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Observation and identification of wood decay fungi from the heartwood of peach tree limbs in central Georgia, USA

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Abstract Peach tree health, longevity, and limb strength can be affected by wood decay fungi, but the extent of the colonization and their identities have not been characterized in Georgia, United States of America. In an incubation experiment, dense white fungal mycelium grew on the cross-cut faces of asymptomatic limb sections sampled from peach scion cultivars, but no mycelium grew on those from rootstock cultivars. Among the scion cultivars, a Chi-square analysis indicated significant differences in the incidence of colonization. A dissection of six broken peach tree scaffold limbs showed symptoms of colonization along the entire length of the limbs. Only 41 % of primary branches had symptoms of wood decay, but no secondary branches appeared to have symptoms. Next generation sequencing (NGS) and metagenomic analysis of a combined DNA sample revealed the contigs were aligned to sequences of *Trametes versicolor* and/or *Schizophyllum commune*, suggesting these two white-rot fungi were present in the sample tested. Sanger sequencing confirmed two fragments of the same size but distinct nucleotide peak intensities were amplified from the sample used for NGS by two pairs of primers, providing supporting evidence that the two fungi were in the bulk sample but might differ in abundance or incidence. Subsequent multiplexing polymerase chain reaction diagnostics of ten scion samples confirmed the dominance of *Trametes* spp. (all ten samples), although *Stereum* spp. (five samples), *Schizophyllum* spp. (three samples) and *Hericium* spp, (one sample) were sometimes found coexisting with *Trametes* spp. Future research needs on this peach-fungus association are discussed.

Keywords White rot · Limb breakage · Tree architecture · *Prunus persica*

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Introduction

Peach (*Prunus persica* [L.] Batsch) tree health and yield can be affected by many biotic and abiotic stresses, including various fungal pathogens that require management to avoid losses in quality and quantity of harvested fruit. Earlier observations and surveys in South Carolina indicated at least 39 species of wood-decay fungi colonizing peach, belonging to 30 genera, adding to a total of 67 wood-decaying fungi already reported on peach or nectarine in the United States and Canada (Petersen 1960, 1961; Adaskaveg and Ogawa 1990; Adaskaveg et al. 1993). Adaskaveg et al. (1993) found the most common white rot fungi in South Carolina were from the genera (in order of decreasing incidence) *Trametes*, *Stereum*, *Ganoderma*, *Laeticorticium*,

Armillaria, *and Oxyporus*, followed by brown rot fungal species, including *Antrodia* and *Fomitopsis*. These lignicolous fungi may be seen growing on limbs and trunks that are of different ages and/or health status. Some of these fungi, such as *Armillaria* spp., are pathogens directly associated with peach tree decline and tree death, whereas the role of many of the other fungal species, such as *Trametes*, *Ganoderma* spp. or *Stereum* spp., remain less well characterized in regard to their impact on peach tree health.

Wounds caused by pruning, and bark damage caused by other orchard operations on peach trees may allow for infestation by certain insects (Cottrell et al. 2008), and various fungi are reported to grow in these wounds (Doepel et al. 1979; Adaskaveg et al. 1993). Furthermore, prevailing weather appears to affect incidence of colonization of wounds (Doepel et al. 1979; Adaskaveg et al. 1993). A recent and preliminary observation found several wood decay fungi grew on cultivated peach but not on wild relatives (*P. angustifolia* Marshall or *P. serotina* Ehrh). The fungi were putatively identified as *Fomes (Fomitopsis) meliae*, *Trametes versicolor*, and/or *Myrothecium* spp., based on a database search of cloned internal transcribed spacer (ITS) sequences (Dr. Harald Scherm, unpublished data).

Once the wound is colonized, the fungus can establish itself within the heartwood, perhaps bypassing blocked cell lumina and tunneling through cell walls, or degrading the polyphenolic occlusions in the cell lumina (Schwarze and Baum 2000). Once established, these fungi pose an imminent threat to the limbs they have colonized and will likely affect the long-term health and survival of the tree, and have been implicated in tree decline and limb breakage (Alconero et al. 1978; Doepel et al. 1979; Cripps et al. 1983; Adaskaveg et al. 1993); furthermore, they are known to reduce the width of sapwood in colonized peach branches (Shigo and Wilson 1982). These fungi not only use the tree's resources, but the decay process weakens limbs and reduces their ability to bear load. Some basic questions remain unanswered. For example, to what extent are these lignicolous fungi growing inside asymptomatic peach branches, and what are the identities of the fungi growing deep within the heartwood of the colonized branches?

In this study, the incidence of fungal growth at the sterile crosscut surfaces of asymptomatic peach limbs was compared from several different cultivars of peach scion and rootstocks, and the extent of colonization was investigated in some limbs of older peach trees through dissection. Fungal DNA was isolated from a sample of the fungus growing from the heartwood for further identification using next-generation sequencing and metagenomic analysis, using Sanger sequencing and PCR diagnosis. Finally, some samples were subject to multiplex PCR diagnostics to further ascertain identity of some of the fungi causing wood decay in peach limbs in central GA.

Materials and methods

Evidence for wood decay fungi in limbs of peach scion and rootstock cultivars

To ascertain limb colonization among different cultivars of peach, both peach scion and rootstock cultivars were sampled in a cultivar block located at the USDA-ARS Southeastern Fruit and Tree Nut Research Laboratory in Peach County, GA (latitude +32° 39′ 54″ N, longitude + 83° 44′ 31″ W, elevation of ≈ 156 m, ≈ a 240 day freeze-free growing period and annual precipitation of ≈ 118 cm). The site has Faceville sandy loam soils [FoA; fine, Kaolintic, thermic Typic Kandiudult soil].

The scion cultivars included two samples from 'Heath Cling' (#1 and #2), and one sample each from 'Oldmixon Free', 'St. John', 'Elberta', 'Cresthaven', 'Fackler', 'Flameprince', 'Gross Mignone', 'Gross Indian', and 'Chinese Cling'; the rootstock cultivars included samples from 'MP-29', 'Guardian', 'Sharpe', 'Lovell', 'Nemared' and 'Nemaguard'. 'Lovell' is a scion cultivar used for processing as a drying peach, but due to seed availability it has become a widely used rootstock where root-knot nematode is not a problem (Okie and Reilly 1983). A sample of black cherry (*P. serotina*) was also included.

All scion cultivars were grafted on 'Guardian' rootstock and planted in 2005 in a randomized complete block design (with three trees per cultivar per block, with eight blocks of each cultivar). All rootstock trees were from two different plantings of different ages. The scion trees received standard pruning to develop and maintain scaffold limbs with an open canopy structure. The rootstock trees received little pruning or canopy management.

To ensure uniformity, one asymptomatic limb (no external evidence of pest/ pathogen or wood decay

damage) from a visually healthy tree in each block was selected for limb sampling. There were two experiments in 2013. All the scion cultivars were included in the 1st experiment, but only 'Fackler', 'Oldmixon Free', 'Flameprince', 'Elberta', 'Gross Mignone', 'Gross Indian', 'Heath Cling #2', and 'MP-29' were sampled in the second experiment. In the first experiment (27 March–26 June 2013) a single limb section was sampled from one tree in each of six blocks (n=6), except for 'Chinese Cling' (n=4) that only had four replicates. Rootstock limb samples were taken from six trees. In the second experiment (14 August 2013), single limb sections were sampled as described, but from one tree in each of eight blocks (n=8), except for 'Heath Cling' (#2), 'Fackler' and 'Flameprince' (n=6), 'Oldmixon Free' and 'Gross Indian' (n=7). In the second experiment, rootstock cultivar 'MP-29' was sampled.

The sampling procedures differed in sterilization assays and limb lengths. In the 1st experiment, the limb sections (20 cm, one per tree) were cut using a saw and placed singly in Ziploc bags and incubated in the dark at 25±1 °C. Between limb cuts in the field, the saw blade was thoroughly rinsed with 70 % ethanol. In the 2nd experiment limb sections (17 cm, one per tree), were cut in the field, as previously described, taken to the lab, and the entire surface sprayed to run-off with 70 % EtOH in a biosafety cabinet. The two exposed ends of each limb were re-cut, removing an additional 0.5 cm from the cut ends. Limbs were placed singly into Ziploc bags and incubated as described for the 1st experiment. All limb sections were observed for fungal growth at 7 and 14 days.

For each experiment, the percentage of colonized limbs from each scion and rootstock cultivar was calculated ([number with fungus ÷ total numbers of limb sections] × 100). Possible differences among scion cultivars were tested using a Chi-square test (no rootstock cultivar had infection, precluding inclusion in the analysis—'0' values are not valid expected proportions in Chi-square tests). Due to the low number of samples in both experiments 1 and 2, a Monte Carlo simulation (a randomization-based test) was run using the incidence data (McDonald 2009). The null hypothesis was that the incidence of infected limb sections should have approximately the same proportions for each cultivar. A total of 100,000 simulations were run, and the proportion of times the simulated chi-squared statistic was smaller than the observed chi-square statistic was calculated. If the randomization process resulted in a chi-square ≥ the observed value less than 5 % of the time, the null hypothesis was rejected. Analysis was performed using SAS V9.3 (SAS Institute Inc., Cary, NC)

Broken peach scaffold limbs, and the extent of internal wood decay

Broken scaffold limbs were observed in a 12-years old planting of peach cultivars and advanced selections at the USDA-ARS farm at Byron, GA subsequent to weather events on 9, 15, 19 and 21 July 2014 (gust wind speeds recorded were up to 8.65 m/s, combined with rainfall up to 48.8 mm—data recorded at the USDA-ARS, Byron by the GA Automated Environmental Monitoring Network [http://www.griffin.uga.edu/aemn/cgi-bin/AEMN.pl?site=GABY]). The trees were pruned annually to maintain 3–4 scaffold limbs at approximately a 50° angle supported on a single short scion-rootstock-joint trunk. Up to several primary branches were maintained on each scaffold limb, on which were produced most of the secondary and tertiary fruiting shoots. Sections were cut through limbs adjacent to the point of the break (immediately below) to ascertain the proportion of cross-sectional area of the limb colonized with any wood decaying fungi.

A total of six limbs were selected (cultivars 'Julyprince' and 'Jefferson', and selections 'BY92P2597n', 'BY88P4040m', 'BY88P2251a', BY88P2251a) and removed from the field for more detailed analysis of colonization. The length of the scaffold limb, and the distance of each primary branch from the point of breakage was measured. The scaffold limbs and primary branches were cut into sections to ascertain the extent of colonization, and the cross-sectional area colonized (% decayed) was estimated visually. The number of secondary branches with symptoms of colonization on each primary branch was counted. The relationship between severities of colonization from the break along the scaffold limb at the consecutive primary branches (distance) was analyzed using linear regression analysis.

Sampling and DNA extraction from the different cultivars

After incubation of tree limb sections, individual specimens of mycelium were taken from those limb sections that grew fungus (25–130 mg of mycelium). Samples were carefully scraped from the cut ends of the limbs

using a sterile scalpel and placed into Eppendorf tubes taking care to avoid any peach tissue. DNA was extracted using a Zymo Research Fungi/Bacteria MiniPrep kit (Zymo Research, Irvine, CA), following the instructions in the kit manual.

Next-generation DNA sequencing and metagenomics analysis

From the second scion and rootstock experiment in 2013, a combined DNA sample from six individual ends of three colonized limb sections of the cultivar Heath Cling of DNA was prepared for sequencing. A combined sample was used to determine the range of fungal species in the specimens, and to save cost. The combined sample was subject to fragment library construction and sequencing using the Ion Torrent sequencing platform (Life Technologies, Grand Island, NY, USA) at the University of Florida (UF), Interdisciplinary Center for Biotechnology Research (ICBR), Gainesville, FL. All reads were extracted into the fastq format for subsequent metagenomics analysis. Assembly was performed using SeqMan NGen (DNASTAR, Madison, WI) after the library adaptor sequences, reads from a peach genome (against the peach reference genome v1.0) and those of low quality score (<20) or short length (<20 bp) were pre-filtered and removed by the program. The minimum match percentage was set to 95 %, and all the other assembly parameters remained default. Contigs were aligned by BLAST to two datasets (Altschul et al. 1997), the non-redundant (nr) nucleotides retrieved from the nr database in the National Center for Biotechnology Information (NCBI) and all plant pathogen ESTs (dbpEST) retrieved from the Comprehensive Phytopathogen Genomics Resource (CPGR) database (Hamilton et al. 2011). The e value cutoff was $1.0 \times e^{-50}$. The BLAST output files were submitted to MEGAN (Huson et al. 2007) to construct taxonomy profiles and to determine taxa identities in the metagenome.

Primers for Sanger sequencing

The primers used for Sanger sequencing are listed in Table 1, including two widely used fungal internal transcribed spacer (ITS) region primers ITS1 and ITS4 (White et al. 1990), two new sequence walking primers ITS1-Ex1 and ITS1-Ex2 designed from the ITS1 sequencing read with aid of the chromatogram and additionally used only to sequence and separate the two fragments in the ITS1-ITS4 amplicon, and six new contig-based primers from the selected contigs aligned

Table 1 The primers used for Sanger sequencing of the bulked DNA extracted from a wood-decay fungus that had colonized peach limb sections

Primer name	Primer sequences	Amplicon size (bp)	Annotated fungus
ITS1	TCCGTAGGTGAACCTGCGG	622	
ITS4	TCCTCCGCTTATTGATATGC		
ITS1-Ex1	CTTGGAGGCTTGCTGGCCCTTGCG		
ITS1-Ex2	CTGGGAGGCTTGCTGGCCCCCGTT		
Contig31025_F	TTTGGGTTTGTTCGTTATGGTTAT	651	*T. versicolor*
Contig31025_R	AGACTCGCTCCTGACTCTCACT		
Contig39317_F	AGGGATGGGGAGTCGTTAATAC	593	*T. versicolor*
Contig39317_R	CAAGGAGCTGCTGAACAAGTC		
Contig60504_F	CGTCAGCACGATATACCTAAAGAA	228	*T. versicolor*
Contig60504_R	GTTATCGCGCGCTATCAGTATTA		
Contig4731_F	GGAGTTCTCGTACATTCCTACCAG	702	*S. commune*
Contig4731_R	TTCGGCTTATACTTAACCTCCATC		
Contig5709_F	GACACCATGACAGGTATAAACACG	684	*S. commune*
Contig5709_R	CGATATACATACGTATCCGCAAAG		
Contig43045_F	CCAACAACAAAGTGAAAGACAAAC	727	*S. commune*
Contig43045_R	ACTGACGTTCGAAATAGCTCTACG		

to genomic and EST sequences from either *T. versicolor* or *Schizophyllum commune* (three primers from each fungus). BatchPrimer3 was used for the primer design (You et al. 2008). Bi-directional Sanger sequencing was performed in the six amplicons larger than 500 bp, and only forward sequencing was done for ITS1-Ex1, ITS1-Ex2, and Contig60504_228, using an ABI genetic analyzer (Life Technologies, Grand Island, NY, USA). Sequencing chromatographs and reads were generated byBioEdit (Tom Hall, Ibis Biosciences, Carlsbad, CA). Sequence reads were aligned using Clustal Omega (Sievers and Higgins 2014).

PCR detection using the ITS1-ITS4 primer and multiplexing primers

Further confirmation of fungal identities was done by sequencing amplicons from an internal transcribed spacer (ITS) region (White et al. 1990; Guglielmo et al. 2007). These have previously been used to develop a multiplex PCR diagnostic test for wood decay fungi (Guglielmo et al. 2007). In an initial PCR test the six samples isolated for NGS were compared to DNA of *T. versicolor* and *S. commune* (obtained from Toni Mohr, Wood Decay and Diagnostics Research Project Lab, University of CA, Berkeley, CA) which were used as positive controls during the ITS1-ITS4 PCR diagnosis. Additional molecular identification were performed by multiplexing PCR assays on samples from the same trees and limbs sampled in 2013, but using fresh samples collected in August of 2014. Inevitably some trees had died, and some branches used for limb sections in the previous experiments were no longer amenable to sampling. If this was the case alternative branches were selected. Where the same branch could be used, prior to sampling a section of the end of the limb (approx. 3 cm) was discarded ensuring a fresh cut on the limb section that was incubated, as described above. A total of ten samples, each from a different tree were tested. DNA was extracted from these samples (as described above) and the samples were sent to the Forest Pathology and Mycology Lab 'Wood Decay and Diagnostics Research Project' services at the University of CA, Berkeley, CA (http://nature.berkeley.edu/garbelottowp/?page_id=146). Details of the recommended sampling protocols, sample preparation and diagnostic systems (DNA-based, multiplex PCR) and PCR conditions are described in detail elsewhere (Guglielmo et al. 2007, 2008, 2010; Nicolotti et al. 2009). The samples of DNA sent to the UC lab each contained 50 ul extracted DNA at a concentration of 2 ng/ul. Each sample was tested using the wood decay assay, which includes three separate multiplex reactions based on the ITS regions (Guglielmo et al. 2007, 2010): M1 (to identify 'fungi' in general, *Ganoderma* spp., and members of the *Inonotus*/*Phelinus* group), M2 (to identify *Armillaria* spp., *Laetiporus* spp., *Plurotus* spp. and *Hericium* spp) and M3 (to identify *P. fraxinea*, *Schizophyllum* spp., *Stereum* spp. and *Trametes* spp.). Following PCR using M1, samples and positive controls were run on a 1.2 % agarose gel at 80v for 40 min. Samples from PCR using M2 and M3 were sized via sequencing and analyzed with Peak Scanner Software v. 1.0. All peaks were confirmed by running samples out on 2 % agarose gels at 80v for 120 min (Toni Mohr, Wood Decay and Diagnostics Research Project Lab, University of CA, Berkeley, CA).

Results

Wood decay fungi in limbs of peach scion and rootstock cultivars

After 2 weeks' incubation, fungal growth was evident on the ends of the cut peach limbs (Fig. 1), and was manifest as a mat of white mycelium on the cut surface of asymptomatic peach limbs. Although not identical, results from both experiments were similar in most of the cultivars. The fungal growth from the heartwood was observed only on limbs from scion material, with colonization of these cultivars ranging from 17 to 67 % of the limb sections (Fig. 2). Although 'Lovell' is considered a rootstock cultivar, it was initially bred and used as a canning peach (i.e. a scion cultivar) from which seeds are easy to collect for the purpose of growing rootstocks.

No limb sections from any rootstock cultivar in either experiment were colonized by a fungus in the heartwood.

Fig. 1 Sections of peach limbs showing the fungal growth on the crosscut faces of the wood (the two leftmost limb sections). Sections to the right show no evidence of colonization with wood a wood decay fungus

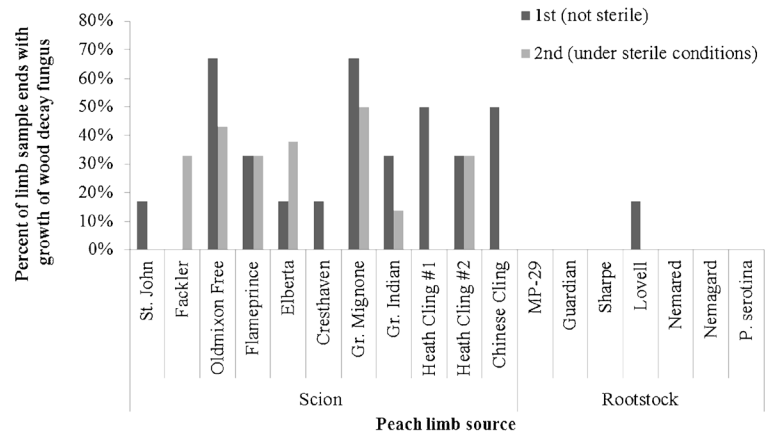

Fig. 2 Incidence (percentage) of crosscut peach limbs showing fungal growth in two separate experiments. All the cultivars were included in the 1st experiment, but only 'Fackler', 'Oldmixon Free', 'Flameprince', 'Elberta', 'Gross Mignone', 'Gr. Indian', 'Heath Cling #2', and 'MP-29' were sampled in the second experiment. *P. serotina* (black cherry) is a wild *Prunus* spp. A Chi-square test comparing infection of limb sections of the scion cultivars in the 1st ($P<0.0001$, $\chi^2=104$, [95 % CIs = 0.0000–0.0001], $n=21$) and 2nd ($P=0.002$, $\chi^2=21.5$ [95 % CIs = 0.001–0.002], $n=19$) experiments indicated significant difference in the incidence of colonization

Among the scion cultivars, the Chi-square test in the both experiments showed significant differences (1st experiment $P<0.0001$, $\chi^2=104$ (95 % CIs = 0.0000–0.0001); 2nd experiment $P=0.002$, $\chi^2=21.5$ (95 % CIs = 0.001–0.002)). Not all cultivars were included in the 2nd experiment, but among those that were common to both, the incidence was similar (for example, cultivar Oldmixon Free had 67 % limb sections with fungal growth in the 1st experiment, and 43 % in the 2nd experiment; the incidence on cultivar Flameprince was 33 % in both experiments). However, the sample sizes were small, and results from a larger experiment may be different.

Broken peach scaffold limbs, and the extent of internal wood decay

The broken limbs had foliage and green, but full-sized, fruit (Fig. 3a). The breaks were invariably low down on the scaffold limb. There was evidence of fungal mycelium growing in the decayed heartwood (Fig. 3b), and in some cases there were fruiting bodies typical of the 'Turkey Tail' fungus (*T. versicolor*), along the scaffold limb proximal to the point of breakage. Cross sectional cuts close to the point of breakage indicated the degree of colonization due to discoloration of the heartwood where the fungus had grown (Fig. 3c and d).

The six broken scaffold limbs sectioned each had 1–4 primary branches (Fig. 4a), with a total of 17 primary branches on all trees combined. The severity estimated at the cross section adjacent to the breakage was not always greater than that at the 1st to 4th primary branches (Fig. 4b). Depending on the tree, the branches on the scaffold were borne at 25 to 245 cm from the break (Fig. 4c). Linear regression analysis showed a weak relationship between distance from the breakage and the severity of colonization on the scaffold at the location of each of the consecutive primary branches on the scaffold (Fig. 4d, $R^2=0.46$), perhaps indicating that initial colonization occurred low down on the scaffold limb, and time allowed greater colonization at the proximal end compared to the distal end (although this relationship might be explained by the amount of suitable heartwood available for colonization in the scaffold limb as it narrows towards the distal end). Only 41 % of primary branches had symptoms of wood decay, and severity on the 1st primary branch was invariably more severe compared with that on the 2nd or 3rd primary branches (Fig. 4e). The 4th primary branch was not colonized in either tree that had four primary branches. No secondary branches on primary branches were observed to have symptoms of colonization by wood decay fungi; perhaps insufficient time had lapsed since they grew to allow for colonization (or there is insufficient heartwood in these young limbs).

Molecular identification of wood decay fungi

Assembly and characterization of metagenomic sequences A total of 3,203,056 Ion Torrent reads were generated. After the adaptor sequences and reads from

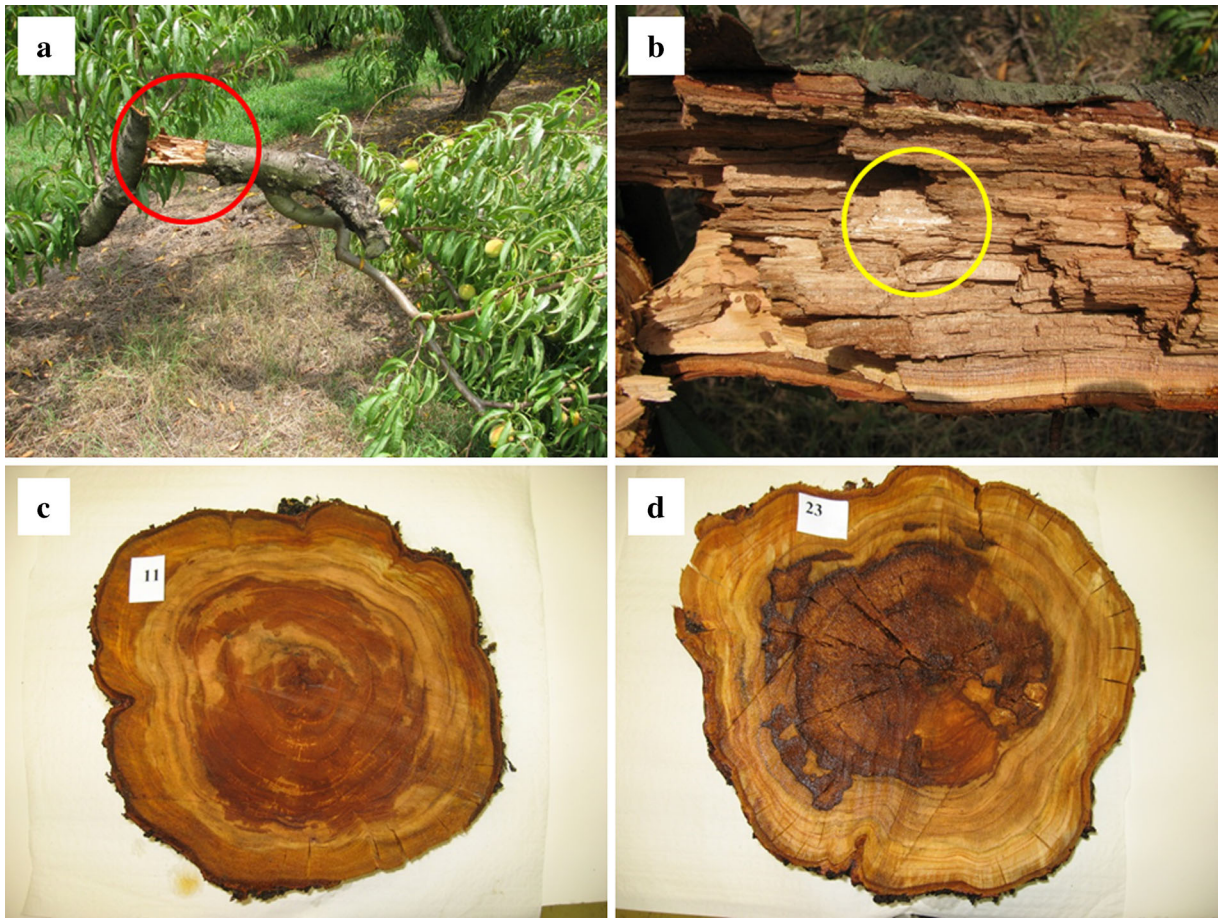

Fig. 3 Newly broken peach scaffold limbs in an orchard. **a** Apparently healthy and actively growing leaves and fruits are evident on the broken scaffolds, where the break point is marked by a *red circle*; **b** A magnified view (marked by a *yellow circle*) of the break point from a, where white fungal growth and discoloration are visible at the broken surface, with the colonized wood being soft and friable; and **c** and **d** Typical discoloration caused by wood decay fungi in the heartwood of cross-cut surfaces of peach scaffold limbs

the peach genome and those reads of low quality or short length were pre-filtered and removed; 3,058,172 of them were used for *de novo* assembly. The average length and G+C content of cleaned reads was 240 bp and 56 %, respectively. A total of 62,113 contigs were assembled, with an average coverage of 10 reads and an average length of 1041 bp. The contig N50 length was 1385 bp, with 4812 contigs generated over 2 kb. The genome length was estimated at 41.9 Mb, according to the assembly program (SeqMan NGen, DNASTAR).

Taxonomical profiling by metagenomic analysis According to the BLAST hits against the non-redundant nucleotide database (nr) from the NCBI and the plant pathogen EST database (dbpEST) from the CPGR at e^{-50}, the taxonomy profiles generated by MEGAN analysis of both the BLAST output results independently identified two fungi, *T. versicolor* and *S. commune*, but they differed in the numbers of hits (Figs. 5a and b and 6). There were 3784 hits with *T. versicolor* ESTs and 84 hits with *S. commune* ESTs at e-50. Based on these hits, it appeared that *T. versicolor* was the most likely (or the most common fungus isolated from the cut peach limbs in this sample, rather than *S. commune* (which might also be present) based on substantially more ESTs (genes) matched. However, there were slightly more sequences (most were rRNA sequences) aligned with *S. commune* in the nr database, along with some aligned ESTs in the dbpEST, further suggesting *S. commune* was growing from the heartwood of colonized, cut peach limbs. Many fewer aligned ESTs might be simply due to fewer

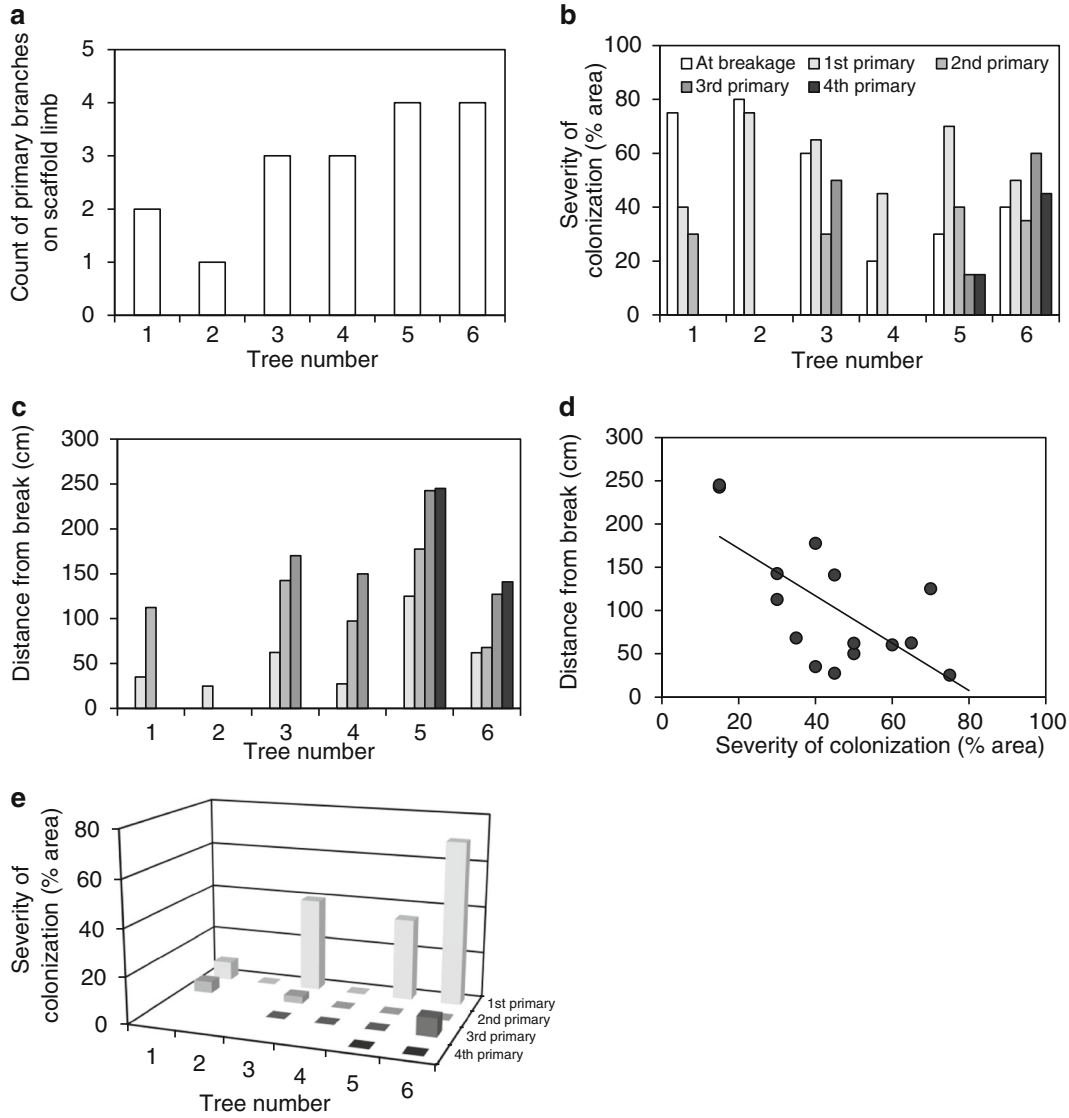

Fig. 4 Extent of colonization of peach limbs with wood decay fungus. **a** The number of primary branches on the broken scaffold limb of each tree; **b** The severity (percent cross sectional area) of the main branch with symptoms of decay at the attachment point of the primary branches; **c** The distance to the point of attachment of the primary branches from point of breakage on the scaffold limb; **d** The relationship between severity of colonization on scaffold limbs at primary branch attachment, and attachment distance of primary branches (y=−2.7 x+226.3 R^2=0.46); and **e** the severity at the base of each of the consecutive primary branches along the scaffold branch on each of the six trees

ESTs from *S. commune* in the database, or a result of less abundant growth. According to the taxonomic profiles based on the BLAST output results against dbpEST under a much less stringent e-value (e^{-10}), a few other fungi, in addition to *T. versicolor* and *S. commune*, were also identified by MEGAN analysis, but the numbers of hits even at this low threshold were limited (Electronic supplementary materials—ESM Fig. 1) and most aligments were short (data not shown), supporting the earlier results at a more conservative e-value that *T. versicolor* and *S. commune* were the two fungi that had colonized the heartwood of the samples taken. In addition, a few dozen short alignment hits were found when a similar analysis was performed against the bacterial genome database at e^{-10} (data not shown), confirming that no bacteria were associated with the isolation.

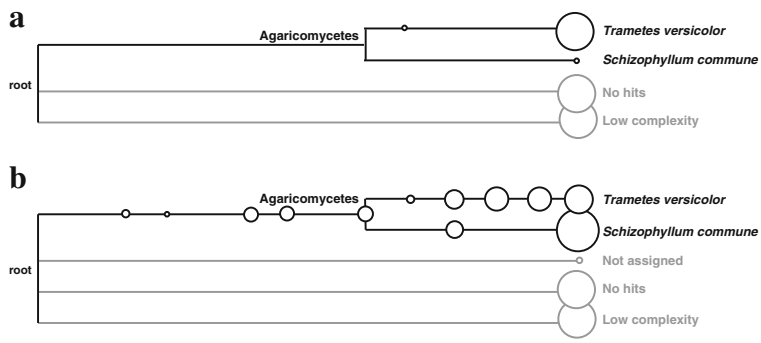

Fig. 5 The taxonomic trees built by MEGAN analysis based on the BLAST output from the metagenomic sequences at an e-value of e-50. **a** Tree derived from the BLAST output data using the plant pathogen EST database (dbpEST); **b** Tree derived from the BLAST output data using the non-redundant nucleotide database (nr)

Validation by sequencing of the PCR product As shown in the Sanger sequencing chromatograph (ESM Fig. 2a and b), single nucleotide peaks were read up to 405 bp by ITS1 and up to 159 bp by ITS4, and after which overlapped nucleotide peaks emerged, respectively. The overlapped portion showed that two fragments (amplicons) of the same or similar sizes were amplified from the DNA extracted from the specimen recovered from the peach limb section, supporting the result that two fungi were identified by metagenomic analysis. The sum of 405 and 159 is very close to the size of the sequenced amplicon that was visualized on an Agarose gel (Lane 0 in Fig. 7), implying the overlapping peaks might result from a deletion-derived shift. This implication was also supported by high-identity alignment of the two Chromas-automatically-generated sequences, in which the ITS-1 and ITS-4 primers were shown at the end of the ITS-4 and ITS-1 sequences, respectively (ESM Fig. 3). The two fragments were separated by additional sequencing, confirming that nucleotide variations (including two single nucleotide deletions) existed in the two sequences and caused the overlapped peaks starting at the read position 406 and 160 in the ITS-1 and ITS-4 sequences, respectively. However, their assignment to the genome sources, *T. versicolor* or *S. commune*, had not been confirmed through BLAST since the two sequences were almost identical.

All six new primers produced an amplicon of expected size, respectively (Lane 1–6 in Fig. 7). Similar to the nucleotide chromatograph pattern of the ITS1-ITS4 amplicon, two fragments were also detected by Sanger sequencing in the amplicon from Contig31025_651 (ESM Fig. 4 a and b). In the Sanger sequencing chromatograph, single nucleotide peaks were read up to 200 bp by Contig31025_651_F (forward primer) and up to 410 bp by Contig31025_651_R (reverse primer), and after which overlapped nucleotide peaks emerged, respectively, which was additional evidence that in the mixed DNA there were two genomic templates, although almost identical at this amplicon region. Sequencing failed to generate

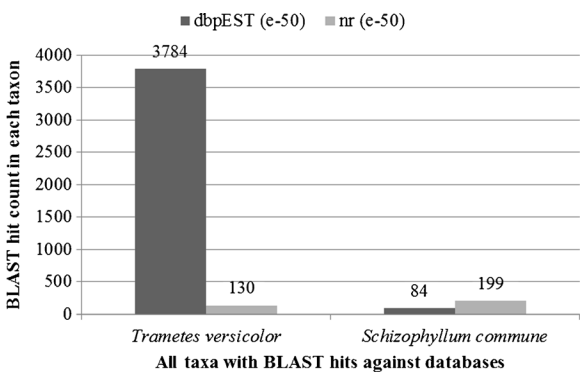

Fig. 6 The taxonomic profiles and hit counts in each taxon generated by MEGAN analysis. At a stringent e-value of e-50, only two fungi, *Trametes versicolor* and *Schizophyllum commune*, were found in the taxon profile. The profiles were based on the output results of BLAST against the plant pathogen EST database (dbpEST) and the non-redundant nucleotide database (nr), respectively

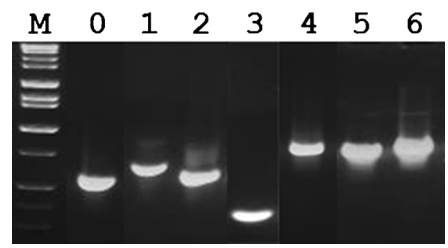

Fig. 7 Fragments amplified and sequenced from the six samples bulked for the next generation sequencing (NGS). M: 50–10,000 bp Hi-Lo™ DNA marker (Bionexus, Oakland, CA); Primers for 0–6 in turn: ITS1-ITS4, Contig31025, Contig39317, Contig60504, Contig4731, Contig5709, and Contig43045

readable nucleotide peaks from the amplicon from Contig39317_593, but succeeded in the amplicons from Contig60504_228, Contig4731_702, Contig5709_684, and Contig43045_727 that were read as a neat single sequence respectively (data not shown). The successful amplification and sequencing confirmed their presence in the DNA template and proved the quality of NGS reads and assembly. But it remained unclear at this stage that the four single sequences resulted from either a single amplicon specific to one genome or two identical amplicons from the two genomes.

PCR detection using multiplexing Not all of the limbs tested in 2014 were the same as those found to be colonized in the two experiments in 2013 (Table 2). Of the ten limb samples that were tested, six came from trees previously confirmed as having a wood decay fungus, while the remaining four came from trees previously screened, but which had limb sections not found to be colonized with a wood decay fungus. With the M1 assay, all samples tested were positive for fungal ITS (data not shown), but the PCR did not produce any other bands. With the M2 assay one sample (from cultivar Gross Mignone rep 7)—was positive for *Hericium* spp, and with the M3 assay, all samples tested positive for *Trametes* spp. There was so much *Trametes* DNA in these samples that the M3 assay was repeated without the *Trametes* primers. In this modified retest, five of the samples were positive for *Stereum* spp., and three were positive for *Schizophyllum* spp. The molecular identity based on the multiplex PCR test from 2014 indicates the ubiquity of *Trametes* spp. in peach limbs at this location.

Table 2 Peach limb sections collected from different cultivars in two experiments showing limb samples with evidence of growth of wood decay fungi

	Tree	Experiment			Multiplex PCR (Y = positive, N = negative, - = not tested)			
Peach cultivar	replicate	2013 - 1st	2013 - 2nd	2014	*Trametes* spp.	*Schizophyllum* spp.	*Stereum* spp.	*Hericium* spp.
Fackler	1	NG	NG	1	Y	N	N	N
	2	NG	1	1	-	-	-	-
	3	NG	1	NG	-	-	-	-
Flame Prince	1	NG	NG	1	Y	Y	Y	N
	2	NG	1	1	-	-	-	-
	3	1	NG	1	Y	N	N	N
	4	NG	NG	1	Y	Y	Y	N
	5	1	1	1	-	-	-	-
Gross Indian	1	NG	1	1	Y	N	Y	N
	2	NG	1	NG	-	-	-	-
	3	1	NG	NG	-	-	-	-
	5	1	1	NG	-	-	-	-
Gross Mignone	2	1	NG	1	Y	N	N	N
	3	1	NG	NG	-	-	-	-
	4	1	NG	NG	-	-	-	-
	5	NG	NG	1	Y	N	N	N
	6	1	NG	NG	-	-	-	-
	7	(no sample)	1	1	Y	N	N	Y
Heath Cling #1	1	1	NG	NG	-	-	-	-
	2	1	1	1	Y	N	Y	N
	4	NG	1	1	Y	Y	Y	N
	6	1	NG	NG	-	-	-	-
Oldmixon Free	2	NG	1	NG	-	-	-	-
	3	1	NG	NG	-	-	-	-
	4	1	1	NG	-	-	-	-
	5	1	1	1	-	-	-	-
	6	1	NG	NG	-	-	-	-

Shaded cells represent those diagnosed using multiplex PCR

1 = mycelial growth present, *NG* = no mycelial growth observed

The multiplex PCR is described elsewhere (http://nature.berkeley.edu/garbelottowp/?page_id=146) (Guglielmo et al. 2007, 2008, 2010; Nicolotti et al. 2009)

Discussion

Colonization of peach wood by wood-decay fungi in peach limbs

Wood decay caused by fungi was observed in scion, but not in rootstock cultivars sampled in this study (Figs. 1 and 2). Weakening of peach tree limbs may have ramifications for load bearing and for long-term vigor and productivity of peach trees in orchards in the southeastern US (Alconero et al. 1978; Doepel et al. 1979; Cripps et al. 1983; Adaskaveg et al. 1993). Commercial peach scion wood is heavily pruned each winter, in order to maintain desirable tree architecture and promote new shoots to sustain cropping and management practice; but pruning creates numerous wounds, which might allow access of wood decay fungi (Doepel et al. 1979; Cripps et al. 1983). Generally there is no pruning of rootstock cultivars. It is also possible that selection of scion cultivars for peach fruit edibility may result in loss of some generic chemical components that are used in resistance to diseases and/or pests. Rootstocks are under no such selection and are constantly subject to both soilborne and aerial pathogens, so any defense components would be maintained in these cultivars, and render them less susceptible to opportunistically invasive agents, including perhaps wood decay fungi. In regard to scion cultivars, adjustment to pruning methods and breeding practices might be required in the future (Doepel et al. 1979; Cripps et al. 1983). Where applied, applications of zinc, copper or manganese sulphates might help protect pruning wounds against wood decay organisms, and training to optimize branch angles might reduce opportunities for colonization (Cripps et al. 1983). Differences observed in incidence of wood decay found in different scion cultivars may be due to a number of factors, including cultivar resistance, ability to heal wounds and local environmental effects (and the limited sample size in this study).

The weakening effect of the wood decay was evident in the orchard of older peach trees subject to a rain and wind event. Many scaffold limbs, supporting foliage and fruit, in the orchard were broken—and all broken limbs had evidence of substantial internal wood decay. The full length of the scaffold branch was colonized, and wood decay was observed in some primary branches on the scaffold limbs. The weak relationship found between distance from the breakage and the severity of colonization on the scaffold may be due to earlier colonization of the proximal end of the scaffold limb, or due to the amount of suitable wood available for colonization in the scaffold limb as it narrows towards the distal end of the branch. Each of the scaffold limbs on a peach tree bear the full load of the leaves and fruit. Their health is thus crucial to minimize yield loss through limb breakage (Petersen 1960, 1961; Alconero et al. 1978; Doepel et al. 1979; Cripps et al. 1983; Adaskaveg et al. 1993). In addition, wood decay may impact overall tree vigor.

Identity of the main fungi colonizing peach wood

According to the metagenomics analysis and Sanger sequencing, *T. versicolor* and *S. commune* were most likely the fungi isolated from the crosscut surface of the peach limbs of the selected peach scion cultivars. Both *T. versicolor* and *S. commune* are ubiquitous degraders of complex biomass, including lignin (Floudas et al. 2012). The multiplex PCR also confirmed the species present, the most commonly diagnosed being *Trametes* spp. which was also the most common species among wood decay fungi found on peach trees in a survey in South Carolina (Adaskaveg et al. 1993). Only a few samples showed evidence of other species too.

The fungi were found growing deep within the heartwood of asymptomatic scaffold limbs of peach scion cultivars. This is indicative of the ability of these fungal species to compromise the health and possibly reduce the longevity of peach trees, in addition to other existing issues that already compromise peach tree health including PTSL and ARR (Beckman et al. 1998; Ritchie and Clayton 1981; Cottrell et al. 2008). More research is needed to confirm these observations, and to understand the epidemiology of these organisms in peach, including the fungal distribution within trees, the process of infection and/or colonization (including the effect of single and multiple infections), the effect of tree and limb age, disease severity on different germplasm, and the effect of colonization on tree limb strength.

Utilization of the metagenome approach to identify the fungi

The next-generation sequencing and metagenome analysis affirmed the capability and efficiency of this approach as a tool for identification of unknown organisms (Huson et al. 2007; Kunin et al. 2008; Schmieder and Edwards 2011). Based on the BLAST data against the

most recent all plant pathogen EST and the non-redundant nucleotide databases, only sequences common to *T. versicolor* and *S. commune* were found. The genome and metagenome sequences of these two fungi (Floudas et al. 2012; Ohm et al. 2010) are valuable resources on which to base a continued investigation of the distribution and abundance of both wood decay species in the limbs of peach. The techniques can be used to help gain knowledge in regard to effects of these fungi on peach tree health, limb strength and tree longevity. However, more work is needed to identify fungal genome specific sequences through whole genome alignment analysis (Floudas et al. 2012), which will facilitate development of species-specific confirmative PCR assays, especially where genomes nucleotide identities are very similar, as found in this study (very limited nucleotide variation was found in the ITS1-ITS4 amplicon of *T. versicolor* and *S. commune*).

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References

Adaskaveg, J. E., & Ogawa, J. M. (1990). Wood decay pathology of fruit and nut trees in California. *Plant Disease, 74*, 341–352.

Adaskaveg, J. E., Miller, R. W., & Gilbertson, R. L. (1993). Wood decay, lignicolous fungi, and decline of peach trees in South Carolina. *Plant Disease, 77*(7), 707–711.

Alconero, R., Campbell, R., Brittain, J., & Brown, B. (1978). Factors contributing to early decline of peach in South Carolina. *Plant Disease Report, 62*, 914–918.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389–3402.

Beckman, T. G., Okie, W. R., Nyczepir, A. P., Pusey, P. L., & Reilly, C. C. (1998). Relative susceptibility of peach and plum germplasm to Armillaria root rot. *HortScience, 33*(6), 1062–1065.

Cottrell, T. E., Fuest, J., & Horton, D. L. (2008). Influence of Prunus spp., peach cultivars, and bark damage on oviposition choices by the lesser peachtree borer (Lepidoptera: Sesiidae). *Environmental Entomology, 37*(6), 1508–1513. doi:10.1603/0046-225x-37.6.1508.

Cripps, J. E. L., Doepel, R. F., & McLean, G. D. (1983). Canning peach decline in Western Australia. II. methods of prevention. *Australian Journal of Agricultural Research, 34*, 517–526.

Doepel, R. F., McLean, G. D., & Goss, O. M. (1979). Canning peach decline in Western Australia. I. Association between trunk cankers, trunk pruning wounds and crotch angles of acaffold limbs. *Australian Journal of Agricultural Research, 30*, 1089–1100.

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R. A., Henrissat, B., et al. (2012). The Paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science, 336*(6089), 1715–1719. doi:10.1126/science.1221748.

Guglielmo, F., Bergemann, S. E., Gonthier, P., Nicolotti, G., & Garbelotto, M. (2007). A multiplex PCR-based method for the detection and early identification of wood rotting fungi in standing trees. *Journal of Applied Microbiology, 103*(5), 1490–1507. doi:10.1111/j.1365-2672.2007.03378.x.

Guglielmo, F., Gonthier, P., Garbelotto, M., & Nicolotti, G. (2008). A PCR-based method for the identification of important wood rotting fungal taxa within Ganoderma, Inonotus s.l. and Phellinus s.l. *FEMS Microbiology Letters, 282*(2), 228–237. doi:10.1111/j.1574-6968.2008.01132.x.

Guglielmo, F., Gonthier, P., Garbelotto, M., & Nicolotti, G. (2010). Optimization of sampling procedures for DNA-based diagnosis of wood decay fungi in standing trees. *Letters in Applied Microbiology, 51*(1), 90–97. doi:10.1111/j.1472-765X.2010.02860.x.

Hamilton, J. P., Neeno-Eckwall, E. C., Adhikari, B. N., Perna, N. T., Tisserat, N., Leach, J. E., et al. (2011). The Comprehensive Phytopathogen Genomics Resource: a web-based resource for data-mining plant pathogen genomes. *Database*. doi:10.1093/database/bar053.

Huson, D. H., Auch, A. F., Qi, J., & Schuster, S. C. (2007). MEGAN analysis of metagenomic data. *Genome Research, 17*(3), 377–386. doi:10.1101/gr.5969107.

Kunin, V., Copeland, A., Lapidus, A., Mavromatis, K., & Hugenholtz, P. (2008). A bioinformatician's guide to metagenomics. *Microbiology and Molecular Biology Reviews, 72*(4), 557–578. doi:10.1128/MMBR.00009-08.

McDonald, J. H. (2009). *Handbook of biological statistics* (2nd ed.). Baltimore: Sparky House Publishing.

Nicolotti, G., Gonthier, P., Guglielmo, F., & Garbelotto, M. (2009). A biomolecular method for the detection of wood decay fungi: a focus on tree stability assessment. *Arboriculture & Urban Forestry, 35*(1), 14–19.

Ohm, R. A., de Jong, J. F., Lugones, L. G., Aerts, A., Kothe, E., Stajich, J. E., et al. (2010). Genome sequence of the model mushroom Schizophyllum commune. *Nature Biotechnology, 28*(9), 957–963. doi:10.1038/nbt.1643.

Okie, W. R., & Reilly, C. C. (1983). Effect of criconemella xenoplax on ninhydrin positive compounds in Lovell and Nemaguard peach rootstocks. *Phytopathology, 73*(5), 830.

Petersen, D. H. (1960). Hymenomycetous fungi except polyporus associated with wood decay of living peach trees in South Carolina. *Plant Disease Report, 44*, 891–893.

Petersen, D. H. (1961). Polyporus spp. Associated with wood decay of living peach trees in South Carolina. *Plant Disease Report, 45*, 186–189.

Ritchie, D. F., & Clayton, C. N. (1981). Peach tree short life—a complex of interacting factors. *Plant Disease, 65*(6), 462–469.

Schmieder, R., & Edwards, R. (2011). Fast identification and removal of sequence contamination from genomic and metagenomic datasets. *PLoS One, 6*(3), e17288. doi:10.1371/journal.pone.0017288.

Schwarze, F. W. M. R., & Baum, S. (2000). Mechanisms of reaction zone penetration by decay fungi in wood of beech (Fagus sylvatica). *New Phytologist, 146*(1), 129–140. doi:10.1046/j.1469-8137.2000.00624.x.

Shigo, A. L., & Wilson, C. L. (1982). Wounds in peach trees. *Plant Disease, 66*, 895–897.

Sievers, F., & Higgins, D. G. (2014). Clustal Omega, accurate alignment of very large numbers of sequences. *Methods in Molecular Biology, 1079*, 105–116. doi:10.1007/978-1-62703-646-7_6.

White, T. J., Bruns, T. D., Lee, S., & Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innes, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols: A guide to methods and applications* (pp. 315–322). San Diego: Academic.

You, F. M., Huo, N., Gu, Y. Q., Luo, M. C., Ma, Y., Hane, D., et al. (2008). BatchPrimer3: a high throughput web application for PCR and sequencing primer design. *BMC Bioinformatics, 9*, 253. doi:10.1186/1471-2105-9-253.