	Phoma	401P	3 (0)	155	2	
	Cercospora	325H	1 (0)	157	2	
Totivirus 1	*A. alternata*	**47P**	2 (12)	1063	196	*Totiviridae*
		559P	5 (3)	231 (507)	7	
Totivirus 2	*A. alternata*	**95.1H**	1 (2)	2684	450	*Totiviridae*
Totivirus 3	*Stemphylium solani*	**46.1H**	7 (3)	721	123	*Totiviridae*
Totivirus 4	*Phoma*	**406.2P**	1 (4)	3628	847	*Totiviridae*
Totivirus 5	*Phoma*	**525P**	2 (0)	727	47	*Totiviridae*
Chrysovirus 1	*A. alternata*	**4P**	2 (5)	231 (632)	10	*Chrysoviridae*
Chrysovirus 2	*A. alternata*	**47P**	2 (0)	339	8	*Chrysoviridae*
	Phoma	**447P**	1 (5)	2218	911	
	Curvularia	**469H**	11 (15)	2319	204	
		551P	1 (0)	241	2	
	S. solani	117.2H	1 (0)	201	2	
Chrysovirus 3	*Alternaria triticina*	**162H**	1 (0)	249	7	*Chrysoviridae*
	S. solani	**218P**	9 (6)	3007	384	
		46.1H	2 (1)	341 (634)	18	
	Curvularia	496H	2 (0)	230	3	
Chrysovirus 4	*Phoma*	401P[g]	1 (0)	201	2	*Chrysoviridae*
Partitivirus 1	*Cladosporium*	**87P**	4 (16)	1754	848	*Partitiviridae*
	Phoma	545P	1 (0)	240	4	
Partitivirus 2	*S. solani*	**117.2H**	2 (0)	1006	40	*Partitiviridae*
Partitivirus 3	*A. alternata*	32.1P[g]	1 (0)	222	2	*Partitiviridae*
	Cercospora	**564.1H**	2 (0)	400	10	
	Cladosporium	87P	1 (0)	205	4	
	Preussia	507H	0 (1)	na (230)	na (5)	
CThTV-like virus 1	*Curvularia*	496H	0 (2)	na (1558)	na (15)	CThTV-like viruses
CThTV-like virus 2	*Cercospora*	**564.1H**	0 (6)	na (414)	na (6)	CThTV-like viruses
Mitovirus 1	*A. triticina*	**162H**	2 (1)	698	9	*Narnaviridae*
Mitovirus 2	*S. solani*	**117.2H**	1 (1)	260	3	*Narnaviridae*
	S. solani	533H	1 (0)	179	2	
Hypovirus 1	*A. alternata*	**46.31H**	5 (3)	3054	134	*Hypoviridae*

a Viruses were lumped if overlapping nucleotide sequences greater than 150 bases long were at least 90 % similar.
b Several samples listed below were discarded from diversity analyses if 454 sequencing did not confirm a virus supported by cloning evidence, or if 454 uncovered a sequence that matched virus sequences, but with low numbers of reads. The letter after each sample number indicates from which plant species the host fungus originated, where 'P' stands for 'parasite' (*Cuscuta cuspidata*), and 'H' stands for 'host' (*Ambrosia psylostachya*).
c The number of contigs from 454 (and from cloning).
d The length of the longest 454 contig in base pairs (and the longest cloning contig). The longest cloning contig is listed only if it is longer than the longest 454 contig.
e The number of reads from the best-supported amplicon from 454 (and from cloning). The number of reads from the best-supported amplicon from cloning is listed only if it is larger than that from the best-supported 454 amplicon.
f Sequences from amplicons matched sequences of viruses in GenBank belonging to these taxonomic groups. The overall amino acid identity ranged from 5 % to 80 %.
g The sequence data are insufficient to determine if this virus is the same as another. In one case (sample 32.1P from *Partitivirus* 3), the virus was lumped with another virus because the small sequence overlapped completely with other sequences from the same putative virus and was 100 % similar. In one case (sample 401P from *Chrysovirus* 4), the sequence did not match sequences from other putative chrysoviruses listed in the table.
Viruses used in analyses are shown in bold.

which may not be the case. Because we were interested in testing the hypothesis that the virus community is more diverse than the fungal community with which it is associated, we 'biased' our test against finding this result by splitting the fungal community as much as possible according to species within genera (as indicated by phylogenetic analyses), by lumping viruses if overlapping portions of their nucleotide sequences were >90 % similar, and by excluding predicted virus taxa that remained unconfirmed (also, we obtain identical results if we lump viruses that are >80 % similar). Even when we split fungi further based upon morphological differences (results not shown), the rescaled rarefaction curve for the virus community was still steeper than that of the fungal community, supporting our hypothesis. As expected, results from additional tests that included all unconfirmed viruses showed the same pattern (analyses not shown).

Our ability to compare fungal and viral communities also depends upon our ability to detect fungi in plants, and viruses in fungi. Because we used culturing techniques to assess the diversity of fungal endophytes, our results are likely to be biased towards faster-growing species, and our diversity estimates are probably lower than actual (Hyde & Soytong 2008; Higgins *et al.* 2011). Even so, if slower-growing or hard to culture endophytes are infected by similarly frequent and diverse mycoviruses, then mycovirus richness should still be greater than fungal richness in a given fungal community. Many factors could explain why mycoviruses were found infrequently in fungal samples: mycoviruses may be uncommon, viruses may be lost during fungal culturing (Márquez *et al.* 2007), or mycoviruses often occur at low titre, so are difficult to detect even when they are present. This latter idea is supported by the viruses detected in pyrosequencing that were not detected in sequences derived from cloning, although our results were similar if we computed rarefaction curves including only viruses detected with cloning (results not shown). Because pyrosequencing has the potential to detect viruses at low titre, this method may be more effective for assessing virus diversity (Roossinck *et al.* 2010). Three viruses (two used in statistical analyses) were found only through pyrosequencing. In addition, through pyrosequencing, we were able to confirm several viruses, previously detected with cloning techniques, in 19 additional fungal isolates and taxa (eight used in analyses; Table 2). The infrequent occurrence of viruses among fungi may in part explain why the total richness we observed for fungi was greater than that for viruses. We might have to sample more than 10X as many fungi before the rarefaction curve for the virus community would show signs of reaching an asymptote.

Soil and plant-associated fungi may be cryptically diverse not only due to difficulties associated with morphological identification, but also due to the lack of obvious symptoms caused by infections. Just as endophytic fungi occur in plants without detectable symptoms of disease, mycoviruses may inhabit cryptic fungi without detectable evidence of pathogenicity (Cole *et al.* 1998). Mycoviruses are found more commonly in some systems than others (van Diepeningen *et al.* 2006), but so far there are no reliable predictors of mycovirus incidence in natural communities. The cryptic virus communities inhabiting these fungi pose further challenges to estimate biodiversity on Earth.

These results suggest that just as fungal communities may be more diverse than their associated plant communities, virus communities may be more diverse than their associated host communities (see also Al Rwahnih *et al.* 2009; Coetzee *et al.* 2010 for example in plant viruses). Data suggest that this pattern holds among many parasite–host systems (Dobson *et al.* 2008). Phylogenetically related groups of viruses of plant-associated fungi also have been found in plants. Moreover, recent evidence suggests that viruses may occasionally move from plants to fungi and back, potentially increasing virus diversity as viruses adapt to their new hosts (Roossinck 2010; Roossinck *et al.* 2011). In addition, four viruses (*Endornavirus*, *Chrysovirus* 2 and 3, and *Partitivirus* 3) were found in fungi isolated from both parasitic and host plants (Table 2).

All of the viruses documented here likely represent newly described viruses, or even new virus genera. Although the confirmed viruses were somewhat similar to viruses described elsewhere, some of them represent groups in which very few members have been described (e.g., hypoviruses and CThTV-like viruses). Completely new mycoviruses that do not match any other viruses, or that match viruses associated with plants have been documented periodically (e.g., Preisig *et al.* 2000). Further, sequences from some fungal samples that yielded strong dsRNA banding patterns were dissimilar to all sequences currently in the GenBank, indicating that these viruses may represent currently unrecognized and uncharacterized groups of viruses.

Greater challenges are posed by the potential diversity of unknown effects of mycoviruses on their hosts. As indicated by previous studies on some of the few described mycoviruses, these diverse and almost completely uncatalogued virus taxa may directly affect the fitness of their hosts (e.g., Nuss & Koltin 1990), may indirectly affect whether their host can tolerate its environment (Márquez *et al.* 2007), may affect host evolution through acquisition of viral gene function (Bae & Kim 2004) or may serve as vectors for horizontal gene transfer (Liu *et al.* 2010).

As habitats are degraded or lost, biodiversity is lost with them, thus we will lose opportunities to fully understand these systems. The tallgrass prairie ecosystem in particular has been degraded or lost over most of its geographical extent in North America (Samson *et al.* 2004). This study provides a basis for comparing virus biodiversity in this endangered ecosystem with diversity in other ecosystems. The additional layer of cryptic diversity demonstrated in this study of tallgrass prairie is likely to occur in other systems as well.

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