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4 For Fungal Ecology
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9 New evidence for broad trophic status of leaf endophytic fungi of *Quercus gambelii*
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Key words: endophyte, fungi, saprotrophy, biotrophy, *Quercus gambelii*, Utah, trophic
plasticity, niche breadth

Introduction

Complex communities of symbiotic, endophytic fungi occur in the living tissues of every plant species investigated thus far (Arnold et al., 2003; Arnold and Lutzoni, 2007; Rodriguez et al., 2009). The consequences to a plant of colonization by endophytic fungi are numerous.

Endophytic fungi may increase seed germination, photosynthesis, growth and reproduction of the plants they colonize (Clay, 1987; 1988; Baynes et al., 2012). In some cases endophytic fungi confer on their hosts increased resistance to herbivory (Clay, 1987, 1988; Carroll, 1988; Cheplick and Clay, 1988;), disease (Carroll, 1988; Arnold et al., 2003), heat stress (Rodriguez et al., 2004; Marquez et al., 2007; Hubard et al., 2013), water stress (Clay and Shardl, 2002; Rodriguez et al., 2004; Peñuelas et al., 2012; Hubard et al., 2013) and increased competitive ability (Aschehoug et al., 2012). In some stressful environments endophytic fungi may even be essential for plant survival (Rodriguez et al., 2004).

Although their biotrophic status has never been demonstrated directly, endophytic fungi are presumed to obtain reduced carbon directly from living host tissues because they persist stably and asymptotically in live organs including leaves, stems and roots. Decomposer fungi, by contrast, are usually saprotrophs and obtain reduced carbon from dead material (litter) rather than from living tissues. However, categorizing fungi on the basis of their trophic status can be problematic (Koide et al., 2006). For example, Lewis (1973) recognized the problem of trophic categorization when he acknowledged the possibility of facultative trophic forms. Some fungi, for example, obtain reduced carbon both biotrophically and saprotrophically.

As with endophytic fungi, ectomycorrhizal fungi have traditionally been assumed to be biotrophic. However, facultative saprotrophy may exist among the ectomycorrhizal fungi (Ferry and Das, 1968; Lamb, 1974; Trojanowski et al., 1984; Haselwandter et al., 1990; Tibbett et al.,

1998; Egger, 2006). If biotrophy evolved from saprotrophy among the endophytic fungi, as hypothesized for ectomycorrhizal fungi (Hibbett et al., 2000), it stands to reason that at least some endophytic fungi continue to possess saprotrophic capacity. Indeed, the endophytic habit could merely represent a small extension of saprotrophy in which the reduced carbon source is colonized before death, rather than after, and in which degradative enzyme production is largely attenuated during the living phase of the host (see Colpaert and van Laere, 1996 for an example of this in ectomycorrhizal fungi).

One can envision the selection pressure responsible for maintaining saprotrophic capacity among the biotrophic endophytes. The domicile of a pure biotroph provides reduced carbon over a limited lifespan, but the domicile of the biotroph/saprotroph continues to provide reduced carbon long after its death. Endophytic fungi with biotrophic and saprotrophic capacity have both the last opportunity to obtain carbon from a living host and the first opportunity to consume its tissues upon its death, as suggested by Porras-Alfaro & Bayman (2011).

We have hypothesized, therefore, that at least some endophytic fungi possess saprotrophic capacity. If this were true, we would expect to find that some fungi present in living leaves persist after leaf death and during leaf decomposition. Another group, that we might term ‘pure biotrophs’, should be present in living leaves but not in decomposing leaves. A third cohort of fungi, the ‘pure saprotrophs’, should only colonize after death of the leaf. We determined whether such expectations were correct for the fungi occupying leaves of gambel oak (*Quercus gambelii*) as they transitioned from living organs to decomposing litter. Gambel oak is a common woody inhabitant of the eastern great basin along the Wasatch front of Utah, USA, the location of Brigham Young University.

Materials and methods

Longitudinal study

Leaf collection, field deployment and DNA extraction, PCR. On 18 October 2014 we collected 101 mature, living leaves from a cluster of about 20 gambel oak (*Quercus gambelii* Nutt.) trees (N 40° 09.797' W 111° 34.040') in a canyon in the Wasatch Range within the limits of Springville City, UT, USA. Twenty leaves were set aside for immediate DNA extraction and analysis of fungal communities to document the taxa present prior to decomposition. Four small disks were removed from each of the 20 leaves using a paper punch. The 4 disks per leaf were pooled and frozen at -20°C for no more than 1 week prior to fungal DNA extraction using Mobio PowerPlant Pro DNA isolation kits and a Biospec Mini-BeadBeater-8. DNA was then amplified using Sigma Jumpstart Taq (Sigma–Aldrich, St. Louis, MO, USA) and ITS1F[Hex] and ITS4 primers (Gardes and Bruns, 1993). The thermal cycling program used to amplify fungal DNA was: 30 cycles of 95°C for 40 s, 49°C for 30 s, and 72°C for 48 s with final elongation at 72°C for 7 min.

The 81 remaining leaves were placed on the lab bench at room temperature for two days following collection to allow the leaves to attain a stable air dry weight without the use of additional heat in order to mimic the conditions likely to be experienced by the fungi in nature. Individual leaves were then weighed and sealed into envelopes made from PVC-coated fiberglass window screen using a heat sealer. Each bag was uniquely numbered. On 21 October 2014, individual bags were randomized and secured to the ground under a stand of gambel oaks (N 40° 09.754' W 111° 34.097') in the same canyon in Springville City, UT, using 7.5 cm long nails in each of the four corners.

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4 We returned to the canyon on three occasions, each time collecting 27 of the
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6 decomposing leaves (5 December 2014, 13 February 2015 and 14 May 2015). On each date,
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8 leaves were air-dried on the laboratory bench at room temperature until they attained a stable
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10 weight. The leaves were then weighed to calculate decomposition rate, and 4 disks were
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12 removed from each leaf using a paper punch for DNA extraction. The 4 leaf disks per leaf were
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14 pooled and then were frozen at -20°C less than 1 week until fungal DNA was extracted as above.
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18 DNA amplification was performed as above.
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21 DNA analysis. We used ARISA (automated ribosomal intergenic spacer analysis) to
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23 characterize the fungal communities (Fisher and Triplett, 1999). ARISA is a method used to
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25 rapidly characterize the structure of microbial communities based on the lengths of polymerase
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27 chain reaction (PCR) amplicons. It has been used for microbial communities in soil (Moll et al.,
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29 2015), water (Lear et al., 2014) and plant material (Purahong et al., 2014). We separated the
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31 hex-labeled PCR products from the 101 samples on an ABI 3730xl genetic analyzer using
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33 MapMarker ROX-1000xl ladder (BioVentures, Inc. Murfreesboro, TN). We used GeneMapper
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35 Software (Applied Biosystems, Grand Island, NY, USA) to analyze the PCR amplicons. The
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37 peaks from all collection dates, corresponding to the amplicons, were binned simultaneously into
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39 operational taxonomic units (OTUs) according to their lengths using a bin width of 5 bp. Each
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41 OTU was designated by the midpoint of the bin (number of base pairs). Bins created by spurious
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43 peaks (usually ladder bleed-through) were manually eliminated prior to subsequent analysis.
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50 Peak areas were Hellinger-transformed in order to relativize the peak areas for each sample
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52 (Yannarell et al., 2014) prior to permutational multivariate analysis of variance and for
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54 ordination (see below). Peak areas were converted to binary (presence/absence) form for
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58 longitudinal (traj) analysis (see below) and for estimation of OTU richness (see below).
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4 Longitudinal analysis. An analysis of taxon frequency of occurrence through time was
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6 used to distinguish between presumptive endophytes (present prior to initiation of
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8 decomposition) and presumptive saprotrophs (present only after initiation of decomposition).
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10 For this longitudinal analysis, we calculated relative frequencies of occurrence over time (binary
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12 data) for each OTU by dividing the number of occurrences at each sampling date by the sum of
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14 the occurrences for all four sampling dates. Thus, for a given OTU, the sum of these relative
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16 frequencies for the multiple sampling dates was always 1. The purpose of this scaling was to
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18 characterize the temporal pattern of occurrence for each species in a manner that was
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20 independent of its absolute frequency of occurrence and independent of all other OTUs in the
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22 community.
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28 To assist us in our effort to distinguish between presumptive endophytes and presumptive
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30 saprotrophs, cohorts of fungal OTUs with similar temporal patterns were obtained using traj, a
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32 semiparametric procedure that fits models to longitudinal data in order to produce data-sequence
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34 groupings (see the traj website: <http://www.andrew.cmu.edu/user/bjones/>; see also Nagin, 1999,
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36 2005; Nagin and Tremblay, 2001). In our case, we used traj to group OTUs according to their
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38 similarity in relative frequency through time. Because traj was applied to the relative
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40 frequencies (above), it separated OTUs into groups according to temporal pattern only (relative
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42 frequency through time), and not on the basis of absolute frequency of occurrences, which varied
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44 markedly among OTUs. We employed censored normal models in traj, specifying second-order
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46 polynomial models and 6 groups, which produced the lowest AIC value. One of the peculiarities
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48 of traj is that all relative frequencies must be expressed to the same degree of precision. In other
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50 words, frequencies of 25 of 100 and 33 of 67 must not be expressed as 0.25 and 0.4925, but
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52 rather as 0.2500 and 0.4925, or as 0.25 and 0.49. For the analyses herein, we arbitrarily
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expressed all relative frequencies to four decimal places. In the model, the timescale was centered about 0 and scaled to be comparable in magnitude to that of the dependent variable (relative frequency) as recommended (D. S. Nagin, pers. comm.). Traj has been used previously for a similar longitudinal analysis of ectomycorrhizal fungal communities (Koide et al., 2007).

We determined the effect of removing from the longitudinal analysis the taxa occurring in fewer than 10 samples. Although this resulted in a reduction of the total number of fungal taxa from 84 to 32 and the elimination of 2 of the 6 original traj cohorts, we present the results of this more conservative analysis because taxa occurring in few samples are more prone to registering a false positive than taxa occurring more frequently, and because it is difficult to determine the longitudinal pattern of frequency of occurrence for rare species.

Decomposition and leaf chemistry

Decomposition (percent of weight loss) was subjected to analysis of variance and means were separated using the Tukey honestly significant difference method. Air-dried leaves were ground prior to chemical analyses. Nitrogen and phosphorus concentrations were determined after $\text{H}_2\text{SO}_4\text{-H}_2\text{O}_2$ digestion at 400°C for 1 h by colorimetry: for nitrogen, the Nessler's method was used (Jensen, 1962), for total phosphorus, a modified version of the molybdi-phosphate method as outlined by Watanabe and Olsen (1965) was used. To analyze total phenolics, 100 mg ground leaf was extracted in 10 ml 70% methanol in 15 ml centrifuge tubes on a vortex for 10 min at room temperature. A 1 ml aliquot was then transferred to a microfuge tube and centrifuged for 1 min at 10,000 G. From the 1 ml aliquot, a 5 μl sample was mixed with 250 μl water, to which was added 125 μl Tanniver reagent (Hach, Loveland, CO, USA) and 625 μl 700mM Na_2CO_3 . Absorbance was determined at 760 nm after 30 min. using triple analytical replicates, which

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4 were averaged to calculate total phenolic concentration expressed as mg tannic acid equivalents
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6 per g leaf dry weight. Phenolics, nitrogen and phosphorus concentrations were subjected to
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8 analysis of variance and were used as supplemental variables in the ordinations (below).
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10 11 12 13 14 Community structure

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16 Significant differences among sampling periods in the structure of communities of presumptive
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18 saprotrophic fungi and presumptive endophytic fungi (performed separately) were determined
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20 using permutational multivariate analysis of variance (PerMANOVA) in the R statistical
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22 environment (R Core Team, 2013) with the Vegan package. Bray-Curtis distances were used.
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24 Variation in structure was visualized using Canoco 5 (Ter Braak and Šmilauer, 2012), which we
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26 employed to performed detrended correspondence analysis with supplementary variables (leaf N,
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28 P and total phenolic concentrations) of log-transformed data with detrending by segments
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30 (DCA).
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36 Fungal OTU diversity was characterized using Fisher's alpha (Hayek and Buzas, 1996),
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38 computed in EstimateS v. 9.1.0 (Colwell, 2013). 95% confidence intervals for Fisher's alpha
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40 were calculated from standard deviations and values from the t distribution.
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45 46 *Sequencing*

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48 For our tentative identification of some of the fungi found in the longitudinal analysis of the oak
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50 leaves (above), we matched their ARISA signatures (PCR product lengths) to those obtained
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52 from fungi cultured from a separate set of oak leaves. This separate set consisted of one hundred
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54 live, mature gambel oak leaves from which we isolated endophytic fungi. These 100 leaves were
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56 surface sterilized by vigorously rubbing with 70% ethanol to kill phylloplane fungi prior to
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4 plating out. A sterile paper hole punch was used to take a small disk from each of the 100
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6 leaves. Disks were incubated at room temperature on half-strength potato dextrose agar (PDA).
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8 As fungi grew out from the leaf pieces, individual colonies were replated onto half-strength PDA
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10 to create pure cultures. DNA was extracted from the pure cultures using Mobio microbial DNA
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12 isolation kits. DNA was amplified using the primers and procedure above. The hex-labeled
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14 PCR products were separated as above, and GeneMapper Software (Applied Biosystems, Grand
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16 Island, NY, USA) was used to analyze the PCR amplicons as above. The raw peaks from all
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18 collection dates were binned into OTUs as above and spurious peaks (ladder bleed-through) were
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20 manually eliminated prior to analysis. DNA was also amplified again with the same primers but
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22 without the Hex tag and sequenced on an ABI 3730xl genetic analyzer. The ends of the
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24 sequences were trimmed in Geneious (<http://www.geneious.com/>) and the sequences were then
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26 matched against existing sequences in the NCBI nucleotide database
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28 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). All accepted matches had maximum (=total) scores
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30 between 928 and 1042, queries of 100%, E values of 0 and identities of 99 or 100%. Because
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32 many results had E values of 0, we chose the result based on maximum score unless this result
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34 was inconsistent with the phylogenetic tree. To check whether assignments of taxonomy based
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36 on blast matches were reasonable, we aligned our trimmed sequences and other representative
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38 sequences of known Ascomycete fungi using SATé (v. 2.2.7,
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40 <http://phylo.bio.ku.edu/software/sate/sate.html>). Uninformative parts of sequences were
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42 removed using Mesquite (v. 3.04, <http://mesquiteproject.wikispaces.com/installation>). GARLI
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44 (v. 0.951, <http://www.bio.utexas.edu/faculty/antisense/garli/garli.html>) was used to construct the
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46 phylogenetic tree, which was visualized in FigTree (v. 1.4.2,
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48 <http://tree.bio.ed.ac.uk/software/figtree/>). We used the Zygomycete genus *Mucor* as the
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4 outgroup. Amplicon lengths of unknown fungi fingerprinted from the leaves from the various
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6 sampling times in the longitudinal study were then matched against the amplicon lengths of the
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8 cultured fungi to establish tentative identities. Because multiple, distinct fungal cultures could
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10 possess PCR product lengths that were the same, in some cases a single taxon in the longitudinal
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12 study could be tentatively assigned to more than one name.
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19 **Results**

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21 The time course of decomposition is expressed in Figure 1. Not surprisingly, the analysis of
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23 variance indicated that time was significant ($P < 0.05$) with respect to decomposition. The most
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25 rapid decomposition was between the first and second collection dates, and the rate declined
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27 between the second and third collection dates. By the third collection date, just over 20% of the
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29 leaves, on average, had decomposed. During the course of decomposition, total phenolics
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31 concentration steadily declined, but the bulk of the decline occurred between the first and second
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33 sampling dates (Table 1). N concentration remained fairly stable for the first three sampling
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35 dates, then increased significantly at the fourth date (Table 1). P concentration declined from the
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37 first to the second date, then remained fairly stable thereafter (Table 1).
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43 When we used all fungal taxa identified in the ARISA (84 taxa), traj produced 6 cohorts
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45 of fungi. We assumed for the purposes of this study that presumptive endophytes were present
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47 on the first sampling before placement on the ground, and that presumptive saprotrophs were
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49 present only after the leaves were placed on the ground. Thus, the six groups included: late
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51 successional saprotrophs (those only present at the last sampling, 31 members), another group of
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53 late successional saprotrophs (those that became more frequent with time but were present before
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55 the last sampling, 14 members), early successional saprotrophs (3 members), mid-successional
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saprotrophs (3 members), non-persistent endophytes (2 members), and a group that was a mixture of persistent endophytes and late successional saprotrophs (31 members).

When we eliminated all taxa occurring on fewer than 10 samples, in order to reduce the probability of including taxa that were either falsely detected (false positives) or of very rare occurrence for which longitudinal patterns cannot be reliably deduced, the total number of fungal taxa was reduced from 84 to 32, and 2 of the 6 original traj cohorts were eliminated. The first cohort (Figure 2A) comprised late successional saprotrophs, the second comprised mid to late successional saprotrophs (Figure 2B), the third comprised early to mid successional saprotrophs (Figure 2C), and the last comprised persistent endophytes (Figure 2D). Notably missing from the results of this analysis of the reduced dataset was the cohort of non-persistent endophytes.

According to the permutational multivariate analysis of variance, time significantly ($P < 0.05$) influenced the structure of both saprotrophic and endophytic fungal communities. Considering only the presumptive saprotrophic fungi, the DCA ordination clearly distinguished among communities of the second, third and fourth sampling dates (Figure 3A). Time generally proceeds from left to right in Figure 3A. As shown by the supplemental data vectors (leaf total phenolics, leaf N, leaf P concentrations), the progression through time was associated with increasing N and P concentrations and decreasing total phenolics concentration (see also Table 1). As seen by the size of the symbol for each sample, there was a significant increase in OTU richness through time, which was verified by Fisher's alpha diversity from FEB to MAY 2015 (Figure 4).

Considering only the presumptive endophytic fungi, the DCA ordination distinguished fairly well among the first, second, third and fourth sampling dates (Figure 3B). However, in contrast with the saprotrophs, the progression through time was associated with increasing N but

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4 decreasing P and decreasing total phenolics concentrations (see also Table 1). Thus the leaf P
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6 vector in Figure 3B points in the opposite direction to that in Figure 3A. These contrasting
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8 results for saprotrophic and endophytic fungal communities were mainly due to the fact that the
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10 saprotrophs were sampled only on the last three dates (as they did not exist in leaves on the first
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12 sampling date, by definition) while the endophytes were present on all four sampling dates. The
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14 largest changes in total phenolics and P concentrations were between the first and second
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16 samplings, while the N concentration changed significantly only between the third and fourth
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18 samplings (Table 1). Time generally proceeds from left to right in Figure 4B. For the
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20 endophytes, in contrast with the saprotrophs, the ordination does not reveal a clear change in
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22 OTU richness with time, but Fisher's alpha diversity decreased significantly from OCT 2014 to
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24 DEC 2014 and then increased slightly but significantly from DEC 2014 to FEB 2015(Figure 4).
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31 Of the approximately 200 distinct fungal isolates obtained from the gambel oak leaves,
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33 the ARISA bins of ten taxa corresponded to those from the experimental leaves (Table 2).
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35 Among those from the group of mid- to late-successional saprotrophs, amplicon 607 matched to
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37 *Alternaria spp.* and amplicon 676 matched to *Aspergillus spp.* Among those from the group of
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39 persistent endophytes, amplicon 584 matched *Botrytis spp.*, amplicon 590 matched
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41 *Cladosporium spp.*, amplicon 595 matched *Cladosporium spp.* or *Phacidium* (formerly
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43 *Ceuthospora) spp.*, amplicon 612 matched *Ulocladium spp.* or *Alternaria spp.*, amplicon 617
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45 matched *Discosia spp.* or *Alternaria spp.*, amplicon 621 matched *Penicillium spp.*, amplicon 639
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47 matched *Alternaria spp.* or *Cladosporium spp.*, and amplicon 688 matched *Mucor spp.*,
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49 *Mortierella spp.*, or *Epicoccum spp.*
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58 **Discussion**

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4 We assumed for the purposes of this study that the OTUs present on the first sampling, when
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6 leaves were alive and asymptomatic, were endophytes, an assumption made by many others
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8 (Petrini, 1991; Arnold et al., 2007). Because endophytic fungi are defined only on the basis of
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10 their existence in living tissues, they do not necessarily share a common function. Thus it seems
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12 quite probable that at least some of the endophyte taxa are incipient pathogens or parasites (in
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14 other words, not necessarily beneficial to the plant). We further assumed that the fungi present
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16 only *after* the first sampling (during the course of decomposition) were saprotrophs, incapable of
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18 living as biotrophs. However, some of these could potentially be parasitic on saprotrophic fungi.
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24 Nevertheless, in a general sense our assumptions used in distinguishing endophytes from
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26 saprotrophs appear to be reasonable. First, the group of fungi we assumed to be saprotrophs
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28 increased in OTU richness and diversity over time, which contributed to significant shifts in
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30 community structure through time as documented in the ordinations and the permutational
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32 analysis of variance. Indeed, one expects saprotrophic species to accrete in the first few months
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34 of litter decomposition (Hogg and Hudson, 1966; Voříšková and Baldrian, 2013). Moreover, the
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36 group of fungi we assumed to be endophytes declined in OTU diversity from the first to second
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38 sampling, again contributing to significant shifts in community structure through time as seen in
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40 the ordinations and permutational analysis of variance. The decline in OTU diversity with time
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42 is consistent with the hypothesis that at least some members of the group are solely biotrophic
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44 and cannot make the switch to saprotrophy or that, even if they do have saprotrophic capacity,
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46 they were competitively excluded by more aggressive saprotrophs.
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53 Second, eight of the fifteen OTUs we presume to be persistent endophytes were
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55 tentatively identified by ITS sequences as taxa previously reported as endophytes. We
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58 were able to provide tentative identities to a number of the OTUs from our experimental
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4 leaves by matching amplicon lengths to those of fungal isolates that we sequenced.
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6 Matching ITS amplicon lengths is certainly not a foolproof method for identifying fungal
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8 species. Indeed, different species may possess the very same ITS amplicon lengths as seen
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10 for our amplicon 688 (see also Liekfeldt and Seifert, 2000). Nevertheless, amplicon length
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12 matching can help us narrow the field of potential species and, indeed, the possibilities we
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14 uncovered were not unexpected. Members of the *Cladosporium*, *Epicoccum*, *Ulocladium*,
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16 *Discosia*, *Mucor*, *Mortierella*, *Alternaria*, *Penicillium*, *Botrytis*, and *Aspergillus* genera, which
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18 we matched to our persistent endophyte taxa, have been previously isolated as endophytes
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20 from broadleaf host plants (Collado et al., 1999; Hata et al., 2002; Fisher et al., 2006; Arnold
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22 et al., 2007; Abdel-Motaal et al., 2010; Vega et al., 2010; Koukol, 2011; Eom, 2012;
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24 Martinez-Alvarez et al., 2012; Peršoh, 2013; Hodgson et al., 2014; Yan et al., 2014; Martín-
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26 Sampedro et al., 2015). Of course we could have utilized high-throughput sequencing in
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28 our analyses had we had sufficient funds. Nevertheless, while it is possible for sequencing
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30 to give us names and evolutionary relationships among fungi, those are currently not
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32 particularly helpful in distinguishing between endophytes and saprotrophs simply because
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34 we do not have a good appreciation for the relationship between lineage and trophic status,
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36 and because a great deal of trophic plasticity exists within a lineage and, indeed, within a
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38 given species. A complementary approach would be to culture all the fungi and determine
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40 their enzymatic capacities in culture in order to distinguish between biotrophs and
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42 saprotrophs, although that approach, too, has its limitations; the physiological behavior of
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44 fungi in culture may not be the same as in nature. And it is certainly possible that at least
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46 some of the fungi growing in leaves are not culturable.
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4 Therefore, in the absence of confirmatory evidence, we acknowledge the possibility of
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6 error. For example, it is possible that none of the endophytes were actually persistent, but that
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8 the DNA of some of them was (England et al., 1998), particularly in the case of highly melanized
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10 fungi (Skucas, 1967). However, because we documented the presence of persistent endophytes
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12 over the course of several months of decomposition, it seems reasonable to assume either that
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14 persistent taxa formed melanized spores or hyphae resulting in persistent DNA, or that they were
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16 truly active as saprotrophs during decomposition.
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21 While the former seems probable, the latter possibility also appears likely (Arnold, 2007;
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23 Porras-Alfaro & Bayman, 2011; Voříšková and Baldrian, 2013). Others have found that at least
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25 some endophytic fungi possess limited saprotrophic capacity (Müller et al., 2001; Hyde and
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27 Soyong, 2008; Sun et al., 2011), demonstrating this by inoculating sterile litter with the
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29 endophytes in question and then documenting litter weight loss. The challenge with that
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31 approach is that when inoculated onto sterile litter, the endophytes in those circumstances were
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33 not in competition with the saprotrophs that would normally be present, and so were functioning
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35 under artificial conditions. Our approach, which involved categorizing fungi based on their
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37 temporal patterns, is also potentially limited as indicated previously, but it is complementary to
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39 the culturing approach previously taken. In a third approach, Hogg and Hudson (1966) observed
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41 on decomposing beech leaves *Cladosporium* spp., *Botrytis* spp. *Mortierella* spp., *Penicillium*
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43 spp. and *Discosia* spp., all of which have members identified as possible persistent endophytes in
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45 the current study. Together, the various approaches provide good evidence that some endophytic
46
47 fungi are capable of persisting during decomposition as facultative saprotrophs. Thus, at least
48
49 some endophytic fungi appear to be rather plastic in their trophic status. Other notable cases of
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51 trophic plasticity have been documented among endophytes. For example, some may have both
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4 an endophytic phase in terrestrial plants as well as an aquatic phase, presumably as saprotrophs
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6 (Selosse et al., 2008). Some fungal species found as leaf endophytes are the same species found
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8 in leaf litter (Unterseher et al., 2013). And endophytes in wood may be latent wood
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10 decomposers (Parfitt et al., 2010). Thus, niche breadths for some endophytic fungi appear to be
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12 much greater than generally believed (Müller et al., 2001; Selosse et al., 2008). Our results,
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14 then, are largely consistent with our hypothesis that some biotrophic endophytes persist in
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16 decomposing leaves via a transition to saprotrophy and thus form communities with purely
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18 saprotrophic species that colonize only upon the death of the leaf. This is consistent with the fact
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20 that some endophytes are evolutionarily closely related to saprotrophs while others are not
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26 (Promputtha et al., 2007).
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29 Among the fungal taxa occurring in at least 10 samples and in addition to the cohort of
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31 persistent endophyte fungi, traj identified 3 cohorts that appear to be saprotrophic including early
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33 to mid-successional, mid to late-successional, and late-successional fungi. Others have also
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35 observed similar waves of succession of fungi on decomposing leaves (Hogg and Hudson, 1966).
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37 The temporal shifts in the structure of endophyte and saprotroph communities were correlated
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39 with changes in leaf chemistry. Litter has frequently been shown to increase in N concentration
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41 as it decomposes (Melillo et al., 1982) due, in part, to the importation of N by saprotrophic fungi,
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43 particularly when C:N ratios are initially high (Boddy and Watkinson, 1995). Total phenolics
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45 concentrations also frequently decline during decomposition due to leaching (Wood et al., 2012;
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47 Wilson et al., 1986). These changes may have contributed to the shifts in the structure of fungal
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49 communities during decomposition (Voříšková and Baldrian, 2013), but it is also probable that
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51 the shifts were at least partly attributable to the simple accretion of species with time as
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53 recruitment naturally occurs on new substrate (Andrews et al., 1987).
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4 Further research is needed to determine the ecological consequences of the persistence of
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6 endophytes during decomposition. For example, if facultative saprotrophic endophytes are
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8 generally less capable of litter decomposition than pure saprotrophs but are nonetheless still
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10 competitive enough to persist throughout decomposition, it is possible that the process of
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12 decomposition and nutrient cycling would be slightly retarded by their presence. This could
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14 have the effect of increasing the size of organic matter pools in soil, thus sequestering more C in
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16 the soil, and reducing nutrient cycling fluxes, possibly reducing primary productivity. Further
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18 research on the consequences of competition between facultative saprotrophic endophytes and
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20 pure saprotrophs appears to be warranted.
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26 The evolution of facultative saprotrophy among endophytic fungi is unknown. Some
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28 have speculated that saprotrophs evolved from biotrophic endophytes (Hyde and Soyong, 2008).
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30 It is also possible that biotrophy among endophytes evolved within a saprotrophic lineage. One
31
32 can envision the selection pressure responsible for maintaining saprotrophy among biotrophic
33
34 endophytes evolving from pure saprotrophs. While pure saprotrophs must wait until the death of
35
36 a tissue before colonizing it, a saprotroph positioning itself among living host cells in
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38 anticipation of their death would have priority over other saprotrophs. Saprotrophic fungi with
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40 some capacity for biotrophy would thus have the last opportunity to obtain carbon biotrophically
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42 and the first opportunity to consume the tissue saprotrophically. The endophytic habit could thus
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44 be, at least in some cases, a special extension of saprotrophy in which the food source is
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46 colonized before death, rather than after, and in which degradative enzyme production is largely
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48 attenuated during the living phase of the host (see Colpaert and van Laere, 1996 for an example
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50 of this in ectomycorrhizal fungi). It is possible, of course, for some endophytes to have evolved
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4 from saprotrophs while some saprotrophs evolved from endophytes. Further research into the
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6 origins of the endophytic fungi is warranted.
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10 11 12 13 **Acknowledgments** 14

15
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Figure 1. Decomposition (dry matter loss) vs. time (5 DEC 2014, 13 FEB 2015 and 14 MAY 2015) for the sampled leaves of *Quercus gambelii*. Different letters indicate means are significantly different according to the Tukey HSD method. n = 27.

Figure 2. The four traj cohorts including only those OTU's occurring at least 10 times, plotted as relative frequency vs. time (18 OCT 2014, 5 DEC 2014, 13 FEB 2015, 14 MAY 2015). A. Late-successional saprotrophs (6 members). B. Mid- to late-successional saprotrophs (9 members). C. Early- to mid-successional saprotrophs (2 members). D. Persistent endophytes (15 members).

Figure 3. Detrended correspondence analysis plots with plots of leaf N, P and total phenolics. The size of the circles is proportional to the sample OTU richness, indicated by the numerals. 18 OCT 2014: yellow, 5 DEC 2014: orange, 13 FEB 2015: blue, 14 MAY 2015: dark green. A. Saprotrophic fungi only. Eigenvalues for axes 1-4 were 0.431, 0.222, 0.162, 0.134, respectively. Only first two axes shown (x is axis 1). B. Endophytic fungi only. The eigenvalues for axes 1-4 were 0.224, 0.145, 0.109, 0.087, respectively. Only first two axes shown (x is axis 1).

Figure 4. Fisher's alpha diversity indices for fungal endophytes and saprotrophs vs. time (18 OCT 2014, 5 DEC 2014, 13 FEB 2015, 14 MAY 2015). Vertical bars define the 95% confidence interval.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1. Mean (standard error of the man) of leaf total phenolics concentration (mg tannic acid equivalents per g), N concentration (% of dry weight) and P concentration (% of dry weight) before and during decomposition. Different letters indicate means are significantly different according to Tukey HSD 95% method. Phenolics: n = 8 except 18 Oct 2014 n=5; N and P: n = 8 except 18 Oct 2014 n = 9 and 5 Dec 2015 n=7.

Sampling date	Total phenolics	Nitrogen	Phosphorus
18 October 2014	92.8 (3.5) A	1.22 (0.059) B	0.140 (0.007) A
5 December 2014	25.7 (2.8) B	1.32 (0.067) B	0.086 (0.008) B
30 January 2015	12.7 (2.8) C	1.36 (0.062) B	0.091 (0.008) B
14 May 2015	3.8 (2.8) C	1.60 (0.062) A	0.091 (0.008) B
Analysis of variance F test	<0.0001	<0.0001	<0.0001

Table 2. List of OTUs (amplicon length), group membership and some tentative identities.

Late successional saprotrophs	Mid to late successional saprotrophs	Early to mid successional saprotrophs	Persistent endophytes
448	560	601	584 <i>Botrytis spp.</i>
465	565	740	590 <i>Cladosporium spp.</i>
569	594		595 <i>Cladosporium spp.</i> <i>Phacidium</i> <i>(Ceuthospora) spp.</i>
574	607 <i>Alternaria spp.</i>		610
579	676 <i>Aspergillus spp.</i>		612 <i>Ulocladium spp.</i> <i>Alternaria spp.</i>
704	682		617 <i>Discosia spp.</i> <i>Alternaria spp.</i>
	695		621 <i>Penicillium sp.</i>
	707		626
	717		632
			635
			639 <i>Alternaria spp.</i> <i>Cladosporium spp.</i>
			643
			661
			672
			688 <i>Mucor spp.</i> <i>Mortierella spp.</i> <i>Epicoccum spp.</i>