REVISED

Title: Comparison of pectin-degrading fungal communities in temperate forests using glycosyl hydrolase family 28 pectinase primers targeting Ascomycete fungi.

Authors: Matthew D Gacura^{a*}, Daniel D Sprockett^{a,b}, Bess Heidenreich^a, and Christopher B Blackwood^a

a. Department of Biological Sciences, Kent State University, Kent OH 44242

b. Present Address: Department of Microbiology & Immunology, Stanford University, Stanford CA 94305

* Corresponding Author email: mgacura@kent.edu

*Corresponding Author phone number: 330-672-8371

Abstract

Fungi have developed a wide assortment of enzymes to break down pectin, a prevalent polymer in plant cell walls that is important in plant defense and structure. One enzyme family used to degrade pectin is the glycosyl hydrolase family 28 (GH28). In this study we developed primers for the amplification of GH28 coding genes from a database of 293 GH28 sequences from 40 fungal genomes. The primers were used to successfully amplify GH28 pectinases from all Ascomycota cultures tested, but only three out of seven Basidiomycota cultures. In addition, we further tested the primers in PCRs on metagenomic DNA extracted from senesced tree leaves from different forest ecosystems, followed by cloning and sequencing. Taxonomic specificity for Ascomycota GH28 genes was tested by comparing GH28 composition in leaves to internal transcribed spacer (ITS) amplicon composition using pyrosequencing. All sequences obtained from GH28 primers were classified as Ascomycota; in contrast, ITS sequences indicated that fungal communities were up to 39% Basidiomycetes. Analysis of leaf samples indicated that both forest stand and ecosystem type were important in structuring fungal communities. However, site played the prominent role in explaining GH28 composition, whereas ecosystem type was more important for ITS composition, indicating possible genetic drift between populations of fungi. Overall, these primers will have utility in understanding relationships between fungal community composition and ecosystem processes, as well as detection of potentially pathogenic Ascomycetes.

Keywords

fungi, PCR primers, forest litter, environmental samples, functional gene, endopolygalacturonase

36

37 **Introduction**

38 Pectin is a complex polysaccharide comprising as much as 35% of plant primary cell
39 walls (Voragen et al. 2009). It is a polymer of up to 17 monosaccharides, but is commonly
40 defined by a backbone of 1,4 linked α -D-galactosyluronic acid monomers (Mohnen 2008). Due
41 to its prevalence and structural diversity, microorganisms have evolved many extracellular
42 enzymes to aid in pectin decomposition (Prade et al. 1999, A. Manucharova 2009 and Glass et al.
43 2013). The gene family glycosyl hydrolase 28 (GH28) codes for a diverse array of hydrolytic
44 enzymes involved in pectin depolymerization, including polygalacturonases,
45 rhamnogalacturonases and xylogalacturonases (Abbott and Boraston 2007 and Markovic and
46 Janecek 2001). Because of its role in decomposition and plant pathogenicity, fungal GH28 gene
47 abundance and diversity is of particular importance in ecosystem functioning (Kjoller and
48 Struwe 2002, Herron et al. 2000 and Reignault et al. 2008). However, effective assays for
49 examining the distribution of GH28 genes in natural communities have not been developed.

50 Development of primers targeting functional genes with key biogeochemical roles
51 provides a unique opportunity to reveal linkages between microbial communities and ecosystem
52 processes (Zak et al. 2006, Peay et al. 2008). For example, PCR based assays have been used to
53 show that gene diversity and abundance are linked to nitrogen fixation, nitrification, and
54 denitrification (Levy-Booth et al. 2014). Primers have also been developed for the analysis of
55 several extracellular enzymes involved in decomposition, such as laccases, cellulases, and
56 chitinases (Williamson et al. 2000, Luis et al. 2004, Edwards et al. 2008 and Takaya et al. 1998).
57 Subsequently, functional gene primers have been used to study how environmental perturbations

such as nitrogen deposition (Hofmockel et al. 2007), agricultural management (Fan et al. 2012), and hydrocarbon contamination (Cebren et al. 2015) influence ecosystem processes through altering distributions of microbial functional genes. Because of the important role played by the distribution of functional genes in constraining ecosystem processes, it is critical to continue development of new primers that can be used in a community context.

In addition to contributing to our understanding of decomposition responses to environmental factors through microbial communities, a pectinase PCR assay may also reveal important insights into the distribution of plant pathogens. Glycosyl hydrolases, including members of the GH28 family, are important virulence factors in plant pathogens (Zhao et al. 2013). The number of GH28 gene homologues in fungal genomes has been found to be reflective of species ecological niches, with biotrophic pathogens having fewer GH28 genes than necrotrophic pathogens or saprotrophic fungi (Sprockett et al. 2011). Pectinase gene distribution, including GH28, can also vary among genomes based upon plant host, with increased pectinase gene numbers in organisms that attack hosts and tissues enriched in pectin (de Wit et al. 2012, King et al. 2011).

The purpose of this project was to develop fungi-specific PCR primers for GH28 genes. We focus on a single clade within GH28 specific for endopolygalacturonases (clade F *sensu* Sprockett et al. 2011). Clade F genes are found in a wide array of dikarya fungi, including many types of plant pathogens and saprotrophs, and are closely related to GH28 genes found in pathogenic *Phytophthora* species (Sprockett et al. 2011). After successful testing using cultured organisms, we further tested the primers by examining GH28 diversity in a limited number of leaf samples, allowing us to obtain a preliminary understanding of the role that environmental

factors have on pectinase gene distribution. We tested the hypotheses that ecosystem type or leaf type influenced GH28 gene community composition and diversity. Finally, pyrosequencing of fungal internal transcribed spacer region (ITS) genes was performed on the leaf samples to determine the taxonomic specificity of the selected primers.

Materials and Methods

Primer Development

Primer design was conducted using a database of 293 fungal GH28 DNA sequences from 40 genome sequences (Sprockett et al. 2011). We focused primer design on GH28 phylogenetic clade F because of a high level of sequence conservation and good representation of major fungal lineages (Sprockett et al. 2011). Primers matching the maximum possible number of sequences were designed to have as few degeneracies as possible, avoid self-priming and primer-dimers, and other appropriate physiochemical properties (e.g., annealing temperatures) (Table 1).

Primer Testing on Isolates

Initial primer testing was performed on *Aspergillus niger* strain NRRL 3 and *Phanerochaete chrysosporium* strain NRRL 6361 (ARSEF Culture Collection, Ithaca, NY). Genomic DNA was extracted from fungal isolates using a modified CTAB method (Griffiths et al. 2004). Samples were frozen in liquid nitrogen, ground and then treated with CTAB buffer and β -mercapthoethanol. This was followed by removal of co-extractants with chloroform and DNA precipitation using isopropanol.

Four different primer pair combinations were tested in PCR reactions. Primers were ordered from Integrated DNA Technologies (IDT; Coralville, IA). PCR was performed under a

variety of conditions to determine an optimal protocol for use in all subsequent tests. Reagent concentrations in the optimal PCR protocol were 0.025 U/μl DNA Taq DNA polymerase (B-Bridge, Santa Clara, CA, USA), 1× standard buffer (B-Bridge), 1.5 mM MgCl₂, 0.16 mM each deoxynucleotide triphosphate, 0.1 μg/μl bovine serum albumin (New England BioLabs, Ipswich, MA), 0.1 μM of each of the forward and reverse primers, and 0.3 ng/ul of template genomic DNA. Optimal PCR conditions were: 95°C denaturation for 3 min, followed by 35 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 1 min 30 s, and final extension at 72°C for 7 min. PCR products were checked on a 1.5% agarose gel stained with ethidium bromide.

PCR products from *A. niger* NRRL3 and *P. chrysosporium* NRRL 6361 were purified using an UltraClean PCR Cleanup Kit (MoBio Laboratories, Carlsbad, CA), quantified using Picogreen fluorescent DNA stain (Life Technologies, Eugene, OR, USA), and ligated into the pGEM-T easy vector (Promega, Madison, WI) following instructions from the manufacturers. Plasmids were then used to transform *Escherichia coli* JM109. PCR was performed on colonies using M13 primers to amplify cloned inserts. Clones with different amplicon insert sizes were grown overnight in 4 ml LB broth with ampicillin and glycerol, and plasmids were isolated using an UltraClean Plasmid Prep Kit (MoBio Laboratories). Sanger sequencing was performed at Ohio University Genomics Facility.

Following these initial tests, one primer pair was selected for further analysis: GH28F-1786F (5'-TRB TGG GAY GGH NWR GG-3') and GH28F-2089R (5'-GCV ABR CAR TCR TCY TGR TT-3') (Table 1). Amplification and analysis was then performed on genomic DNA extracted from additional well-characterized fungi shown in Table 2. The one or two most

intense bands were gel-purified using an UltraClean GelSpin DNA Extraction kit (MoBio Laboratories) and sequenced at Ohio University.

Sequences from cultured organisms were trimmed for quality using the program Sequencher® version 5.4.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI, USA). The program BLAST (Altschul 1990) was used to find the closest matches in the NCBI nucleotide database. All hits with the best bit scores and e-values were examined to look for consistency in their functional and taxonomic annotation. Results were further confirmed by classifying sequences in the Protein family database (Pfam) (Bateman et al. 2004).

Testing Primers on Metagenomic DNA from Environmental Leaf Samples

Senesced leaf material was gathered from three forest stand replicates (sites) of each of two ecosystems in Manistee National Forest in Northwest Michigan: sugar maple/basswood (SMBW) and black oak/white oak (BOWO) (Zak et al. 1989). These ecosystems differ based upon their dominant overstory tree coverage, topography and soil characteristics. Sites were separated by varying distances, with a maximum distance of 56 km. At each site, leaf samples were collected for each of the two dominant tree species (e.g., black oak and white oak from a BOWO site). Leaves were freeze dried, homogenized in a Genogrinder 2000 (SPEX Certiprep; Metuchen, NJ, USA) for two minutes at 1000 beats per minute, and then stored at -80°C. Genomic DNA was extracted from the microbial community of each leaf using the CTAB DNA extraction following Wu et al. (2011). PCRs were performed on metagenomic DNA and cloned into *E. coli* JM109 as described above. Inserts were sequenced at the University of Kentucky Advanced Genetic Technologies Center. Sequences were processed and taxonomically and functionally identified as described above. Sequences were then clustered into operational

taxonomic units (GH28-OTUs) with cut offs from 70% to 90%-similarity using the software CD-Hit (Huang et al. 2010). Non-GH28 clusters (generated from non-specific amplification) were removed from the data set before further community analysis. No differences in results were observed with different cut offs, so only results using the 90% cut off are reported here.

Pyrosequencing Fungal ITS Region from Leaf Samples

To determine phylogenetic composition of fungal communities on leaves used for GH28 primer testing, the fungal ITS region was amplified using barcode-labeled primers ITS1F and ITS4, as described in Chattopadhyay et al. (2014). Amplicons were gel purified using the UltraClean GelSpin DNA Extraction kit, followed by Agencourt Ampure XP (Beckman Coulter, Brea, CA, USA) purification. Afterwards, DNA concentrations were quantified using the PicoGreen dsDNA Assay Kit. Samples were sequenced in a GS Junior 454 Pyrosequencer (Roche) as in Chattopadhyay et al. (2014). The program QIIME (Caporaso et al. 2010) was used for demultiplexing, denoising and clustering into 97% similarity ITS-OTUs. Phylogenetic identification was performed using BLAST of sequences representing each OTU against the databases Unite and Genbank. BLAST results were then parsed using the last common ancestor algorithm using the software MEtaGenome ANalyzer (MEGAN) 5.0 (Huson and Mitra 2012). Unite (Kõjalg et al. 2005) and Genbank results were then consolidated by comparing the taxonomic strings output from MEGAN. We used the finest taxonomic assignment (down to genus) that did not result in higher level disagreement between databases. In cases of disagreements, we used the assignment from the curated UNITE database.

Statistical Analysis of Leaf Fungal GH28 and ITS Composition

Statistical analyses were performed in the R statistical analysis program (R core team 2014) using the vegan package (Oksanen et al. 2013). OTU frequencies were rarefied to equal sampling depth, converted to relative abundance, and Hellinger transformed (Legendre and Gallagher 2001) before analysis. Rarefaction and analyses were repeated 10 times to account for variation among simulations of even sampling effort. Redundancy analysis (RDA) (Legendre and Alexander 1999) was utilized to test for effects of ecosystem, site, and leaf type. Adjusted R-squared values (Peres-Neto et al. 2006) were obtained from each RDA and interpreted as variation explained by each factor.

Sequence Availability

Sequences obtained from GH28 primers have been submitted to GenBank under accession numbers KU664125 - KU664184.

Results

Primer Testing on Isolates

Four conserved regions of GH28-clade F were chosen as potential primer sites based on different physicochemical properties. Amplification with primers GH28F-1786f/GH28F-2089r resulted in the most consistent and distinct visible product by gel electrophoresis. These primers were selected for testing on a collection of fungal cultures from a variety of different taxa (Table 2). Clear bands were produced by all of the isolates except *Rhodotorula graminis*. Amplification from six Ascomycota isolates resulted in sequences that matched endopolygalacturonases in Genbank and Pfam databases (Table 2). However, inconsistent results were found in Basidiomycota, with GH28 sequences obtained from three of seven isolates (five of nine bands

sequenced). GH28 sequences were variable in length (~300-500bp) due to the presence of introns.

Pectinase gene diversity found on decomposing leaves

A total of 901 clones were sequenced from GH28 PCRs of 12 leaves. These were clustered at 90% similarity into 456 GH28-OTUs. After deletion of singleton OTUs, it was found that 77% of the sequences matched fungal endopolygalacturonases (GH28 clade F sequences) in NCBI. The majority (61%) of GH28 sequences were located in the twelve largest GH28-OTUs (Table 3). Representative sequences from these GH28-OTUs matched endopolygalacturonases found in a variety of fungal taxa, representing saprotrophs and major plant pathogens. All GH28 sequences obtained from leaves had best matches to database GH28 genes obtained from Ascomycota (Table 3).

Several leaves were dominated by very few GH28-OTUs; for example, OTU 1 made up 60% of the clone library from sample BO-U and OTU 3 made up 40% of the clone library for sample BW-6. However OTU richness, Shannon diversity and Simpson diversity were not significantly impacted

by ecosystem or leaf type. Some GH28-OTUs were found only in single ecosystems or sites

Figure 1. RDA ordination of GH28 endopolygalacturonase OTUs. Each point indicates an individual leaf. Ecosystem is indicated by color (yellow=SMBW, black=BOWO). Symbols are different for each forest stand. Leaves from the same forest stand are connected by a line.

(Table 3). This is reflected in results of RDAs performed on GH28-OTU relative abundances, with OTU composition significantly affected by both ecosystem (Adj. R-square= 0.075, P -value= 0.023) and site (Adj. R-square=0.142, P -value= 0.035). The larger role of site in structuring GH28-OTU composition, instead of ecosystem, can be seen in the RDA ordination in Figure 1. Other factors such as leaf species, leaf size, and location in the litter layer were found to be non-significant.

Taxonomic Structure of Fungal

Communities

Pyrosequencing was performed to determine if leaf communities were indeed dominated by Ascomycota, as indicated by GH28 clone libraries, or if primers were biased against Basidiomycota. A total of 56,134 fungal ITS sequences were obtained from the leaf samples described above. These sequences were clustered, with a 97% cut off, into 251 ITS-OTUs.

For individual leaves, Basidiomycota abundance varied from 0% to 39% of ITS sequences. Communities were dominated by Ascomycota, with an average relative abundance of 90% Ascomycota sequences in BOWO leaves and 98% in SMBW leaves. Redundancy analysis on ITS-OTU profiles indicated that ecosystem was the most important factor explaining variation in

Figure 2. RDA ordination of ITS-OTUs (taxonomic community composition). Each point indicates an individual leaf. Ecosystem is indicated by color (yellow=SMBW, black=BOWO). Symbols are different for each forest stand. Leaves from the same forest stand are connected by a line.

232 fungal communities (Adj. R-Square= 0.167, *P*-value= 0.011), with site not having a significant
233 effect (Adj. R-square= 0.08, *P*-value= 0.101). Ecosystem and site together explained more
234 variation in taxonomic community composition than in GH28-OTU distribution (Figure 2). The
235 ordination plot indicates a very strong separation of communities between ecosystem types
236 (Figure 2). Fungal communities in BOWO ecosystems were grouped more closely together in the
237 RDA plot than the SMBW communities. As in GH28 OTUs, other factors such as leaf species,
238 leaf size, and location in the litter layer were found to be non-significant in ITS sequences.

240 **Discussion**

241 This study, to our knowledge, is the first report of general primers for fungal pectinase
242 genes, providing a valuable tool to link community composition to decomposition and plant
243 pathogenicity. Testing of pure culture organisms confirmed that these primers will amplify
244 GH28 clade F genes from several different taxonomic and functional groups of fungi, but are
245 particularly useful for Ascomycete fungi. Also, the primers amplified Ascomycete GH28 genes
246 from environmental samples, which will be useful for determining major environmental factors
247 that drive GH28 gene distribution.

248 On senesced leaves there is a rich community of potentially pathogenic and saprotrophic
249 fungi (Gessner et al. 2010 and Hattenschweiler 2011). Using our GH28 clade F primers, we
250 detected endopolygalacturonases from Ascomycota fungi on all leaves tested. Although we were
251 able to successfully quantify GH28 composition and diversity, fine taxonomic assignments (e.g.,
252 genus-level) are likely unreliable because sequence matches generally had low confidence values
253 and fungal GHs in Genbank are not representative of the fungal diversity in leaves. Novel fungi

may also be present, as fungal diversity is high in temperate ecosystems (Schmit and Mueller et al. 2007) and many ITS sequences could not be identified past the order level.

The dominance of Ascomycota sequences in leaf samples was unexpected because Basidiomycota are thought to be common decomposers of plant tissue in forests (Heilmann-Clausen et al. 2001, Osono 2007 and Voriskova and Baldrian 2013). However, analyses of ITS sequences as well as GH28 sequences clearly indicated fungal communities on leaves from our sites were dominated by Ascomycota. Ascomycota fungi include diverse, highly prevalent saprotrophic, pathogenic, and endophytic organisms, and it is thus perhaps not surprising that they dominate communities on senesced leaves (Schoch et al. 2009). Leaves that came from tree species with more lignified tissue (i.e. oaks) did have a higher percentage of Basidiomycota (up to 39% of sequences), as found previously in forests dominated by oak species (Blackwood et al. 2007). The absence of Basidiomycete GH28 sequences even from oak leaves confirms the primer bias for Ascomycota GH28 genes.

There is a high degree of variation in dominant tree species and soil properties between different ecosystem types in Manistee National Forest (Host et al. 1988). This environmental variation appears to have had a strong influence on the structure of saprotrophic fungal communities as indicated by ITS sequences. This could be the result of differences in plant tissue chemistry between the two ecosystems, which has been shown to play a large role in structuring fungal communities (Schneider et al. 2012 and Hanson et al. 2008). In contrast, GH28 sequences were mainly influenced by site-to-site heterogeneity, whereas ecosystem type played a negligible role. The high level of taxonomic similarity but lower level of functional gene similarity among sites of the same ecosystem type is unexpected under the dominant paradigm that microbial

communities harbor a high level of functional redundancy (Wardle et al. 2006 and Nielson et al. 2011). One explanation is that genetic drift between sub-populations of fungi in separate forest stands could cause divergence in GH28 composition. Necrotrophic plant pathogens have a high degree of genomic plasticity for genes coding for virulence factors as a result of transposable elements, translocation and duplication events, and mutations arising from compensatory variation in host defense genes (Raffaele and Kamoun 2012). Sexual reproduction within a species will also result in new combinations of GH28 alleles, after which new GH28 combinations can become prevalent in a site through asexual reproduction (McDonald and Linde 2002). Differences between GH28 and ITS datasets may also have arisen from different numbers of sequences generated by different sequencing methods (cloning and Sanger sequencing for GH28, pyrosequencing for ITS). It is possible that differences in GH28 composition between ecosystems could have been masked by increased error due to smaller number of sequences.

Primers developed here will be suitable for amplification of GH28 genes in Ascomycota fungi, but not Basidiomycota. The primers should be useful because Ascomycota appear to dominate decomposer communities in leaf tissue, and many of the most prevalent plant pathogens are Ascomycota that depend on GH28 genes (Klosterman et al. 2011). GH28 genes are particularly prevalent in necrotrophic plant pathogen genomes (Floudas et al. 2012, Sprockett et al. 2011) and are important virulence factors in necrotrophic plant pathogens (van Kan 2006, Hematy et al. 2009 and Zhao et al. 2013). Our results indicate that these primers will be most useful when used in sequencing based assays because non-GH28 sequences can be removed from datasets before further analysis. Direct sequencing methods have been used for rapid screening of fungal isolates for functional genes (Lee et al. 2001 and Pointing et al. 2005) and for

detecting the presence of pathogens in plant hosts (McCartney et al. 2003). Hence, these primers should be a valuable new molecular tool to complement other methods for both detection of pathogens and better understanding the roles that fungi play in ecosystem processes.

Acknowledgements

This research was supported by grants from the US National Science Foundation (DEB-0918240 and DEB-0918878) and US Department of Energy (DE-SC000433).

References

- Abbott D.W. and Boraston A.B. 2007. The Structural Basis for Exopolygalacturonase Activity in a Family 28 Glycoside Hydrolase. *Journal of Molecular Biology*, Vol. **368**:1215-1222.
- Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, Vol. **215**:403-410.
- Bateman A., Coin L., Durbin R., Finn R.D., Hollich V., Griffiths-Jones S., Khanna A., Marshall M., Moxon S., Sonnhammer E.L.L., Studholme D.J., Yeats C. and Eddy S.R. 2004. The Pfam protein families database. *Nucleic Acids Research*, Vol. **32**:138-141.
- Blackwood C.B., Waldrop M.P., Zak D.R. and Sinsabaugh R.L. 2007. Molecular analysis of fungal communities and laccase genes in decomposing litter reveal differences among forest types but no impact of nitrogen deposition. *Environmental Microbiology*, Vol. **9**:1306-1316.
- Caporaso J.G., Kuczynski J., Stombaugh J., Bittinger K., Bushman F.D., Costello E.K., Fierer N., Peña, A.G., Goodrich J.K., Gordon J.I., Huttley G.A., Kelley S.T., Knights D., Koenig J.E., Ley R.E., Lozupone C.A., McDonald D., Muegge B.D., Pirrung M., Reeder J., Sevinsky J.R., Turnbaugh P.J., Walters W.A., Widmann J., Yatsunenko T., Zaneveld J. & Knight R.

2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, Vol. 7: 335-336.

Cebren A., Beguiristain T., Bongoua-Devisme J., Denonfoux J., Faure P., Lorgeoux C., Ouvrard S., Parisot N., Peyret P. and Leyval C. 2015. Impact of clay mineral, wood sawdust or root organic matter on the bacterial and fungal community structures in two aged PAH-contaminated soils. *Environmental Science and Pollution Research*: 1-15

Chattopadhyay, Suhana. 2014. Ecosystem Controls on Soil Microbial Guilds: dynamics and carbon sequestration. Electronic Thesis or Dissertation. Kent State University. *OhioLINK Electronic Theses and Dissertations Center*. 03 Jul 2015.

de Wit P.J.G.M., van der Burgt A., Okmen B., Stergiopoulos I., Abd-Elsalam K.A., et al. 2012. The Genomes of the Fungal Plant Pathogens *Cladosporium fulvum* and *Dothistroma septosporum* Reveal Adaptation to Different Hosts and Lifestyles But Also Signatures of Common Ancestry. *PLoS Genet* 8(11): e1003088. doi:10.1371/journal.pgen.1003088

Edwards I.P., Upchurch R.A. and Zak D.R. 2008. Isolation of Fungal Cellobiohydrolase I Genes from Sporocarps and Forest Soils by PCR. *Applied Environmental Microbiology*, Vol. 74: 3481-3489.

extraction from soil. In F.J. de Bruijn, I.M. Head, A.D.L. Akkermans, and J.D. van Elsas, eds. *Molecular Microbial Ecology Manual*. Norwell, MA: Kluwer Academic Publishers.

Fan F., Li Z., Wakelin S.A., Yu W. and Liang Y. 2012. Mineral fertilizer alters cellulolytic community structure and suppresses soil cellobiohydrolase activity in long-term fertilization experiment. *Soil Biology & Biochemistry*, Vol. 55:70-77

342 Floudas D., Binder M., Riley R., Barry K., Blanchette R.A., Henrissat B., Martinez A.T. Otilar
 343 R., Spatafora J.W., Yadac J.S., Aerts A., Benoit I., Boyd A., Carlson A., Copeland A.,
 344 Coutinho P.M., et al. 2012. The Paleozoic Origin of Enzymatic Lignin Decomposition
 345 Reconstructed from 31 Fungal Genomes. *Science*, Vol. **336**:1715-1719.
 346 Gessner M.O., Swan C.M., Dang C.K., Mckie B.G., Bardgett R.D., Wall D.H. and
 347 Hattenschwiler S. 2010. Diversity meets decomposition. *Trends in Ecology and Evolution*,
 348 Vol. **25**:372-380.
 349 Glass N.L., Schmoll M., Cate J.H.D. and Coradetti S. 2013. Plant Cell Wall Deconstruction by
 350 Ascomycete Fungi. *Annual Reviews in Microbiology*, Vol. **67**:477-498
 351 Griffiths, R.I., Manefield M., Whiteley A.S. and Bailey M.J. (2004) DNA and RNA
 352 Hanson C.A., Allison S.D., Bradford M.A., Wallenstein M.D. and Treseder K.K. 2008. Fungal
 353 Taxa Target Different Carbon Sources in Forest Soil. *Ecosystems*, Vol. **11**:1157-1167.
 354 Hattenschweiler S., Fromin N. and Barantal S. 2011. Functional diversity of terrestrial
 355 microbial decomposers and their substrates. *Comptes Rendus Biologies*, Vol. **334**:393-402.
 356 Heilmann-Clausen J. 2001. A gradient analysis of communities of macrofungi and slime moulds
 357 on decaying beech logs. *Mycological Research*, Vol. **105**:575-596
 358 Hematy K., Cherk C. and Somerville S. 2008. Host-pathogen warfare at the plant cell wall.
 359 *Current Opinion in Plant Biology*, Vol. **12**:406-413.
 360 Herron, S.R., Benen, J.A.E., Scavetta, R.D., Visser, J., Jurnak, F., 2000. Structure and function
 361 of pectic enzymes: virulence factors of plant pathogens. *Proceeding of the National Academy*
 362 *of Science*, Vol. **97**: 8762–8769.

363 Hofmockel K.S. Zak D.R. and Blackwood C.B. 2007. Does atmospheric N deposition alter the
 364 abundance and activity of ligninolytic fungi in forest soils? *Ecosystems*, Vol. **10**:1278-1286
 365 Host G.E., Pregitzer K.S., Ramm C.W., Lusch D.T. and Cleland D.T. 1988. Variation in
 366 overstory biomass among glacial landforms and ecological land units in northwestern Lower
 367 Michigan. *Canadian Journal of Forest Research*, Vol. **18**:659-668.
 368 Huang Y., Niu B., Gao Y., Fu L. and Li W. 2010. CD-HIT Suite: a web server for clustering and
 369 comparing biological sequencing. *Bioinformatics*, Vol. **26**:680-682
 370 Huson D.H. et al. 2012. Integrative analysis of environmental sequences using MEGAN 4.
 371 *Genome Research*, Vol. **21**: 1552-1560.
 372 King B.C., Waxman K.D., Nenni N.V. Walker L.P., Bergstrom G.C. and Gibson D.M. 2011.
 373 Arsenal of plant cell wall degrading enzymes reflects host preference among plant
 374 pathogenic fungi. *Biotechnology for Biofuels*, Vol. **4**:1-14.
 375 Kjølner, A.H., Struwe, S., 2002. Fungal communities, succession, enzymes, and decomposition.
 376 In: Burns, R.G., Dick, R.P. (Eds.), *Enzymes in the Environment: Activity, Ecology, and*
 377 *Applications*. Marcel Dekker, New York, pp. 267–284.
 378 Klosterman S.J., Subbarao K.V., Kang S., Veronese P., Gold S.E., Thomma B.P.H.J., Chen Z.,
 379 Henrissat B., Lee Y., Park J., Garcia-Pedrajas M.D., Barbara D.J., Anchieta A., et al. 2011.
 380 Comparative Genomics Yields Insights into Niche Adaptation of Plant Vascular Wilt
 381 Pathogens. *PLOS Pathogens*, Vol. **7**:1-19.
 382 Kõjalg U, Larsson K-H, Abarenkov K, Nilsson RH, Alexander IJ, Eberhardt U, Erland S,
 383 Høiland K, Kjølner R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo L, Vrålstad T,

384 Ursing BM. 2005. UNITE: a database providing web-based methods for the molecular
 385 identification of ectomycorrhizal fungi. *New Phytologist*, Vol. **166**: 1063-1068
 386 Lee T., Oh D., Kim H., Lee J., Kim Y., Yun S. and Lee Y. 2001. Identification of
 387 Deoxynivalenol- and Nivalenol-Producing Chemotypes of *Gibberella zeae* by Using PCR.
 388 *Applied and Environmental Microbiology*, Vol. **67**:2966-2972.
 389 Legendre P. and Alexander M.J. 1999. Distance-Based Redundancy Analysis: Testing
 390 MultiSpecies Responses in Multifactorial Ecological Experiments. *Ecological Monographs*,
 391 Vol. **69**:1-24.
 392 Legendre P. and Gallagher E.D. 2001. Ecologically meaningful transformations for ordination of
 393 species data. *Oecologia*, Vol. **129**:271-280
 394 Levy-Booth D.J., Prescott C.E. and Grayston S.J. 2014. Microbial functional genes involved in
 395 nitrogen fixation, nitrification and denitrification in forest ecosystems. *Soil Biology and*
 396 *Biochemistry*, Vol. **75**:11-25.
 397 Luis P. Walther, G., Kellner H., Martin F. and Buscot F. 2004. Diversity of laccase genes from
 398 basidiomycetes in a forest soil. *Soil Biology and Biochemistry*, Vol. **36**:1025-1036.
 399 Manucharova N.A. 2009. The Microbial Destruction of Chitin, Pectin and Cellulose in Soils.
 400 *Eurasian Soil Science*, Vol. **42**: 1526-1532
 401 Markovič, O. and Janeček, Š., 2001. Pectin degrading glycoside hydrolases of family 28:
 402 sequence-structural features, specificities and evolution. *Protein Engineering*: **14**:615–631.
 403 McCartney H.A., Foster S.J., Fraaije B.A. and Ward E. 2003. Molecular diagnostics for fungal
 404 plant pathogens. *Pest Management Science*, Vol. **59**:129-142.

405 McDonald B.A. and Linde C. 2002. Pathogen Population Genetics, Evolutionary Potential, and
 406 Durable Resistance. *Annual Reviews in Phytopathology*, Vol. **40**:349-379.
 407 Mohnen D. 2008. Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, Vol.
 408 **11**:266-277.
 409 Nielson U.N., Ayres E., Wall D.H. and Bardgett R.D. 2011. Soil biodiversity and carbon cycling:
 410 a review and synthesis of studies examining diversity-function relationships. *European*
 411 *Journal of Soil Science*, Vol. **62**:105-116.
 412 Oksanen J., Blanchet F.G., Kindt R., Legendre P., Minchin P.R., O'Hara R.B., Simpson G.L.,
 413 Solymos P., Henry M., Stevens H. and Wagner H. 2015. Vegan: Community Ecology
 414 Package. R package version 2.3-0.
 415 Osono T. 2007. Ecology of ligninolytic fungi associated with leaf litter decomposition.
 416 *Ecological Research*, Vol. **22**:955-974.
 417 Peay K.G., Kennedy P.G. and Bruns T.D. 2008. Fungal Community Ecology: A Hybrid Beast
 418 with a Molecular Master. *21st Century Directions in Biology*, Vol. **58**: 799-810.
 419 pectinolytic enzymes in plant invasion, host adaptability and symptom type. *Eur J Plant Pathol*
 420 Vol. **120**: 1-11.
 421 Peres-Neto P.R., Legendre P., Dray S. and Borcard D. 2006. Variation Partitioning of Species
 422 Data Matrices: Estimation and Comparison of Fractions. *Ecology*, Vol. **87**:2614-2625.
 423 Pointing S.B., Pelling A.L., Smith G.J.D., Hyde K.D. and Reddy C.A. 2005. Screening of
 424 basidiomycetes and xylariaceous fungi for lignin peroxidase and laccase gene-specific
 425 sequences. *Mycological Research*, Vol. **109**:115-124.

426 Prade R.A., Zhan D., Ayoubi P. and Mort A.J. 1999 Pectins, Pectinases and Plant-Microbe
 427 Interactions. *Biotechnology and Genetic Engineering Reviews*, Vol. **16**:361-392

428 R core team. 2014. R: A language and environment for statistical computing. R foundation for
 429 Statistical Computing, Vienna Austria. URL <http://www.R-project.org/>.

430 Raffaele, S., & Kamoun, S. (2012). Genome evolution in filamentous plant pathogens: why
 431 bigger can be better. *Nature Reviews Microbiology*, **10**:417-430.

432 Reignault, P., Valette-Collet O. and Boccara M. 2008. The importance of fungal
 433 Schmit J.P. and Mueller G.M. 2007. An estimate of the lower limit of global fungal diversity.
 434 *Biodiversity & Conservation*, Vol. **16**:99-111.

435 Schneider T., Keilblinger K.M. Schmid E., Sterflinger-Gleixner K., Ellersdorfer G., Roschitzki
 436 B., Richter A., Elberl L., Zechmeister-Boltenstern S. and Riedel K. 2012. Who is who in
 437 litter decomposition? Metaproteomics reveals major microbial players and their
 438 biogeochemical functions. *The ISME Journal*, Vol. **6**:1749-1762.

439 Schoch, C. L., Sung, G. H., López-Giráldez, F., Townsend, J. P., Miadlikowska, J., Hofstetter,
 440 V., ... & Sugiyama, J. (2009). The Ascomycota tree of life: a phylum-wide phylogeny
 441 clarifies the origin and evolution of fundamental reproductive and ecological
 442 traits. *Systematic biology*, Vol. **58**:224-239.

443 Sprockett D.D., Piontkivska H. and Blackwood C.B. 2011. Evolutionary analysis of glycosyl
 444 hydrolase family 28 (GH28) suggests lineage-specific expansions in necrotrophic fungal
 445 pathogens. *Gene*, Vol. **479**: 29-36.

446 Sprockett, Daniel. 2009. The Evolution of Fungal Pectinases in Glycosyl Hydrolase Family 28
 447 and Their Association with Ecological Strategy. Electronic Thesis or Dissertation. Kent State
 448 University. *OhioLINK Electronic Theses and Dissertations Center*. 03 Jul 2015.

449 Takaya N., Yamazaki D., Horiuchi H., Ohta A. and Takagi M. 1998. Cloning and
 450 Characterization of Chitinase-encoding Gene (*chiA*) from *Aspergillus nidulans*, Disruption of
 451 Which Decreases Germination Frequency and Hyphal Growth. *Bioscience, Biotechnology*
 452 *and Biochemistry*, Vol. **62**: 60-65.

453 van Kan J.A.L. 2006. Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in*
 454 *Plant Science*, Vol. **11**:247-253.

455 Voragen A.G.J., Coenen G.J., Verhoef R.P. and Schols H.A. 2009. Pectin, a versatile
 456 polysaccharide present in plant cell walls. *Structural Chemistry*, Vol. **20**:263-275.

457 Voriskova J. and Baldrian P. 2013. Fungal community on decomposing leaf litter undergoes
 458 rapid successional changes. *The ISME Journal*, Vol. **7**:477-486.

459 Wardle D.A. 2006. The influence of biotic interactions on soil biodiversity. *Ecology Letters*,
 460 Vol. **9**:870-886.

461 Williamson N., Brian P. and Wellington E.M.H. 2000. Molecular detection of bacterial and
 462 streptomycete chitanases in the environment. *Antonie van Leeuwenhoek*, Vol. **78**: 315-321.

463 Wu L., Feinstein L.M., Valverde-Barrantes O., Kershner M.W., Leff L.G. and Blackwood D.B.
 464 2011 Placing the Effects of Leaf Litter Diversity on Saprotrophic Microorganisms in the
 465 Context of Leaf Type and Habitat. *Microbial Ecology*, Vol. **61**:399-409.

466 Zak D.R., C.B. Blackwood and M.P. Waldrop. 2006. A molecular dawn for biogeochemistry.
 467 *Trends in Ecology and Evolution*, Vol. **21**:288-295.

468 Zak D.R., G.E. Host and K.S. Pregitzer. 1989. Regional variability in nitrogen mineralization,
469 nitrification, and overstory biomass in northern Lower Michigan. *Canadian Journal of*
470 *Forest Research*, Vol. **19**:1521-1526.

471 Zhao Z., Liu H., Wang C. and Xu J. 2013. Comparative analysis of fungal genomes reveals
472 different plant cell wall degrading capacity in fungi. *BMC Genomics*, Vol. **14**:274.

473

474 **Table 1.** Primer regions and examples of conserved sequences from fungal genomes.
475

	GH28F-1786f region	GH28F-2089r	Genbank assembly accession number
Primers	TRBTGGGAYGGHNWRGG	AAYCARGAYGAYTGYVTBGC	
<i>Phanerochaete chrysosporium</i>	TACTGGGATGGCCAAGG	AACCAGGACGACTGCCTCGC	GCA_000167175.1
<i>Aspergillus niger</i>	TACTGGGACGGCGAGGG	AACCAGGACGACTGTGTTGC	AM270980 – AM270998
<i>A. niger</i>	TGGTGGGATGGAGAGGG	AACCAAGATGACTGCGTTGC	
<i>A. niger</i>	TGGTGGGATGGCGAGGG	AACCAAGATGACTGTGTGGC	
<i>A. niger</i>	TGGTGGGATGGCAAGGG	AACCAGGATGACTGTCTTGC	
<i>A. niger</i>	TGGTGGGACGGTGAGGG	AACCAGGACGACTGCGTCGC	
<i>Sclerotinia sclerotiorum</i>	TATTGGAATGGGTATGG	AATCAGGATGACTGCGTAGC	GCA_000146945.1
<i>Pyrenophora tritici-repentis</i>	TGGTGGGATGGACTCGG	AACCAAGATGATTGCGTTGC	GCA_000149985.1
<i>Laccaria bicolor</i>	TATTGGGATGGTACGGG	AATCAAGACGATTGCCTTGC	GCA_000143565.1
<i>Botrytis cinerea</i>	TATTGGGATGGTCTTGG	AATCAGGATGATTGCGTCGC	GCA_000143535.1

476

477

478 **Table 2.** List of fungal isolates used to test GH28 clade F primers, and results from PCR reactions using selected primers.
 479

Species Name	Phylum ¹	Ecological Niche	GH28 Copy Number ²	Amplify	Band	Size (bp)	GH28 (NCBI) ³	GH28 Pfam ⁴
<i>Aspergillus niger</i> (ATCC1015 and FGSC A1513)	A	Pathogen	23	Yes	1	300	Yes	Yes
					2	350	Yes	Yes
<i>Cenococcum geophilum</i> (ATCC 52041)	A	Mycorrhizal	4	Yes	1	350	Yes	Yes
					2	450	Yes	Yes
<i>Neurospora crassa</i> (OR74A)	A	Saprotroph (not wood)	2	Yes	1	300	Yes	Yes
<i>Saccharomyces paradoxus</i> (NRRL 17217)	A	Saprotroph (not wood)	Not Available	Yes	1	350	Yes	Yes
<i>Talaromyces stipitatus</i> (ATCC 10500)	A	Saprotroph (not wood)	8	Yes	1	350	Yes	Yes
<i>Trichoderma reesei</i> (QM6a)	A	Saprotroph (not wood)	4	Yes	1	350	Yes	Yes
<i>Agaricus bisporus</i> (var. burnettii JB137-S8)	B	Saprotroph (not wood)	6	Yes	1	300	No	No
<i>Coprinopsis cinerea</i> (FGSC 9003)	B	White Rot Saprotroph	3	Yes	1	350	No	No
<i>Laccaria bicolor</i> (ATCC 4686)	B	Mycorrhizal	6	Yes	1	300	Yes	Yes
					2	500	No	No
<i>Mycena galopus</i> (ATCC 62051)	B	White Rot Saprotroph	Not Available	Yes	1	350	Yes	No

					2	500	Yes	Yes
<i>Phanerochaete chrysosporium</i> (RP-78)	B	White Rot Saprotroph	4	Yes	1	350	No	No
<i>Rhodotorula graminis</i> (FGSC 10291)	B	Saprotroph (not wood)	1	No	N/A	N/A	N/A	N/A
<i>Schizophyllum commune</i> (H4-8)	B	White Rot Saprotroph	3	Yes	1	350	Yes	Yes
					2	450	Yes	No

¹ A indicates Ascomycota and B indicates Basidiomycota.

² GH28 copy number indicates the number of genes present in an organism's genome (if data is currently available) (Sprockett et al. 2011 and Floudas et al. 2012).

³ Indicates whether sequences were successfully matched to a GH28 sequence in BLAST analysis of the NCBI database.

⁴ Indicates whether sequences were successfully matched to a GH28 sequence in the PFAM database.

486

487 **Table 3.** Taxonomy of closest matches in the NCBI Genbank database for the twelve GH28-OTUs with the greatest number of
 488 sequences (all closest matches were endopolygalacturonase sequences).

489

OTU #	Sequences	Ecosystem ¹	Leaf Species ²	Organism (Closest Match)	BLASTn Score	e-value	Genbank Accession
OTU1	48	BOWO and SMBW	SM and BO	<i>Cochliobolus carbonum</i>	62.6	0.000001	KU664125
OTU2	43	SMBW	SM and BW	<i>Aspergillus fumigatus</i>	62.6	0.000002	KU664126
OTU3	33	SMBW	SM and BW	<i>Colletotrichum lindemuthianum</i>	163	6.00E-37	KU664127
OTU4	28	BOWO	BO and WO	<i>Botryotinia calthae</i>	170	4.00E-39	KU664128
OTU5	19	BOWO	BO	<i>Colletotrichum lupine</i>	95.1	3.00E-16	KU664129
OTU6	16	BOWO and SMBW	SM, BO and WO	<i>Botryotinia fuckeliana</i>	167	5.00E-38	KU664130
OTU7	15	SMBW	SM and BW	<i>Aspergillus niger</i>	156	9.00E-35	KU664131
OTU8	14	SMBW	SM and BW	<i>Thielavia terrestris</i>	118	3.00E-23	KU664132
OTU9	13	BOWO	BO and WO	<i>Aspergillus oryzae</i>	176	1.00E-40	KU664133
OTU10	12	SMBW	SM and BW	<i>Pyrenophora teres</i>	57.2	0.00009	KU664151
OTU11	12	SMBW	SM and BW	<i>Botryotinia convolute</i>	210	5.00E-51	KU664145
OTU12	10	BOWO and SMBW	SM, BW, BO and WO	<i>Botryotinia fuckeliana</i>	192	1.00E-41	KU664165

490

491 ¹ BOWO indicates black oak/ white oak and SMBW indicates sugar maple/basswood forest stands

492 ² For leaf species SM indicates sugar maple, BW indicates basswood, BO indicates black oak

493