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Research Article

Direct PCR detection, cloning, and characterization of fungi communities in soils and compost

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Abstract. A procedure was developed to detect and identify fungi in garden soil, forest soil, and compost samples. Microbial DNA was extracted using the Zymo Microbe DNA MiniPrep protocol. Fungi ribosomal internal transcribed space sequences (ITS) were amplified by PCR using primers ITS1 and ITS4. DNA fragments of approximately 640 base pair were detected in all positive samples. Clone libraries were constructed with amplified DNA by ligating the detected fragments with vector pCR®4-TOPO. Transformations were performed using competent Mix and Go *Escherichia coli* cells. Plasmids were isolated from each clone using the Zyppy plasmid miniprep protocol and inserts were screened by PCR using M13 DNA primers. Most of the identified fungal species were aligned to the phyla Ascomycota and Basidiomycota. BLAST analysis of clone libraries showed that sequences from compost samples were only comprised of the Ascomycota species *Thermomyces lanuginosus*. Clones from garden soils were mostly unidentified species closely aligned to Ascomycota and Zygomycota while the most abundant sequences in forest soils were related to the Basidiomycota species *Cortinarius flexipes*. In garden soil more than 50% of ITS sequences belonged to the Kingdom Plantae with species such as *Cardamine hirsute, Stellaria media,* and *Cerastium dinaricum*. However, there were no plant ITS sequences in compost and forest soil. Fungal species belonging to the phylum Ascomycota were widely distributed in the 3 environments studied with the forest soil showing the highest fungal diversity.

Introduction

Fungi communities in soils are responsible for carbon cycling, supporting plant nutrition, and pathology (Tedersoo et al., 2014). Fungi are also major sources of biologically active compounds. Most fungi are mesophilic and grow in temperatures ranging from 5°C to extreme temperatures (e.g., 55°C). However, some thermophilic fungi can grow at 55°C. Because of the functionality of fungal communities, they are widely distributed in different terrestrial environments (Hibbett, 2016). There is a poor understanding of community structure regarding phyla, species, and functional groups (cellulose degradation, lignin degradation, etc.). The first studies on fungal ecology were culture-based, where samples were grown using selective media such as Potato Dextrose Agar (PDA) or Sabouraud Dextrose Agar (SDA; Vancov and Keen, 2009). Some fungi are hard to isolate using growth media, with the great majority of fungal species being unculturable (Tedersoo et al., 2014). Furthermore, when isolated, fungal identification

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using phenotypic analysis is inaccurate, slow, and extremely subjective (Rittenour et al., 2012).

Accurate fungi identification can be performed using ribosomal internal transcribed spacer regions (ITS) within rRNA genes. The ITS that separate the small-subunit (SSU) rRNA and large subunit (LSU) rRNAs in eukaryotes mutates at a faster rate and can be used to identify fungi to genus and species level. Furthermore, large ITS databases are publicly available from diverse fungal taxa (Cole et al., 2014; Kõljalg et al., 2013). However, some databases have not been curated and may contain sequences with annotation mistakes, leading to misidentification of environmental isolates.

Fungal DNA can be extracted from environmental matrices with ITS sequences amplified by polymerase chain reaction (PCR) and cloned (O'Brien et al., 2005; Rittenour et al., 2012). The retrieved ITS sequences are identified by high similarities to genetic sequences on databases. Previous studies using PCR and cloning of ITS sequences detected in environmental samples provided a more complete description of fungal community structure and diversity (Hultman et al., 2009; Menkis et al., 2014; O'Brien et al., 2005). Cloning libraries have been constructed to study the diversity and distribution of fungal species in soils and compost. The environmental ITS sequences found did not align to any known fungal phyla, genera, or species. These studies revealed that climatic factors were able to predict fungal richness and community composition (Tedersoo et al., 2014).

The objective of this study was to develop a rapid culture-independent procedure using direct DNA extraction, PCR amplification, cloning, and Sanger sequencing of fungal ITS sequences to ascertain the structure and diversity of fungal communities present in compost, forest soil, and garden soil.

Materials and Methods

Three environmental samples were analyzed to determine the structure and diversity of fungal communities. The compost sample was obtained from an in-vessel compost system described by Jimenez et al. (2015). The garden soil sample was obtained at Bergen Community College (BCC) in Paramus, New Jersey. Undisturbed conifer forest soil sample (elevation 3,700 feet) came from the Balsam Lake Mountain Wild Forest in the Catskill Mountains, Hardenburgh, New York.

DNA extraction

DNA was extracted from 300 mg of soils and compost using the ZR Soil Microbe DNA MiniPrep protocol (Zymo Research, Irvine, CA). Fungal DNA was also extracted from environmental isolates randomly isolated from contaminated microbiological media. These fungal DNA samples were used as positive controls for PCR and sequencing reactions.

PCR amplification

The suitability of extracted soil and compost DNA for PCR analysis was determined by running different dilutions to determine the presence of inhibitors. The reactions target a fragment of approximately 640 base pair (bp) of fungal ITS using primers ITS1 and ITS4 (Ferrer et al., 2001). The reaction conditions were 95°C for 3 min, and 30 cycles of 95°C for 30 sec, 65°C for 30 sec., 72°C for 30 sec, with a final extension of 10 min at 72°C. Ready-To-Go (RTG) PCR beads (GE Healthcare, Buckinghamshire, UK) were used for each PCR reaction. For fungal ITS amplification from soil and compost DNA, 2 beads were used in a total reaction volume of 50 μL.

PCR amplicon detection was carried out by gel electrophoresis using the FlashGel™ system (Lonza Inc., Rockland, ME) with FlashGel DNA cassettes containing 1.2% agarose. A FlashGel DNA marker (Lonza Inc., Rockland, ME) with fragment sizes ranging from 100 bp to 4 kb was used to determine the presence of amplified DNA gene fragments.

Cloning libraries

The DNA fragments from Fungi ITS genes detected in environmental samples were ligated using plasmid pCR®4-TOPO (Life Technolo-

gies, Thermo Fisher Scientific, Grand Island, NY) according to the manufacturer's instructions. Transformations were performed using Mix and Go competent *Escherichia coli* strains (Zymo Research, Irvine, CA). The ligation mixture was added to *E. coli* followed by incubation on ice for 10 min. After transformation, *E. coli* was plated on Luria Bertani Agar (LBA) with ampicillin (50 μg/mL). Plates were incubated for at least 18 hours at 37°C. White colonies (clones) grown on LBA with ampicillin (50 μg/ml) were transferred to LB broth containing ampicillin (50 μg/mL). Samples were incubated for at least 18 hours at 37°C with shaking at 200 rpm.

Plasmids were isolated from each clone using the Zyppy Plasmid Miniprep Kit (Zymo Research, Irvine, CA). Cloned inserts were reamplified using M13 DNA primers. PCR reactions screening for inserts were performed using 1 PCR bead in a total volume of 25 μL. DNA sequencing reactions of pure fungal cultures and amplified PCR fragments from clone libraries were performed by Genewiz, Inc. (South Plainfield, NJ). Homology searches were performed using the GenBank server of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the BLAST algorithm (Altschul et al., 1997). Homology values equal or greater than 97% were used for fungal species level identification. Multiple sequences alignment and phylogenetic analyses of fungal ITS sequences were performed using Clustal Omega and ClustalW (McWilliam et al., 2013) from the European Bioinformatics Institute (EMBL-EBI; https://www.ebi.ac.uk/Tools/msa/clustalo/). Further identification of compost ITS sequences was performed using the Unite Fungal ITS trainset 07-04-2014 and Warcup Fungal ITS trainset 2 located on the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/index.jsp; Cole et al., 2014).

Results

DNA was extracted from randomly isolated fungi from contaminated microbiological media found in a teaching microbiology laboratory.

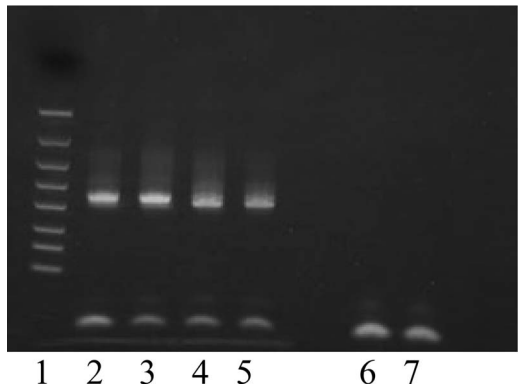


Figure 1. PCR detection of ITS fungal sequences in environmental samples. Lane 1, Molecular weight marker, bp (from top to bottom, 4000, 2000, 1250, 800, 500, 300, 200, 100); lane 2, forest soil; lane 3, forest soil; lane 4, garden soil; lane 5, garden soil; lane 6, negative control; lane 7, negative control.

These fungal isolates were used to determine the sensitivity and accuracy of the ITS primers to accurately identify fungal isolates and environmental ITS sequences. PCR analysis resulted in the amplification of ITS fragments ranging from 550 to 770 bp. After DNA sequencing and BLAST analysis, one of the isolates was identified as the Basidiomycota species *Irpex lacteus* (99%). However, the other isolate was identified as the Ascomycota species *Cladosporium herbarum* (99%). After extracting microbial DNA from soil and compost samples, PCR amplification using ITS1 and ITS4 primers generated PCR fragments ranging from 640 to 740 bp (Fig. 1). Three clone libraries were developed from ITS sequences in compost and soil samples by cloning all fragments with plasmid pCR®4-TOPO. Five hundred clones were screened using M13 primers to determine the presence of fungal ITS fragments.

In the compost library, 41 clones were found to have fragment inserts ranging from 800 to 900 bp. The higher molecular weight of the clone fragments compared to the amplified sequences from environmental samples was due to the presence of vector sequences. Randomly chosen clones containing DNA fragments between 800 bp and 900 bp did not show any similarities with fungal ITS sequences. All clones generated from compost samples were found to belong to the

Table 1. Molecular identification of fungal clones from compost samples.

Clone number	Phylum	Genera	Species	Percent of DNA homology
1	Ascomycota	*Thermomyces*	*lanuginosus*	97
2	Ascomycota	*Thermomyces* Uncultured fungal clone	*lanuginosus*	75
3	Ascomycota	*Thermomyces*	*lanuginosus*	100
4	Ascomycota	*Thermomyces* Uncultured fungal clone	*lanuginosus*	99
5	Ascomycota	*Thermomyces*	*lanuginosus*	99
6	Ascomycota	*Thermomyces* Uncultured fungal clone	*lanuginosus*	99
7	Ascomycota	*Thermomyces*	*lanuginosus*	99
8	Ascomycota	*Thermomyces*	*lanuginosus*	99
9	Ascomycota	*Thermomyces*	*lanuginosus*	99
10	Ascomycota	*Thermomyces*	*lanuginosus*	99
11	Ascomycota	*Thermomyces*	*lanuginosus*	100
12	Ascomycota	*Thermomyces*	*lanuginosus*	99
13	Ascomycota	*Thermomyces*	*lanuginosus*	99
14	Ascomycota	*Thermomyces*	*lanuginosus*	100
15	Ascomycota	Thermomyces	*lanuginosus*	99
16	Ascomycota	Thermomyces	*lanuginosus*	99
17	Ascomycota	*Thermomyces*	*lanuginosus*	99
18	Ascomycota	*Thermomyces*	*lanuginosus*	99
19	Ascomycota	*Thermomyces*	*lanuginosus*	99
20	Ascomycota	*Thermomyces*	*lanuginosus*	99
21	Ascomycota	*Thermomyces*	*lanuginosus*	99
22	Ascomycota	*Thermomyces*	*lanuginosus*	99
23	Ascomycota	*Thermomyces*	*lanuginosus*	99
24	Ascomycota	*Thermomyces*	*lanuginosus*	100
25	Ascomycota	*Thermomyces*	*lanuginosus*	100
26	Ascomycota	*Thermomyces*	*lanuginosus*	100
27	Ascomycota	*Thermomyces*	*lanuginosus*	99
28	Ascomycota	*Thermomyces*	*lanuginosus*	100
29	Ascomycota	*Thermomyces*	*lanuginosus*	100
30	Ascomycota	*Thermomyces*	*lanuginosus*	100
31	Ascomycota	*Thermomyces*	*lanuginosus*	99
32	Ascomycota	*Thermomyces*	*lanuginosus*	100
33	Ascomycota	*Thermomyces*	*lanuginosus*	100
34	Ascomycota	*Thermomyces*	*lanuginosus*	100
35	Ascomycota	*Thermomyces*	*lanuginosus*	100
36	Ascomycota	*Thermomyces*	*lanuginosus*	99
37	Ascomycota	*Thermomyces*	*lanuginosus*	100
38	Ascomycota	*Thermomyces*	*lanuginosus*	100
39	Ascomycota	*Thermomyces*	*lanuginosus*	99
40	Ascomycota	*Thermomyces*	*lanuginosus*	100
41	Ascomycota	*Thermomyces*	*lanuginosus*	99

phylum Ascomycota (Table 1). Within the Ascomycota phylum all 41 clones showed closer homology values to the species *Thermomyces lanuginosus*. No other fungal species were found in compost samples. BLAST analysis of clone sequences showed similarity values ranging from 75 to 100%. Ninety-eight percent of the clones showed a 97-100% homology with *T. lanuginosus*. Three clones (7%) were closely aligned to uncultured *T. lanuginosus* sequences detected in soils. Two of these clones showed 99% homology. The other clone, number 2, showed the lowest homology value, 75%.

The forest soil clone library contained a total

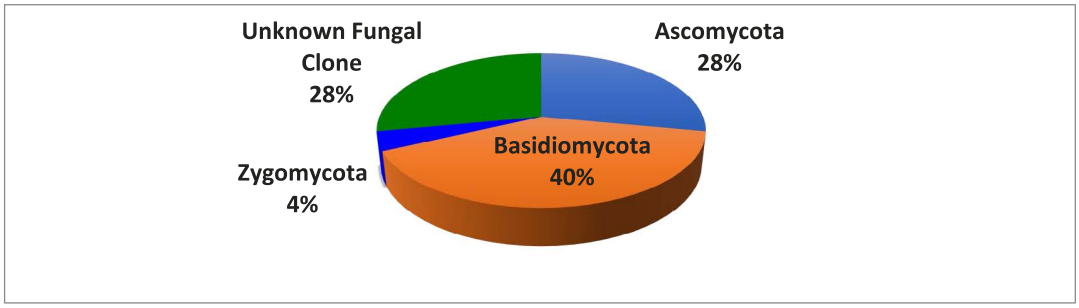
Table 2. Molecular identification of fungal clones from forest soil.

Clone number	Phylum	Genera	Species	Percent of DNA homology
1	Ascomycota	*Phialocephala*	*virens*	94
2	Ascomycota	*Chalara*	*longipes*	98
3	Basidiomycota	*Cortinarius*	*flexipes*	100
4	Basidiomycota	*Tylopilus*	*felleus*	99
5	Basidiomycota	*Cortinarius*	*flexipes*	100
6	Basidiomycota	Uncultured *Piloderma* clone		98
7	Basidiomycota	*Cortinarius flexipes*	*flexipes*	100
8		Uncultured fungal clone		96
9	Basidiomycota	*Tylopilus*	*felleus*	99
10	Ascomycota	*Cenococcum*	*geophilum*	91
11	Basidiomycota	*Cortinarius*	*grosmorneensis*	99
12	Ascomycota	*Penicillium*		99
13	Ascomycota	*Cenococcum*	*geophilum*	100
14	Ascomycota	*Cenococcum*	*geophilum*	100
15		Uncultured fungal clone		99
16		Uncultured fungal clone		99
17	Basidiomycota	*Cortinarius*	*flexipes*	99
18		Uncultured fungal clone		99
19	Basidiomycota	*Cortinarius*	*flexipes*	100
20		Uncultured fungal clone		98
21	Ascomycota	*Elaphomyces*	*muricatus*	99
22		Uncultured fungal clone		99
23	Zygomycota	Uncultured *Mortierella* clone		99
24		Uncultured ectomycorrhiza clone		98
25	Basidiomycota	*Cortinarius*	*flexipes*	99

of 30 clones. However, after BLAST analysis 5 clones did not show any homology with known fungal sequences or any DNA sequence and were removed from the library. The remaining 25 clones showed similarity values ranging from 91 to 100% (Table 2). Fungal clones belonging to the phylum Basidiomycota accounted for 40% of the ITS sequences found in forest soil followed by Ascomycota with 28% (Fig. 2a). The other phylum found was the Zygomycota with only 4% of sequences. The number of fungal species found was 8, of which the Basidiomycota species *Cortinarius flexipes* were the most abundant with 24% of clones showing a 99-100% homology. The second most abundant fungal species were closely aligned to the Ascomycota species *Cenococcum geophilum* with 12% of ITS sequences showing similarity values ranging from 91 to 100%. The Basidiomycota species *Tylopius felleus* were found in 8% of the clones with similarity values of 99%. Other fungal spp. found were *Phialocephala virens*, *Chalara longipes*, *Piloderma* sp., *Cortinarius grosmorneensis, Penicillium* sp., *Elaphomyces muricatus*, *Mortierella* sp., and an uncultured ectomycorrhiza species. Of the 25 clones, 7 (28%) were reported to be never isolated on growth media (uncultured) and did not show any alignment to known fungal phyla or species. However, similarity values ranging from 96 to 99% were obtained with previously detected uncultured fungal sequences. Phylogenetic analysis of uncultured ITS sequences showed that 7 clones aligned with clones identified as *C. flexipes* and *T. felleus* (Fig. 3). Other clones clustered with *C. geophilum*, *P. virens* and *C. longipes*.

More than 100 clones were screened by PCR using M13 primers to develop libraries with ITS sequences amplified and cloned from garden soil. After PCR screening, 51 clones showed fragments ranging from 800 to 900 bp. However, BLAST analysis indicated that 59% of the fungal clones in garden soils showed 95-100% similarity with plant species *Cardamine hirsute* (47%), *Stellaria media* (6%), and

a.



b.

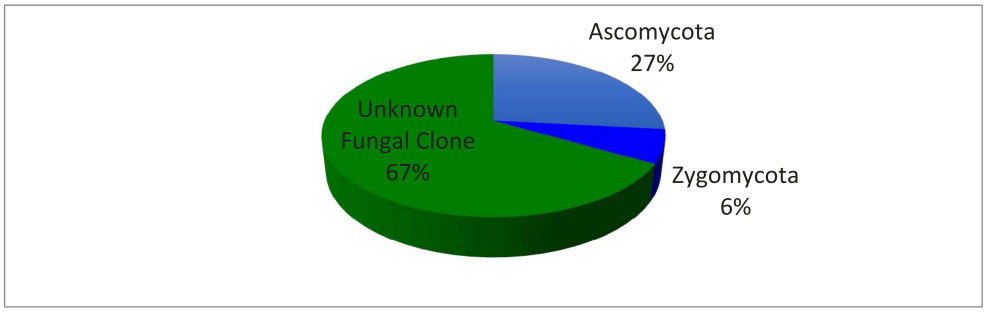


Figure 2. Identified fungal phyla in forest and garden soil. a) Identified phyla in forest soil. b) Identified phyla in garden soil.

Cerastium dinaricum (6%; Table 3). Based upon those results, only 15 of the 51 clones showed similarity with fungal ITS sequences. Of the 15 fungal clones, 67% were uncultured fungal sequences previously detected in soils (Fig. 2b). Two of the uncultured clones showed similarity to the phylum Zygomycota and genera *Alternaria*. However, others did not

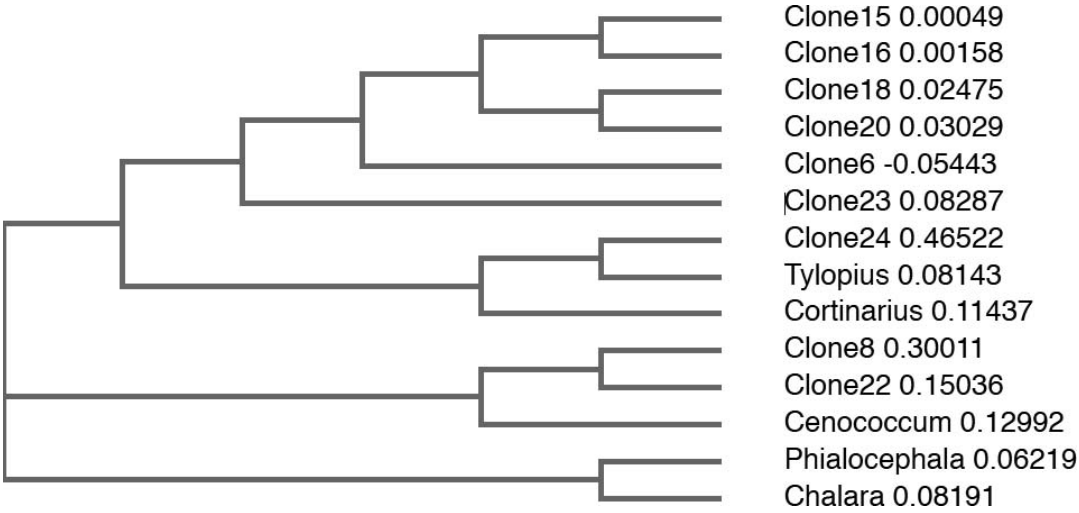


Figure 3. Phylogenetic analysis of unknown clones detected in forest soil. Numbers represent the pairwise scores calculated for every pair of sequences that is to be aligned. Pairwise scores are the number of identities between the two sequences, and divided by the length of the alignment.

Table 3. Molecular identification of fungal clones from garden soil.

Clone number	Phylum	Genera	Species	Percent of DNA homology
1	Ascomycota	*Penicillium*	*restrictum*	100
2		*Cardamine*	*hirsute*	99
3		*Cardamine*	*hirsute*	99
4		*Cardamine*	*hirsute*	98
5		*Cardamine*	*hirsute*	99
6		Uncultured fungal clone		99
7	Zygomycota	Uncultured fungal clone 96%		96
8		*Cardamine*	*hirsute*	99
9		*Cardamine*	*hirsute*	99
10		*Stellaria*	*media*	99
11		*Cardamine*	*hirsute*	99
12		*Stellaria*	*media*	99
13		*Cardamine*	*hirsute*	99
14		*Stellaria*	*media*	99
15		Uncultured fungal clone		94
16		*Cardamine*	*hirsute*	99
17		Uncultured fungal clone		100
18		Uncultured fungal clone		91
19		*Cardamine*	*hirsute*	99
20		*Cerastium*	*dinaricum*	95
21	Ascomycota	*Curvularia*	*trifolii*	100
22		Uncultured fungal clone		92
23		*Cardamine*	*hirsute*	99
24		Uncultured fungal clone		99
25		*Cardamine*	*hirsute*	99
26		*Cardamine*	*hirsute*	99
27		*Cardamine*	*hirsute*	99
28		*Cardamine*	*hirsute*	99
29		*Coccomyxa*	*viridis*	99
30		*Cardamine*	*hirsute*	99
31		Uncultured fungal clone		99
32		*Cardamine*	*hirsute*	99
33		*Cerastium*	*dinaricum*	95
34		*Cardamine*	*hirsute*	99
35		*Cerastium*	*dinaricum*	94
36		*Cardamine*	*hirsute*	99
37		Uncultured fungal clone		94
38		*Cardamine*	*hirsute*	99
39		Uncultured fungal clone		99
40		*Cardamine*	*hirsute*	99
41	Ascomycota	*Phoma*	*herbarum*	100
42		*Cardamine*	*hirsute*	99
43		*Cardamine*	*hirsute*	99
44		Uncultured fungal clone		80
45		*Cardamine*	*hirsute*	99
46		Vector sequence		99
47		*Cardamine*	*hirsute*	98
48		*Cardamine*	*hirsute*	98
49		*Cardamine*	*hirsute*	99
50	Ascomycota	Uncultured Alternaria clone		92
51		*Cardamine*	*hirsute*	99

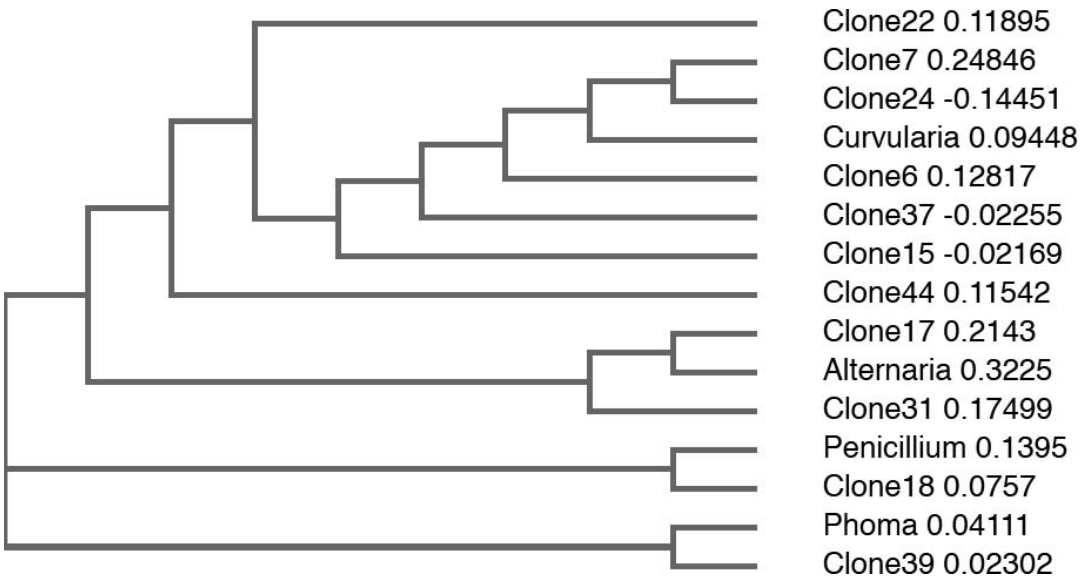


Figure 4. Phylogenetic analysis of unknown clones detected in garden soil. Numbers represent the pairwise scores calculated for every pair of sequences that is to be aligned. Pairwise scores are the number of identities between the two sequences, and divided by the length of the alignment.

have any association with known fungal phyla. Of the 15 clones, 27% belonged to the phylum Ascomycota and 6% to the Zygomycota (Fig. 2b). The only fungal species detected in garden soils were *Curvularia trifolii*, *Penicillium restrictum*, and *Phoma herbarum*. When phylogenetic analyses were completed, the uncultured ITS fungal sequences in garden soil suggested closer similarities to genera or species related to the phyla Ascomycota and Zygomycota (clone 7; Fig. 4). Two of the uncultured clones were clustering with the Zygomycota clone while other showed higher similarities to Ascomycota fungal types such as *P. restrictum, P. herbarum, C. trifolii,* and *Alternaria* sp.

Discussion

The composition and structure of fungi communities present in compost, garden soil, and forest soil was ascertained using direct DNA extraction and PCR amplification of ITS sequences using primer pair ITS1 and ITS4. The suitability and accuracy of the primers used in this study was ascertained by identifying environmental isolates from contaminated microbiological media. Both isolates were accurately identified as the species level with similarity values of 99%. ITS homology values of >97% are commonly accepted for species level identification (Kõljalg et al., 2013). However, for environmental clones, if a value was below 97%, the closest homology value was reported.

Cloning libraries were developed from amplified fungal ITS to screen the identity of fungal phyla and species from compost and soils. Fungal species belonging to the phylum Basiodiomycota were absent in compost and garden soil, while Ascomycota species were found in compost, garden soil, and forest soil. Zygomycota fungi were only found in garden and forest soils. ITS sequences analysis suggested that fungal community diversity in compost was very low, with all sequences belonging to phylum Ascomycota and species *T. lanuginosus*. *T. lanuginosus* has been previously isolated from compost samples where temperatures were up to 60°C (Hultman et al., 2009). They reported a higher fungal diversity in their compost samples by analyzing 2,986 ITS sequences. The three different phyla found were the Ascomycota, Basidiomycota, and Zygomycota. However, only the genera *Ther-*

momyces, *Candida*, and *Rhizomucor* were found in the thermophilic stages. The compost sample analyzed in this study did not show any other fungal genera or species. However, our clone library was comprised of only 41 clones.

Temperature was likely a major factor limiting the numbers and types of fungal species in compost samples to thermophilic fungi. The samples tested were taken from a compost system previously described where ITS sequences were present in only 8% of the compost samples tested (Jimenez et al., 2015). However, the forest soil from New York was found to have a higher fungal diversity than compost samples. While the compost samples have only one fungal species, the forest soil showed 9 known species.

Most species in forest soils belonged to the phylum Basidiomycota. *C. flexipes* clones were the predominant species in forest soils. *C. flexipes* is a mushroom commonly found in North American and European soils. The second most abundant fungal species in forest soils were *C. geophilum*, an Ascomycota ectomycorrhizal species in forest soils. They are important symbionts of many trees helping in the acquisition of soil nutrients in exchange for carbohydrates produced by plants. *T. felleus* was the third most common fungal species found in forest soils. *T. felleus* is a mycorrhizal species with a more cosmopolitan distribution covering Asia, Europe, and eastern North America. Other fungal clones were related to mushrooms or mycorrhizal species such as *P. virens*, *C. longipes*, *Piloderma* sp., *C. grosmorneensis, E. muricatus*, and *Mortierella* sp. Buee et al. (2009), using 454 pyrosequencing, reported Ascomycota and Basiodiomycota to be the most abundant fungi (81%) in forest samples from Burgundy, France. The average length of the ITS sequences analyzed was 252 bp, with a clone library comprised of 166,350 sequences showing the Basidiomycota to be the predominant fungal phylum. The number of unclassified fungi was limited to 11%, while in this study 28% of the sequences were classified as uncultured fungal clones.

The garden soil clone library from BCC did not show a greater fungal diversity than the forest soil since only three known species were detected. However, diversity was higher than compost. Identified fungal species were *C. trifolii*, *P. restrictum*, and *P. herbarum*. Most of the fungal sequences detected in garden soil were related to uncultured fungal clones (67%). Previous studies have shown a high number of unknown fungi ITS sequences not matching any sequences in public databases (Tedersoo et al., 2014). The garden soils samples were obtained from locations where landscaping, including fungicidal application, was used to promote the growth of plants. The application of fungicidal agents to the garden soil may have limited the diversity of fungi by providing an additional environmental stress to the fungal populations.

There were no common fungal genera or species between compost, forest, and garden soil samples. Phylogenetic analysis of unknown sequences showed they aligned closer to the phyla Zygomycota and Ascomycota. However, species level identification was not possible due to the limitations of the fungal databases used to ascertain the alignment with known fungal sequences. Most of the uncultured ITS fungal sequences were found to be in the garden soil. Unclassified fungal ITS sequences were commonly found in soil surveys (Buee et al., 2009; Tedersoo et al., 2014; Vancov and Keen, 2009). Most clones in previous studies were closely associated to the phylum Ascomycota but the lack of taxonomic annotation and errors in taxonomic assignments in international databases are major limitations to accurate identification. Plant ITS sequences were only detected in the garden soil. DNA primers ITS1 and ITS4 have been previously reported to detect plant ITS sequences in environmental samples (O'Brien et al., 2004; Tedersoo et al., 2014; Toju et al., 2012). However, they provided a higher detection and coverage of broad fungal phyla providing an accurate representation of the diversity and community structure of fungal communities present in environmental samples. When it comes to DNA primers for environmental studies, a trade-off exists between specificity for fungi by excluding plant taxa but sacrificing the coverage of important fungal phyla (Toju et al., 2012).

Although more than 300 clones were analyzed, there was a limitation with the standard cloning method which did not cover some groups from environmental samples (Buee et al., 2009). The total number of reliable fungal clones for all three sites was 81. Previous studies have shown that larger ITS clone libraries by Sanger sequencing provided a more complete community structure and higher sample diversity (Hultman et al., 2009; Rittenour et al., 2012). Unfortunately, random sequencing of clones is expensive, labor intensive, and time consuming. Furthermore, PCR amplification conditions, DNA extraction procedures, and primer composition have been shown to affect fungal community composition (Rittenour et al., 2012; Toju et al., 2012). However, when compared to culture studies, cloning of ITS sequences provided a better understanding of fungal diversity in environmental samples. For instance, the compost samples tested for this study showed the presence of *T. lanuginosus* only when fungal ITS sequences were amplified, cloned, and sequenced from extracted compost DNA. Different selective fungal media, e.g., SDA and PDA, were used at different temperatures but no culturable fungi were detected (Jimenez et al., 2015). Only two samples out of 26 showed fungal ITS sequences. However, when the sequences were cloned, Sanger sequenced, and analyzed by BLAST analysis, all clones were found to belong to bacteria and not fungi. Nevertheless, after analyzing the same sequences two years after publication, the ITS sequences were found to belong to *T. lanuginosus*. Further cloning was performed with the same compost DNA to verify the presence of *T. lanuginosus* and validate the new identification information. Expanding the numbers of clones from four to 41 did confirm the identity of the fungal ITS sequences. Evidently, the taxonomic reliability of the ITS sequences in a given database can affect the results of the study (Kõljalg et al., 2013). We confirmed the identity of all compost clones by comparing identification results obtained from two different databases, Unite Fungal ITS trainset 07-04-2014 and Warcup Fungal ITS trainset 2 located on the RDP web site. All clones were found to belong to the Ascomycota species *T. lanuginosus*.

Another way to increase the resolution and accuracy of fungal community composition is by using next generation (Next Gen) sequencing of fungal ITS sequences amplified from soils. Next Gen analysis applied to ITS sequences recovered from environmental samples showed an even higher coverage and diversity than cloning and Sanger sequencing (Buee et al., 2009). Thousands of ITS sequences per sample were generated with a higher resolution of community structure and diversity. A recent global survey of fungal communities demonstrated the high diversity of fungi phyla distributed across continents and environmental factors (Tedersoo et al., 2014). The largest fungal community study analyzed 1.4 million ITS sequences from 365 sites worldwide. Several unknown phyla were discovered with unique sequences demonstrating 97% similarities between unculturable fungi. The Ascomycota and Basidiomycota represented the two major phyla and classes of fungi worldwide. For comparison purposes, future studies will use Next Gen sequencing analysis to determine the fungi communities in the same compost, garden, and forest soil samples.

In conclusion, we were able to describe the fungal communities present in soils and compost samples. Fungal species belonging to the phylum Ascomycota were widely distributed in all three environments. A large number of uncultured fungal sequences were detected in garden and forest soil indicating the undiscovered fungal diversity present in environmental samples. Future identification of these unknown ITS sequences will provide a better understanding of the diversity and structure of fungal communities in soils.

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