Characterization of *Pythium* spp. Associated with Asymptomatic Soybean in Southeastern Pennsylvania

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Abstract

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Soybean production in Pennsylvania has increased substantially over the past 20 years and is a highly valued field crop, together with corn. Soilborne pathogens such as *Pythium* spp. can contribute to soybean stand establishment issues, particularly under the conservation tillage practices that are common in the state. In this study, we collected soil samples from eight asymptomatic soybean-corn rotation fields across six counties in southeastern Pennsylvania between May and June 2012. *Pythium* spp. were isolated via baiting, and tested for aggressiveness on both soybean and corn using laboratory assays. In addition to our culture-based survey, we also assessed the use of cytochrome oxidase subunit 1 pyrosequencing as a culture-independent method for measuring *Pythium* spp. diversity from environmental samples. Diversity estimates were consistent between the culture-based and pyrosequencing datasets; however, important methodological biases inherent to culture-independent methods may have led to some differences. Our results show that several *Pythium* spp. previously characterized as soybean or corn pathogens are present in southeastern Pennsylvania, including *Pythium irregulare*, *P. sylvaticum*, and *P. ultimum* var. *sporangiiferum*, with isolates showing aggressive phenotypes in lab assays.

Seed and seedling diseases of soybean (*Glycine max* (L.) Merr.) are important factors in stand establishment and can be influenced by tillage practices and earlier planting dates (Broders et al. 2007a,b; Workneh et al. 1999). In Pennsylvania, soybean production has increased 10-fold in the past 20 years, and over 90% of the acreage in 2013 utilized no-till or other conservation tillage practices (PSU 2015). Although dwarfed in comparison with soybean production in Midwestern states such as Illinois and Iowa, over 600,000 acres were planted in 2014; with an economic value of $290 million, soybean was the third most valuable crop in Pennsylvania, behind hay and corn (USDA-NASS 2015). The adoption of fungicide seed treatments can often reduce the prevalence of early-season diseases, depending on environmental conditions (Bradley 2008; Bradley et al. 2001; Dorrance et al. 2003; Guy and Oplinger 1989), but monitoring soilborne pathogens such as *Pythium* spp. can better inform management decisions for minimizing sublethal infections that may ultimately affect yield (Broders et al. 2007a; Broders et al. 2009; Schroeder et al. 2013).

Previous surveys of both diseased seedlings (Broders et al. 2007a; Brown and Kennedy 1965; Rizvi and Yang 1996; Zhang and Yang 2000; Zitnick-Anderson and Nelson 2015) and soils (Broders et al. 2009; Dorrance et al. 2004; Griffin 1990; Jiang et al. 2012; Marchand et al. 2014; Murillo-Williams and Pedersen 2008; Zhang and Yang 2000; Zhang et al. 1998) have recovered a number of *Pythium* spp., although aggressiveness on soybean varies among species and, occasionally, among isolates (Broders et al. 2007a; Zhang and Yang 2000); species of particular importance for soybean given their frequent occurrence in field sampling and aggressiveness in laboratory assays include *Pythium irregulare*, *P. sylvaticum*, *P. ultimum* var. *ultimum*, and *P. ultimum* var. *sporangiiferum*. Other species such as *P. heterothallicum* (van der Plaats-Niterink 1981) and several *Phytopythium* spp. (Marano et al. 2016) are commonly isolated from soils but are thought to act as saprotrophs or weak secondary pathogens. Corn is a common rotational partner with soybean, and several species of *Pythium* are significant corn pathogens, including *P. arrhenomanes* and *P. graminicola* (Rao et al. 1978; Sumner et al. 1990; van der Plaats-Niterink 1981). In this study, we sampled soybean-corn rotation fields with no self-reported history of seedling diseases in order to assess *Pythium* spp. diversity under asymptomatic conditions. Considering the complex interactions among environmental conditions, host susceptibility, and biotic diversity of pathogens as well as saprotrophs, our goal was to discover the underlying potential for seed or seedling disease given the *Pythium* spp. diversity present in our sampled locations. For comparison and also as a test of our collection methods, we additionally sampled forest soil and stream water from a local nature conservancy.

Culture-based surveys allow for the characterization of important traits such as virulence or fungicide resistance. However, it has been well documented that these methods underestimate the true species diversity present within a sample (Bik et al. 2012; Cuadros-Orellana et al. 2013; Zinger et al. 2012). The use of culture-independent methods, while not without their own potential biases, offers an alternative approach for studying species assemblages. The study by Arcate et al. (2006) was one of the first to show that the inferred oomycete community (primarily *Pythium* spp.) differed greatly between hemp-seed-baited samples and DNA extracted directly from rhizosphere soils; the authors found lower sequence diversity in the baited communities, as well as little overlap in the particular species recovered by each sampling method (Arcate et al. 2006). More recently, next-generation sequencing methods have been used to study fungal and oomycete species diversity from several environments, including forests (Català et al. 2015; Coince et al. 2013; Vannini et al. 2013) and agricultural soils (Sapkota and Nicolaisen 2015; Sugiyama et al. 2010). These recent studies have utilized the nuclear internal transcribed spacer region of the ribosomal DNA (ITS1) as their sequencing target; however, other loci have been suggested for species identification, such as the mitochondrial cytochrome c oxidase (COI) subunit 1 (Robideau et al. 2011) or subunit 2 (Choi et al. 2015) genes. Given the availability of oomycete-specific primers (Robideau et al. 2011) and its general acceptance as a standard barcode locus (Hajibabaei et al. 2007; Ratnasingham and Hebert 2007), here we have used COI pyrosequencing as a culture-independent approach for estimating *Pythium* spp. diversity. Sequences

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*The ***e*-X**tra logo stands for “electronic extra” and indicates that two supplementary figures are published online.

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generated via pyrosequencing can be 3 to 10 times longer than those of other next-generation sequencing methods, which can be advantageous for sequence-based biodiversity studies of environmental DNA (Shokralla et al. 2012).

The objectives of this study were to (i) isolate and identify *Pythium* spp. present in soils of asymptomatic soybean fields in southeastern Pennsylvania, (ii) characterize the aggressiveness of each isolate on soybean and corn in laboratory assays, and (iii) evaluate the use of COI pyrosequencing for estimating *Pythium* spp. diversity from environmental samples.

Materials and Methods

Site selection and sampling. Soil samples were collected from eight soybean-corn rotation fields in southeastern Pennsylvania between May and June 2012 (Fig. 1). Information on rotation history and previous crop disease was self-reported by the landowner or local extension agent. At each field, soil cores 2 cm in diameter and approximately 20 cm deep were taken at 5-m intervals along a 100-m transect, starting from the edge of the field. Soil temperature was recorded at each core and averaged across all samples for each field; soybean growth stage was also recorded at each field (Norby 2004, adapted from Fehr et al. 1971). Soil cores were bulked and transferred to the lab on ice. Bulked samples were well mixed and approximately 300 g of soil was dried overnight for soil fertility testing (Agricultural Analytical Services Lab, Penn State University, University Park, PA). Total DNA was extracted, in duplicate, from 10 g of bulked soil using the PowerMax Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA), following the manufacturer's instructions.

For comparison, soil and water samples were also collected from an 85-acre nature conservancy in Lancaster County, PA in May 2012 (Fig. 1). Soil cores were taken as described above from the east-facing slope of a second-growth conifer forest. Water (4 liters) was collected from a restored section of a cold-water stream (Lititz Run, Conestoga River watershed) approximately 0.5 miles downstream of the forest soil sampling location. Soil and water samples were transferred to the lab on ice, and bulk soil was processed as described above. Total DNA was extracted, in duplicate, from filter membranes (0.22 μm, 1 liter of water filtered) using the UltraClean Water DNA Isolation Kit (Mo Bio Laboratories Inc.), following the manufacturer's instructions.

Isolate identification and phylogenetics. Isolates were collected from soil and water samples using a baiting method (Erwin and Ribeiro 1996). Several baits were used for all samples to maximize the isolation of different *Pythium* spp., including *Rhododendron* leaf disks (7 mm in diameter), soybean seed and pods, and hemp seed; all baits were surface sterilized with 70% ethanol and rinsed thoroughly in purified water. For the soil samples, approximately 15 g of soil was spread on a 100-mm petri dish and flooded with 20 ml of purified water; for water samples, approximately 25 ml of sample was poured into a petri dish. Baits were then floated on the water, and plates were incubated in the dark at room temperature (25°C) for 3 to 7 days. Colonized baits were rinsed and transferred, in duplicate, to selective cV8-PARP (5% clarified V8 juice [1 g of calcium carbonate per 100 of ml V8 juice, clarified via centrifugation], bacto-agar [15 g/liter], pimaricin [5 mg/liter], sodium ampicillin [250 mg/liter], rifamycin-SV sodium salt [10 mg/liter], and pentachloronitrobenzene [50 mg/liter]) and cV8-PARPH (cV8-PARP plus hymexazol [50 mg/liter]) media (Jeffers and Martin 1986). Once hyaline hyphae had emerged into the agar from the bait, a 5-mm sterile cork borer was used to remove an agar plug from the growing edge of the culture and was placed onto a fresh selective media plate. For long-term storage, agar plugs were transferred to potato-carrot agar slants (Dhingra and Sinclair 1995) in 20-ml scintillation vials.

Isolates were identified via sequencing of the mitochondrial COI barcode locus (Robideau et al. 2011). For genomic DNA extraction, a 5-mm agar plug was taken from the growing edge of a culture, placed in 30 ml of sterile 10% cV8 broth, and grown on an orbital shaker (120 rpm) for 2 to 5 days at room temperature. Mycelial mats were collected via vacuum filtration, and DNA was extracted with either the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) or the UltraClean Tissue & Cells DNA Isolation Kit (Mo Bio Laboratories Inc.), following the manufacturer's instructions. DNA was eluted in sterile ultrapure water and stored at −20°C. The COI locus was amplified using the barcode primers OomCoxI-Levup and OomCoxI-Levlo (Robideau et al. 2011) in a reaction volume of 20 μl containing final concentrations of 1× reaction buffer, 3.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM each forward and reverse primer, 1 unit Taq DNA Polymerase (USB, Affymetrix Inc., Santa Clara, CA), and approximately 20 ng of template DNA. Reactions were brought up to volume with sterile ultrapure water. The thermocycler protocol for COI amplification was as follows: 94°C for 2 min; followed by 45 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min; with a final extension at 72°C for 10 min. Reactions were run on a 1% agarose gel to confirm amplicon size (expected 680 bp). In addition, nuclear ITS and large ribosomal subunit (LSU) amplicons were generated to confirm the identification of some isolates (including fungal contaminants). For the ITS, primers ITS-1 and ITS-4 were used (White et al. 1990); for LSU, LROR and LR6 were used (Blair et al. 2008). Reaction conditions were the same as above except 2.5 mM $MgCl_2$ was used with the following thermocycler protocol: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 90 s, with a final extension at 72°C for 5 min. PCR products were cleaned using an Exo-SAP protocol (USB, Affymetrix Inc., Santa Clara, CA) at half the concentration suggested by the manufacturer; Sanger sequencing was performed using BigDye Terminator chemistry and run on a AB3730XL (Genomics Core Facility, Huck Institutes of the Life Science, Penn State University). Trace files were edited using Sequencher v5.1 (Gene Codes Corp., Ann Arbor, MI); primer sequences were removed and heterozygous sites (for the nuclear loci) were coded using IUPAC nomenclature. COI sequences for each *Pythium* isolate have been deposited in GenBank under accession numbers KT692744 to KT692919.

Consensus sequences were compared with the nonredundant GenBank nucleotide database at the National Center for Biotechnology Information (NCBI) using megaBLAST (Boratyn et al. 2013) for species identification. Sequences matching a vouchered isolate with >98% identity were considered a valid identification; sequences matching with 98% identity or less were labeled as species affinis (aff.). Several closely related *Pythium* spp. cannot be resolved based on COI sequences (Robideau et al. 2011); in these cases, the species epithet published first was used. Sequences were aligned using ClustalX v2.1 (Larkin et al. 2007) and alignments were adjusted manually as needed in MEGA v5.2 (Tamura et al. 2011). Reference sequences from genera *Pythium*, *Phytopythium*, *Phytophthora*, and *Saprolegnia* vouchered isolates were added to alignments to assess

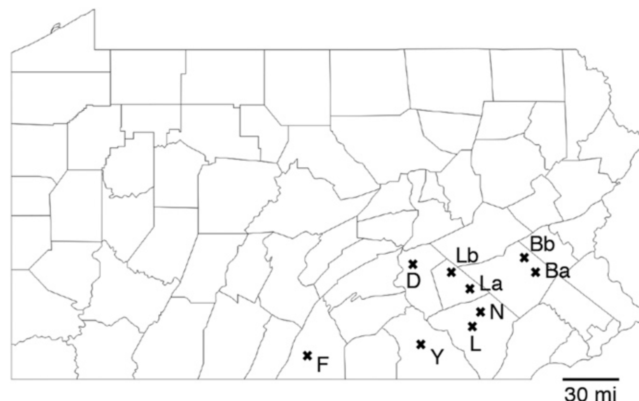

Fig. 1. Soybean sampling locations in southeastern Pennsylvania. Soil samples were collected between May and June 2012. Abbreviations: Berks County Farm A (Ba), Berks County Farm B (Bb), Dauphin County (D), Franklin County (F), Lancaster County (L), Lebanon County Farm A (La), Lebanon County Farm B (Lb), and York County (Y). Forest soil and stream water were also collected from a nature conservancy in Lancaster County (N) in May 2012.

any ambiguous or novel species identifications. Neighbor-joining phylogenies were estimated in MEGA under a Kimura 2-parameter model and pairwise deletion of alignment gaps. Alignments and tree files are available from the Dryad Digital Repository (http://datadryad.org/resource/doi:10.5061/dryad.3s4d3).

Isolate pathogenicity assays. A modified seed-rot assay (Zhang and Yang 2000) was used to assess the aggressiveness of each isolate. Soybean ('Viking 2265', certified organic; Johnny's Selected Seeds, Winslow, ME) and corn ('Luscious F1', certified organic; Johnny's Selected Seeds) seed were surface sterilized in 80% ethanol for 3 min, followed by 2% sodium hypochlorite solution for 15 min; seed were then rinsed five times in ultrapure water, with a final rinse in 95% ethanol, and dried overnight in a sterile hood. Each isolate was grown for approximately 1 week on cV8-PARP. A 5-mm agar plug from the growing edge was transferred to the center of a 2% water agar plate (bacto-agar at 20 g/liter) and incubated in the dark at room temperature. After 24 h, eight sterile seeds were equally spaced around the plate approximately 1 cm from the edge. Sterile seed were also placed on water agar plates with no isolates as uninoculated controls. Plates were then incubated in the dark at room temperature and scored after 6 days. The following rating scale was used to characterize each assay: 0 = no hyphal colonization of seed, 1 = very light hyphal colonization of some seed, 2 = light hyphal colonization of most seed, 3 = dense hyphal growth on all seed, and 4 = dense hyphal growth across plate with blackening of seed. Two replicate assays were performed for each isolate for both soybean and corn seed. Ordinal-scale aggressiveness ratings were analyzed via nonparametric methods as described by Shah and Madden (2004), using a modified R program based on the original NPAR SAS script initially developed by Brunner et al. (2002). Species and isolates were treated as fixed effects in separate one-way analyses; the relative aggressiveness among species and isolates within species were compared using contrasts. Species with a mean rating ≥3 were considered aggressive, while those with ratings between 2 and 3 were considered moderately aggressive.

COI pyrosequencing and molecular operational taxonomic units assignment. Amplicon libraries were constructed from bulk DNA extractions from each of the sampling locations. Initially, each library was prepared with the COI barcoding primers OomCoxI-Levup and OomCoxI-Levlo (Robideau et al. 2011) fused to the pyrosequencing primers (forward: CCATCTCATCCCTGCGTGTCTCC GACTCAG-MID; reverse: CCTATCCCCTGTGTGCCTTGGCAG TCTCAG), with the forward primer containing the sample-specific 10-base multiplexed identifier (MID). A second library was also constructed using the OomCoxI-Levup forward and FM85mod reverse primers (Martin and Tooley 2003; Robideau et al. 2011). Each reaction (25 μl volume) contained 5 pmol each forward and reverse primer, 5 to 10 ng of template DNA, 5 nmol dNTP, and 1.25 U of Fast Start High-Fidelity Taq DNA Polymerase with 1× reaction buffer (Roche Life Science, Indianapolis, IN). The thermocycler protocol was as follows: 94°C for 3 min; followed by 45 cycles of 94°C for 15 s, 56°C for 45 s, and 72°C for 60 s; with a final extension at 72°C for 8 min. PCR products were purified using the Agencourt AMPure XP system (Beckman Coulter, Inc., Brea, CA), as described in the 454 Technical Bulletin number 2011-002 (Short Fragment Removal Procedure), then quantified by both Qubit (Life Technologies, Carlsbad, CA) and quantitative PCR using the KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA). Products were pooled based on molar amounts, run on a 1% agarose gel, and extracted for the expected band size. Extractions were cleaned using the QIAquick PCR purification kit (Qiagen) and assessed using a DNA 7500 assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Sequencing was performed on the Roche GS 454 FLX+ (Roche Diagnostics, Indianapolis, IN). All amplicon preparation, purification, and sequencing was performed at the Genomics Core Facility, Huck Institutes of Life Science, Penn State University.

Preprocessing of SFF files was performed using NextGen Workbench (Heracle BioSoft SRL, Piteşti, Romania) with the following filters: remove reads with an average quality value (QV) under 15; trim low-quality ends until QV > 20 in a 15-bp window; remove reads if 50% of bases are either A, T, C, or G; and remove reads with GC content less than 15% or greater than 85%. Sequence files were then exported in FASTA format for additional processing using custom Python scripts. Sequences were sorted by sampling location using their corresponding MID, with up to one mismatch allowed; the MID plus an additional 23 bp (the length of the OomCoxI-Levup forward primer) were trimmed from the 5′ ends. Only sequences greater than 100 bp in length (after trimming) were retained in each dataset, and the mean, median, and maximum length were calculated. Molecular operational taxonomic units (MOTU) were identified for each dataset by clustering sequences with 100% identity using the USEARCH v7.0 software package (Edgar 2010); input sequences were sorted by decreasing length, and default termination settings were disabled by setting maxaccepts and maxrejects equal to zero. A custom database was constructed for taxonomic assignment of MOTU; COI sequences from vouchered heterokont isolates were obtained from the BOLD Systems database (www.boldsystems.org; 4,687 sequences) and NCBI (1,652 sequences). Sequences longer than 660 bp (which eliminated most nonoomycete heterokonts, whose COI barcode is approximately 658 bp) were clustered at 100% identity and combined with those generated from our in-house culture collection, yielding a final custom database of 1,094 sequences. The usearch_global algorithm was then used to classify MOTU with ≥98.5% identity to sequences in the custom database; alignments <100 bp were discarded. To identify MOTU that represent unknown and potentially novel phylogenetic lineages, a second search was made against the custom database, with a minimum identity of 90% and a maximum identity of 98.4%. MOTU clusters with >3 sequences were retained and combined with vouchered reference sequences for phylogenetic analysis. Data files for each sampling location have been deposited in the Sequence Read Archive under BioProject IDs PRJNA294588 for the soybean-corn rotation fields and PRJNA294606 for the nature conservancy stream water sample.

Diversity analysis. Species accumulation curves were generated for both the culture-based and pyrosequencing results by applying the *rarefaction* method of the function *specaccum*, as implemented in the vegan R package (Oksanen et al. 2015). Mantel's tests for the correlation between community similarity and physical distance were performed with the *mantel* function in the vegan package, using the Spearman method. Similarity matrices were computed from the observed species matrices using the Sørensen index $[2 \times J/(A + B)]$, where J represents the shared number of species between two sites, and A and B represent the number of species in each site; the *designdist* function (vegan package) was used for this calculation. Matrices of Euclidean distance were generated from geographical coordinates with the *dist* function of the stats package in R. A correspondence analysis (CoA) of the species data from soybean-corn field samples was performed using the *cca* function in the vegan package. The effect of soil physical and chemical features on the pattern of community composition revealed by CoA was determined with the *envfit* function.

Results

Sampling locations. Soil samples were collected from eight soybean-corn rotation fields across six counties in southeastern Pennsylvania (Fig. 1). One location (Y) reported a wheat/double crop soybean rotation during the previous year, and two reported a rye (Ba) or barley (Bb) cover crop. No incidents of early-season Pythium diseases (damping off or root rot) were self-reported over the previous 5 years, although this does not confirm that fields were disease free. Comparison of soil nutrient data showed general consistency in micronutrient profiles among sampling locations (Table 1), with pH and Mg^{2+} showing the strongest associations ($P < 0.05$) with the patterns of community similarity, as indicated by the correspondence analysis (Supplementary Fig. S1). Forest soil from the nature conservancy was cooler and more acidic, with a higher C/N ratio but lower micronutrient content (Table 1). Stream water collected from the nature conservancy had an average temperature of 17.8°C and pH 8.0.

Isolate identification and aggressiveness. In total, 199 isolates were obtained and identified molecularly. Twenty were identified as *Mortierella* fungal contaminants based on LSU sequencing, and were removed from the collection. Of the remaining 179 isolates, 3 failed to produce unambiguous sequences and were considered mixed cultures. Using a cut-off of >98% identity, 22 known species were identified based on COI data (Table 2); 3 isolates were similar to unclassified *Pythium* (sp. CAL-2011c voucher Lev2168, GenBank accession HQ708842) and *Phytopythium* (sp. AL-2010 voucher CBS45562, GenBank accession HQ708448) collected elsewhere. One isolate

Table 1. Soybean growth stage and soil properties for eight soybean-corn rotation fields and forest soil (Forest) from a nature conservancy sampled in southeastern Pennsylvania in 2012

	Soybean-corn rotation fields per soybean stage[a]								
	Ba	**Bb**	**D**	**F**	**L**	**La**	**Lb**	**Y**	
Parameter[b]	**V1**	**VE**	**V3**	**VC**	**V1**	**R1**	**R1**	**V3**	**Forest**
Temp (°C)	21.3	24.8	25.0	23.5	19.9	20.5	21.9	26.7	15.8
pH	7.0	6.8	6.1	6.8	6.3	6.6	5.9	7.3	4.6
C (%)	2.39	1.90	1.11	1.39	1.28	1.53	1.88	1.52	4.61
N (%)	0.27	0.23	0.13	0.16	0.15	0.16	0.22	0.15	0.38
C:N	8.85	8.26	8.54	8.69	8.53	9.56	8.55	10.13	12.13
P (ppm)	191	49	51	44	48	102	70	118	62
NO_3-N (ppm)	28.3	9.6	20.4	15.1	15.1	15.1	16.1	24.9	8.2
K (ppm)	364	215	116	174	174	303	219	202	78
Mg (ppm)	231	256	83	201	155	162	176	295	48
Ca (ppm)	2,620	1,651	1,362	1,492	1,568	1,489	1,868	2,170	280
Cu (ppm)	4.1	5.3	4.2	2.9	4.4	13.3	8.1	7.3	2.2
Zn (ppm)	12.1	4.3	2.3	3.3	4.0	4.8	6.8	8.1	3.5
S (ppm)	18.2	8.8	8.0	8.4	10.8	8.9	10.8	8.6	…

[a] Location abbreviations: Berks County Farm A (Ba), Berks County Farm B (Bb), Dauphin County (D), Franklin County (F), Lancaster County (L), Lebanon County Farm A (La), Lebanon County Farm B (Lb), and York County (Y). Soybean growth stages, adapted from Fehr et al. (1971): emergence (VE), cotyledon (VC), first trifoliate (V1), third trifoliate (V3), and beginning bloom (R1).

[b] Temp = average temperature and ppm = parts per million.

Table 2. *Pythium* spp. and number of isolates collected from eight soybean-corn rotation fields and a nature conservancy in southeastern Pennsylvania in 2012

		Soybean-corn rotation fields[a]										
Species[b]	**ID (%)[c]**	**Ba**	**Bb**	**D**	**F**	**L**	**La**	**Lb**	**Y**	**FS**	**SW**	**Total**
Phytopythium sp. CBS45562	99	…	…	…	…	…	…	…	…	2	…	2
Phytopythium citrinum	100	…	…	…	…	…	…	…	…	…	1	1
P. litorale	100	…	…	…	…	…	…	…	…	…	1	1
P. vexans	99–100	…	…	…	…	…	…	…	4	5	…	9
Pythium aff. *acanthophoron*[d]	97	…	…	…	…	…	…	…	11	…	…	11
Pythium aff. *intermedium*[d]	98	1	…	…	…	…	…	…	…	…	…	1
Pythium aff. *undulatum*[d]	98	…	…	…	…	…	…	…	…	1	…	1
Pythium afertile	99	…	…	…	3	…	…	…	…	…	…	3
P. attrantheridium	100	1	…	…	…	…	…	…	…	2	…	3
P. sp. *balticum*/aff. *attrantheridium*	100	…	…	…	…	…	…	…	…	1	…	1
P. conidiophorum	100	…	…	…	1	…	…	…	…	…	…	1
P. dissotocum	100	…	…	…	3	…	…	…	…	…	…	3
P. heterothallicum	99–100	3	4	…	1	6	4	…	2	…	…	20
P. intermedium	100	3	…	…	…	…	…	…	…	…	…	3
P. irregular	99–100	…	4	14	1	…	…	8	…	…	…	27
Pythium sp. JN-1a[e]	100	…	…	…	…	…	…	…	…	…	2	2
Pythium sp. JN-1b[e]	100	…	…	…	…	…	…	…	…	…	5	5
Pythium sp. Lev2168	99	…	…	…	…	1	…	…	…	…	…	1
P. mamillatum	100	…	…	3	…	…	…	…	…	…	…	3
P. nodosum	99–100	8	10	…	8	…	1	…	…	…	…	27
P. perplexum	100	1	…	…	1	1	…	…	3	…	…	6
P. pleroticum	99–100	1	2	…	5	1	3	1	1	…	…	14
P. sylvaticum	99–100	3	2	1	…	6	7	7	…	…	…	26
P. torulosum	99	…	…	…	…	…	1	…	…	…	…	1
P. ultimum var. *sporangiiferum*	99	…	…	…	…	…	1	3	…	…	…	4
Total number of isolates	…	21	22	18	23	15	17	19	21	11	9	176
Total number of species	…	8	5	3	8	5	6	4	5	5	4	25

[a] Location abbreviations: Berks County Farm A (Ba), Berks County Farm B (Bb), Dauphin County (D), Franklin County (F), Lancaster County (L), Lebanon County Farm A (La), Lebanon County Farm B (Lb), and York County (Y). Forest soil (FS) and stream water (SW) were collected from a nature conservancy in Lancaster County.

[b] Isolates were identified via cytochrome c oxidase (COI) sequencing and comparison with vouchered matches in the National Center for Biotechnology Information (NCBI).

[c] Percent identity between the COI sequences generated from the collected isolates and vouchered matches in NCBI.

[d] Species affinis (aff.) epithets were inferred based on the highest scoring match in NCBI.

[e] Identification based on COI sequences generated from the original JN isolates (provided by J. Nechwatal).

collected from the forest soil showed 100% identity to the vouchered isolate of *Pythium* sp. *balticum* (CBS122649, GenBank accession HQ708525), although no formal description exists for this close relative of *P. attrantheridium*. In addition, 13 isolates with COI matches below the cut-off resolved into three novel phylogenetic lineages and were labeled as species affinis (Table 2). There was little overlap in species profiles between the soybean-corn fields and the nature conservancy; all four species identified in the water sample and three of the five species isolated from the forest soil were unique to the nature conservancy location (Table 2). Among the soybean-corn fields, *P. pleroticum* was the most commonly isolated species (seven of eight locations), followed by *P. heterothallicum* and *P. sylvaticum* (six of eight locations). COI sequence diversity was present across isolates of both *P. heterothallicum* and *P. irregulare*; all *P. irregulare* isolates were grouped as sensu lato, following Spies et al. (2011).

Across species, aggressiveness ratings obtained from the soybean and corn seed assays showed a high positive correlation (Spearman's correlation: $\rho = 0.827$, $n = 326$, $P < 0.001$). Several species were considered aggressive on both, including *P. intermedium*, *P. irregulare*, *P. mamillatum*, *P. sylvaticum,* and *P. ultimum* var. *sporangiiferum* (Fig. 2). The novel isolate closely related to *P. intermedium* also showed an aggressive phenotype on both soybean and corn seed. Significant variation was observed among isolates of *P. heterothallicum* ($P = 0.095$ for soybean and $P = 0.025$ for corn), and these differences did not appear to be correlated with patterns of genetic divergence in the COI phylogeny.

Pyrosequencing and MOTU identification. Two separate libraries were constructed from the bulk DNA extractions. The original library constructed with the COI barcoding primers (OomCoxI-Levup and OomCoxI-Levlo) had low yield and resulted in a limited dataset (35,735 raw reads, with a range of 127 to 8,303 reads per sampling location). Therefore, we focused our diversity analysis on the pyrosequencing results generated from the second library (OomCoxI-Levup and FM85mod reverse), although this dataset contained only seven of the eight soybean-corn rotation fields and the stream water sample from the nature conservancy. In total, 263,722 raw reads were obtained but the distribution of reads was unequal across the eight locations (Table 3). After processing, the mean read length across datasets was approximately 390 bp, with some reads up to 750 bp. The percentage of clustered reads matching known species within the custom database was generally low (1 to 2%), although Lebanon County Farm B and the nature conservancy stream water sample showed considerably more matches (13.4 and 26.3%, respectively); these two datasets also showed longer mean read lengths (approximately 430 bp).

Based on the COI pyrosequencing results, 49 MOTU corresponding to known species were identified across the eight sampling locations (Table 4); 3 additional MOTU were designated species affinis based on their closest matches. Similar to the culture-based results, there was almost no overlap in species profiles between the soybean-corn fields and the water sample from the nature conservancy; only one MOTU identified as *Achlya bisexualis* was shared (Table 4). The most common MOTU, present in all seven samples from the soybean-corn fields, were identified as *P. attrantheridium* and the related lineage *Pythium* sp. *balticum*/aff. *attrantheridium*. The next most common MOTU, identified as *P. heterothallicum* and *P. sylvaticum*, were present in six of seven soybean-corn fields; interestingly, these species were also absent from the corresponding locations in the culture-based analysis. In all, 17 MOTU were labeled unclassified *Pythium* spp., given their phylogenetic position within the genus, but they could not be affiliated with any particular species; an additional 24 MOTU were phylogenetically ambiguous and labeled unclassified Oomycota.

Diversity analysis. Species accumulation curves for the culture-based data indicated that we did not exhaustively sample the *Pythium* spp. diversity at any location, although the Berks County Farm B sample appeared to approach an asymptote (Fig. 3A). Accumulation curves for the pyrosequencing data were highly influenced by sequencing depth, with the stream water sample from the nature conservancy and the Lebanon County Farm B sample (the two largest pyrosequencing datasets) showing strong signs of saturation (Fig. 3B). Measures of community similarity were not influenced by the geographic proximity of sampling locations (Supplementary Fig. S2), and soil characteristics are likely to be the main driver of community composition, as suggested by our correspondence analysis and noted previously by Broders et al. (2009).

Discussion

Soybean production in Pennsylvania has increased substantially over the past 20 years. Although midseason diseases such as white mold (Sclerotinia stem rot) and frog-eye leaf spot (*Cercospora*

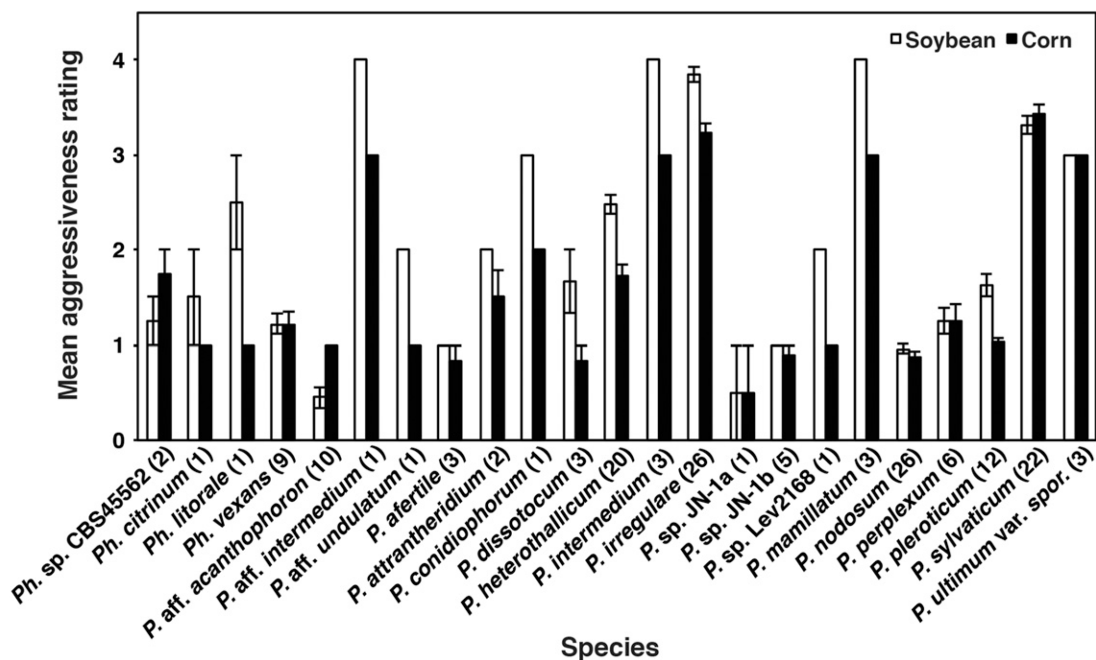

Fig. 2. Mean aggressiveness rating (± standard error) per species on soybean and corn seed. An ordinal scale was used to rate aggressiveness, where 0 = no hyphal colonization of seed, 1 = very light hyphal colonization of some seed, 2 = light hyphal colonization of most seed, 3 = dense hyphal growth on all seed, and 4 = dense hyphal growth across plate with blackening of seed. Assays were conducted at room temperature (25°C). The number of isolates tested is shown in parentheses next to each species name. Results from the single isolate of *Pythium torulosum* are not shown because all assays were rated 0.

sojina) have been more prevalent across the state (PSU 2015), seed and seedling diseases can significantly affect yield, especially when environmental or cultivation conditions undermine the protective effects of seed treatments. Here, we have focused on *Pythium* spp., but soilborne fungi such as *Rhizoctonia solani* (Dorrance et al. 2003) and *Fusarium* spp. (Arias et al. 2013) are also important contributors to soybean stand establishment issues, either individually or as a disease complex. Our culture-based approach identified 18 *Pythium* spp. across eight soybean-corn rotation fields in six counties, with an additional 7 species isolated from a local nature conservancy. Using COI pyrosequencing as a culture-independent method, 26 MOTU identified as *Pythium* spp. or related lineages were present in our soybean-corn samples, with an additional 16 MOTU representing other oomycetes. Similar to other studies (Marchand et al. 2014; Spies et al. 2011; Weiland 2011), we have focused on molecular methods for species identification in order to avoid the ambiguity of morphological traits. However, morphological observations can play a vital role in confirming the presence of novel or unexpected species within a sampling location (Zitnick-Anderson and Nelson 2015).

Although we sampled fields with no self-reported history of stand establishment problems due to seed or seedling diseases, we cannot assume that all fields were disease free, and several potential pathogens were, in fact, identified. Similar to previous studies, *P. intermedium*, *P. irregulare*, *P. sylvaticum*, and *P. ultimum* var. *sporangiiferum* were aggressive on soybean and corn seed in laboratory assays. Our isolates identified as *P. mammilatum* were also aggressive; although this species has been shown to be pathogenic on other hosts (Bahramisharif et al. 2014; Weiland et al. 2013), including other legumes (Li et al. 2014; Matoba et al. 2008) and grasses (van der Plaats-Niterink 1981), we believe that this is the first report of aggressiveness on soybean and corn seed, at least under laboratory conditions. Two additional species, *P. conidiophorum* and *Phytopythium litorale*, were considered moderately aggressive, although only a single isolate was tested for each; *Pythium conidiophorum* has been shown to be aggressive on common bean (Li et al. 2014) while *Phytopythium litorale* isolated from irrigation ponds caused damping off, root rot, and fruit lesions on squash (Parkunan and Ji 2013). Our pyrosequencing results suggest that other, known soybean or corn pathogens are also present in our sampled locations, including *Pythium arrhenomanes* (Sumner et al. 1990; van der Plaats-Niterink 1981), *P. ultimum* var. *ultimum* (Broders et al. 2007a; Jiang et al. 2012; Rizvi and Yang 1996), and *Phytophthora sansomeana* (Hansen et al. 2009; Hartman et al. 2015).

Our pathogenicity assay results for *Pythium heterothallicum* were particularly interesting because isolates of this species showed a range of aggressive phenotypes. Although COI sequence variation was present among isolates, differences in phenotype did not appear correlated with phylogenetic distance. Although this species is typically considered a common soilborne saprophyte, previous studies have shown some pathogenic abilities on wheat (Chamswarng and Cook 1985), lentil (Ingram and Cook 1990), and corn (Gan et al. 2010). Additionally, a recent study by Zitnick-Anderson and Nelson (2015) showed that field-collected isolates of *P. heterothallicum* had some impact on soybean seedling emergence and survival in laboratory assays. In this study, we did not attempt to differentiate between *P. heterothallicum* and the closely related *P. glomeratum*; these two species likely represent a complex (Robideau et al. 2011) but morphological differences exist (Paul 2003). Nothing is known about the pathogenicity of *P. glomeratum*.

Consistent with previous studies, several nonpathogenic species were also collected from our sampling locations, including *P. nodosum* (Paul et al. 1998), *P. perplexum* (Li et al. 2014), and *P. pleroticum* (Abdelzaher et al. 1994; van der Plaats-Niterink 1981). *Phytopythium vexans* has been associated with disease (van der Plaats-Niterink 1981) but is likely an opportunistic pathogen instead of a primary agent (Marano et al. 2016). Our results showed a nonaggressive phenotype for *Pythium afertile*, *P. dissotocum*, and *P. torulosum*, although others have shown these species to be aggressive on various hosts (Rao et al. 1978; van der Plaats-Niterink 1981; Weiland et al. 2013), including soybean (Broders et al. 2007a; Dorrance et al. 2004; Zitnick-Anderson and Nelson 2015). Studies have shown that assay temperature can influence aggressive phenotypes in the laboratory (Matthiesen et al. 2016; Thomson et al. 1971; Wei et al. 2010), which could also explain differences in our results observed at room temperature. Our novel isolates labeled *Pythium* aff. *acanthophoron* and *Pythium* sp. Lev2168 also showed a nonaggressive phenotype. The presence of *P. acanthicum* and *P. nunn* in our pyrosequencing dataset is interesting given their suggested antagonism of other, pathogenic *Pythium* spp. (Ali-Shtayeh and Saleh 1999; Lifshitz et al. 1984a; Ribeiro and Butler 1995). The interactions

Table 3. Number of cytochrome c oxidase pyrosequencing (Pyro) reads and molecular operational taxonomic units (MOTU) identified from seven soybean-corn rotation field soil samples and a stream water sample collected in southeastern Pennsylvania in 2012

	Soybean-corn rotation fields[a]							
Reads, clusters	**Ba**	**Bb**	**D**	**L**	**La**	**Lb**	**Y**	**Stream**
Reads								
Total pyro	48,584	13,366	4,448	64,431	17,731	29,275	76,371	9,516
Total HQ[b]	47,321	13,054	4,019	62,848	12,323	28,034	74,075	9,314
Mean (median)[c]	374 (372)	379 (373)	382 (398)	385 (386)	360 (368)	437 (454)	387 (412)	429 (452)
Maximum[c]	750	675	639	672	689	755	705	622
Clusters								
Total 100% ID[d]	31,449	8,948	2,925	38,034	9,123	18,823	43,038	6,635
ID ≥ 98.5 (%)[e]	1.4	1.0	1.0	2.1	0.9	13.4	0.3	26.3
Assigned[f]	11	9	5	13	8	21	9	35
90 to 98.5 (%)[g]	1.3	1.3	0.6	3.3	0.6	13.6	0.3	41.3
Unclassified[h]	3	1	0	7	1	8	2	22

[a] Location abbreviations: Berks County Farm A (Ba), Berks County Farm B (Bb), Dauphin County (D), Lancaster County (L), Lebanon County Farm A (La), Lebanon County Farm B (Lb), and York County (Y). Stream water was collected from a nature conservancy in Lancaster County.

[b] Number of high quality (HQ) reads retained after applying the following filters: remove reads with an average quality value (QV) under 15; trim low-quality ends until QV > 20 in a 15-bp window; remove reads if 50% of bases are either A, T, C, or G; and remove reads with GC content less than 15% or greater than 85%. Only reads >100 bp after removing the multiplexed identifier and primer sequence were retained in each dataset.

[c] Mean (median) and maximum read lengths.

[d] High-quality reads were clustered at 100% identity (ID) to reduce the search time against the custom database. The number of reads within each cluster was retained in order to calculate the total number of matched reads.

[e] Percentage of clusters matching a sequence in the custom database with ≥98.5% ID.

[f] Number of assigned MOTU; clusters with ID ≥ 98.5%.

[g] Percentage of clusters matching a sequence in the custom database with 90 to 98.4% ID (90% ≤ ID < 98.5%).

[h] Clusters matching a sequence in the custom database with 90 to 98.4% ID that could not be phylogenetically resolved to species were considered unclassified MOTU (90% ≤ ID < 98.5%).

Table 4. Molecular operational taxonomic unit (MOTU) identification and relative abundance from seven soybean-corn rotation field soil samples and a stream water sample (Stream) collected in southeastern Pennsylvania in 2012[a]

	Soybean-corn rotation fields[b]							
MOTU	Ba	Bb	D	L	La	Lb	Y	Stream
Achlya bisexualis	…	…	…	…	…	0.1	…	5.1
A. caroliniana	…	…	…	…	…	0.3	…	…
Aphanomyces laevis	…	…	…	…	…	…	…	0.1
Dictyuchus monosporus	…	…	…	…	…	…	…	1.5
Leptolegnia sp. CBS58285	…	…	…	…	…	…	…	1.0
Phytophthora cactorum	…	…	…	…	…	…	…	0.5
P. citricola	…	…	…	…	…	…	…	1.1
P. cryptogea	…	…	…	…	…	…	…	0.3
P. gonapodyides	…	…	…	…	…	…	…	0.9
P. lacustris	…	…	…	…	…	…	…	40.4
P. parsiana	…	…	…	…	…	…	…	0.3
P. pini	…	…	…	…	…	…	…	0.1
P. plurivora	…	…	…	…	…	…	…	0.1
P. sansomeana	…	…	…	…	…	1.5	…	…
Phytopythium litorale	…	…	…	…	…	…	…	0.9
Pythium acanthicum	9.5	39.1	…	…	…	3.6	3.2	…
Pythium aff. BR6555[c]	…	…	…	…	…	…	…	8.9
Pythium aff. *sukuiense*[c]	…	…	…	…	…	…	…	6.4
Pythium aff. UASW0227[c]	…	…	…	…	…	…	…	0.1
P. aquatile	…	…	…	…	…	…	…	0.1
P. arrhenomanes	…	…	…	…	…	…	…	…
P. attrantheridium	12.7	9.0	16.7	46.3	39.6	14.2	46.4	…
P. sp. *balticum*/aff. *attrantheridium*	2.9	3.2	16.7	1.6	8.3	8.7	5.6	…
P. capillosum	…	…	…	…	…	…	…	0.1
Pythium sp. CBS23294	…	…	…	…	…	…	…	0.4
Pythium sp. CBS63385	…	…	…	…	…	…	…	0.4
P. conidiophorum	…	…	22.2	…	…	6.6	…	…
P. dissotocum	…	…	…	…	…	0.5	…	…
P. heterothallicum	39.9	21.8	…	16.7	20.8	1.6	6.8	…
P. hypogynum	0.8	…	…	…	…	…	…	…
P. irregular	0.7	…	…	…	…	2.8	…	…
Pythium sp. Lev2156	…	…	…	0.5	…	…	…	…
Pythium sp. Lev3106	…	…	…	…	…	0.1	…	…
P. longisporangium	…	…	…	0.3	…	…	…	…
P. monospermum	2.1	…	…	0.3	…	…	2.8	…
P. nunn	0.4	…	…	…	…	0.5	…	…
P. oopapillum	…	…	…	…	…	…	…	0.1
Pythium sp. P8207	…	…	…	…	…	…	…	2.7
P. recalcitrans	…	…	…	1.3	5.2	…	8.0	…
P. rostratifingens	…	…	…	…	…	1.0	…	…
Pythium sp. JN-1a	…	…	…	…	…	…	…	2.6
Pythium sp. JN-1b	…	…	…	…	…	…	…	1.9
P. sylvaticum	2.9	1.9	44.4	5.9	17.7	22.4	…	…
P. ultimum var. *ultimum*	…	…	…	…	5.2	0.5	…	…
P. ultimum var. *sporangiiferum*	…	…	…	…	…	17.7	…	…
P. viniferum	…	…	…	0.2	…	0.1	…	…
Saprolegnia diclina	…	…	…	…	…	…	…	0.7
S. ferax	…	…	…	…	…	…	…	1.5
S. lapponica	…	…	…	…	…	…	…	1.1
S. mixta	…	…	…	…	…	…	…	0.7
S. parasitica	…	…	…	…	…	…	…	1.6
Saprolegnia sp. CBS63285	…	…	…	…	…	…	…	2.1
Unclassified *Pythium* spp.[d]	5.4	…	…	12.0	…	10.8	…	13.3
Unclassified Oomycota[d]	12.7	22.4	…	14.9	3.1	7.1	27.2	2.8
Number of matched reads	755	156	36	1678	96	5411	250	3504
Total number of MOTU	12	7	4	14	7	21	8	41

[a] Relative abundance was calculated as the number of reads per MOTU divided by the total number of matched reads for each dataset.

[b] Location abbreviations: Berks County Farm A (Ba), Berks County Farm B (Bb), Dauphin County (D), Lancaster County (L), Lebanon County Farm A (La), Lebanon County Farm B (Lb), and York County (Y). Stream water was collected from a nature conservancy in Lancaster County.

[c] Designations of species affinis (aff.) were transferred based on matches to cytochrome c oxidase sequences generated from cultured isolates collected in 2013; these cultures were labeled *affinis* based on their highest scoring match in the National Center for Biotechnology Information.

[d] MOTU that matched sequences in the custom database with 90 to 98.4% identity and could not be phylogenetically resolved to species were considered unclassified.

among soilborne *Pythium* spp. and other fungal saprotrophs and pathogens are complex, and can influence the development (or not) of disease independent of environmental conditions or host physiology (Lamichhane and Venturi 2015; Manandhar et al. 1987; Martin and Loper 1999).

The goal of culture-independent methods is to sample species diversity that is unlikely to be recovered using traditional baiting or other culture-based approaches. We identified several MOTU in our pyrosequencing dataset that correspond to species likely to be present in our sampling locations. For example, previous studies have shown that *P. arrhenomanes*, a known corn pathogen identified in two of our soybean-corn rotation fields, is difficult to isolate directly from soil (Sumner et al. 1990). Other species may be present in an environment at low frequency and, therefore, are less likely to be sampled; such may be the case for those MOTU identified from a single sampling location. There may have also been a mismatch between our baiting conditions and those necessary for the production of sporangia or zoospores; *P. monospermum* (MOTU in three locations) and *P. acanthicum* (four locations) may only produce zoospores at cooler temperatures (van der Plaats-Niterink 1981). Similarly, zoospores have not been observed for *P. recalcitrans* (three locations), *P. nunn* (two locations), and *P. viniferum* (two locations) (Lifshitz et al. 1984b; Moralejo et al. 2008; Paul et al. 2008). Although our bait plates were flooded with sterile water, baits were sometimes in direct contact with soil, which may have allowed for hyphal colonization of baits; this could explain how we were able to isolate *P. irregulare* and *P. intermedium* even though these species seldom form sporangia (van der Plaats-Niterink 1981).

The absence of cultured species from our pyrosequencing datasets illustrates that methodological biases specific to culture-independent approaches can also influence estimates of species diversity. Bias can be introduced as early as the DNA extraction and purification steps (Koid et al. 2012; Rajendhran and Gunasekaran 2008). Additionally, the PCR amplification necessary for template preparation is a well-known source of bias due to primer-template mismatches, template GC content, or nonoptimal amplification conditions (Geisen et al. 2015; Pawluczyk et al. 2015; Pinto and Raskin 2012). Preliminary analyses of our pyrosequencing data using the program MEGAN (Huson et al. 2011) did reveal some off-target amplification, particularly of bacterial DNA, suggesting that our template preparation conditions were not ideal. Recently, Choi et al. (2015) showed empirically that the COI primer combination used here for pyrosequencing (OomCoxI-Levup and FM85mod) was unable to amplify samples from several oomycete genera (although their *Pythium* sp. did amplify). The species isolated from our soil and water samples but absent from our pyrosequencing datasets did not follow any particular phylogenetic distribution and, in some cases, closely related species were present in both datasets; for example, *P. afertile* (culture only) versus *P. conidiophorum* (both members of clade B1), and *P. intermedium* (and presumably *Pythium* aff. *intermedium*, culture only) versus *P. sylvaticum* and *P. irregulare* (clade F). These findings suggest that primer-template mismatch may not have played a major role in our amplification conditions, assuming similar substitution patterns among species in the same clade. Sequencing errors have also been associated with inflated diversity estimates in pyrosequencing datasets (Gomez-Alvarez et al. 2009; Kunin et al. 2010), and MOTU represented by a single sequence (singletons) are commonly discarded from analyses. Here, we used a cut-off of three reads, and only 7 of the 52 phylogenetically resolved MOTU (Table 4) were represented by fewer than five sequences. Studies of mock communities have repeatedly shown that, due to these methodological biases, read counts should not be interpreted directly as species abundances (Amend et al. 2010; Geisen et al. 2015; Sapkota and Nicolaisen 2015; Vettraino et al. 2012). Here, we have been conservative by focusing on species or MOTU presence or absence and not abundance when comparing diversity among sampling locations.

The choice of COI pyrosequencing as our culture-independent method was advantageous for several reasons. Of all the next-generation sequencing methods, pyrosequencing produces the longest reads (up to 10× longer than other methods) with a short run time (approximately 24 h) (Shokralla et al. 2012); longer reads improve the reliability of MOTU identification and allow for more robust phylogenetic analysis of sequences. As a coding region, COI has advantages over the more commonly used nuclear ITS1 region (Català et al. 2015; Coince et al. 2013; Sapkota and Nicolaisen 2015; Vannini et al. 2013); although ITS shows higher interspecific variation than COI (Robideau et al. 2011), sequence alignments (and, thus, phylogenetic analysis) beyond the clade-level can be highly ambiguous and arbitrary. Sequencing errors resulting from the incorrect extension of homopolymer repeats, which can be common with pyrosequencing methods (Gilles et al. 2011; Huse et al. 2007), were easily detected as insertions or deletions in our COI sequence alignments. Substitution errors are less common with pyrosequencing methods (Gilles et al. 2011); although we did not attempt to correct for these potential errors here, a systematic analysis of COI substitution patterns per codon position generated from a mock community would allow us to empirically determine substitution error rates. Sequencing errors of both types (indels and substitutions) are more difficult to detect, with confidence, in noncoding regions such as ITS, thus reducing the reliability of this locus for estimating sequence-based biodiversity in environmental samples.

Here, we have shown that there is potential for the continued development of culture-independent methods such as COI pyrosequencing for diversity analysis as long as the underlying methodological

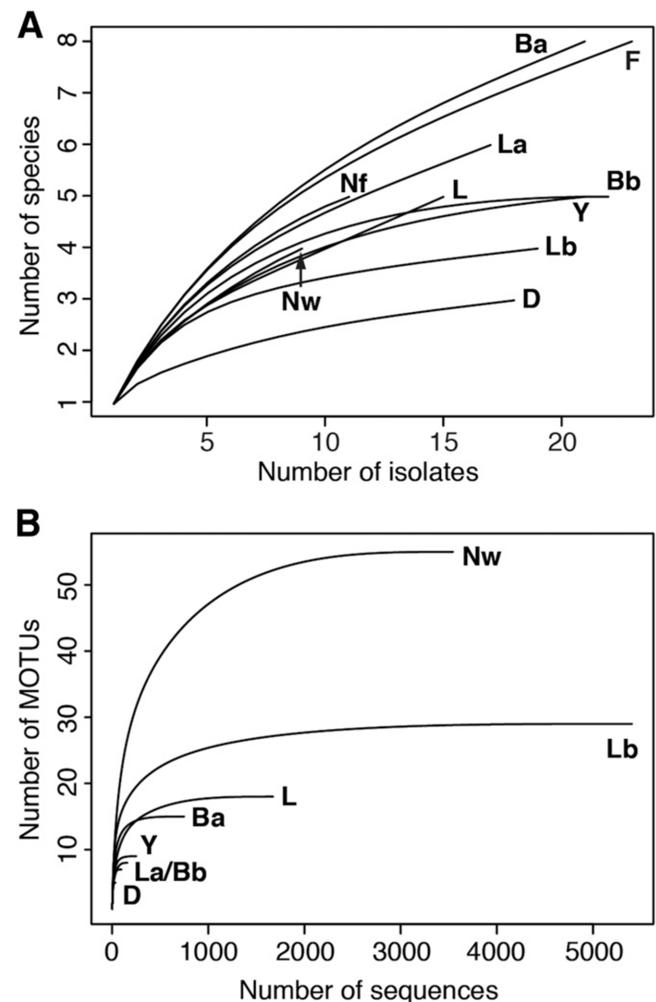

Fig. 3. Species accumulation curves for **A,** culture-based and **B,** pyrosequencing (B) datasets. Location abbreviations: Berks County Farm A (Ba), Berks County Farm B (Bb), Dauphin County (D), Franklin County (F), Lancaster County (L), Lebanon County Farm A (La), Lebanon County Farm B (Lb), York County (Y), nature conservancy forest soil (Nf), and stream water (Nw). Pyrosequencing data were not available for the F and Nf locations.

biases can be addressed. However, culture-based surveys will continue to play an important role for determining phenotypes such as fungicide resistance, as well as for the description of new species. Our analysis of asymptomatic soybean fields compliments other studies that have isolated *Pythium* spp. from fields with a history of stand establishment issues, or directly from diseased seedlings. Together, these data can help inform management decisions by providing a more complete picture of the complex interactions among hosts, pathogens, and saprotrophs that can lead to a disease state under particular environmental conditions.

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