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Pervasive effects of wildfire on foliar endophyte communities in montane forest trees

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Abstract

Plants in all terrestrial ecosystems form symbioses with endophytic fungi that inhabit their healthy tissues. How these foliar endophytes respond to wildfires has not been studied previously, but is important given the increasing frequency and intensity of severe wildfires in many ecosystems, and because endophytes can influence plant growth and responses to stress. The goal of this study was to examine effects of severe wildfires on endophyte communities in forest trees, with a focus on traditionally fire-dominated, montane ecosystems in the southwestern USA. We evaluated the abundance, diversity, and composition of endophytes in foliage of *Juniperus deppeana* (Cupressaceae) and *Quercus* spp. (Fagaceae) collected contemporaneously from areas affected by recent wildfire and paired areas not affected by recent fire. Study sites spanned four mountain ranges in central and southern Arizona. Our results revealed significant effects of fires on endophyte communities, including decreases in isolation frequency, increases in diversity, and shifts in community structure and taxonomic composition among endophytes of trees affected by recent fires. Responses to fire were similar in endophytes of each host in these fire-dominated ecosystems and reflect regional fire-return intervals, with endophytes after fire representing subsets of the regional mycoflora. Together these findings contribute to an emerging perspective on the responses of diverse communities to severe fire, and highlight the importance of considering fire history when estimating endophyte diversity and community structure for focal biomes.

Keywords

Ascomycota; diversity; Dothideomycetes; Eurotiomycetes; fire history; forest management; Leotiomyces; Pezizomycetes; phenology; Sordariomycetes; symbiosis

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Introduction

Wildfire is a major ecological and evolutionary force that has shaped plant traits, community structure, and ecosystem processes in tropical, temperate, and boreal biomes [1,2]. Modern wildfires in ecosystems ranging from grasslands to forests reflect complex interactions of biogeography, climate, land use, and fire management [3-6]. For centuries, coniferous woodlands of western North America have been shaped by episodic, widespread, low-to-moderate intensity surface fires [7]. However, more than a century of fire suppression, coupled with widespread drought, has increased the incidence of highly intense fires that pervasively affect plant communities at local and regional scales [5,8,9].

Especially in the context of regional and global climate change, effects of altered fire regimes on forest ecosystems are of growing concern [4,6,10,11]. In addition to affecting vegetation and soil directly (e.g., [12-15]), fires profoundly influence the diverse microbial communities that shape plant health and soil traits [16-19], and thus can influence forest regeneration. Forest trees form symbioses with fungi that can mitigate responses to the biotic and abiotic stresses that result from major fire events (e.g., [20-22]). Severe fires can impact these fungal communities directly, or can alter the capacity of plants to re-establish or maintain symbioses by altering plant physiology, phenology, or tissue chemistry [17,23-27]. Such effects can reflect fire-return intervals, in some cases consistent with the historic fire regime under which members of these communities evolved (see [28]). Although responses of root-associated fungi to fire have been characterized in some ecosystems [29-31], effects of fire on communities of foliar symbionts in forest trees have not been studied in detail.

Recent studies have revealed a high diversity of fungi in symptomless foliage of trees in diverse ecosystems, including traditionally fire-dominated forests of the southwestern USA [20,32-39]. These fungal endophytes (class 3 endophytes, sensu [40]; hereafter, endophytes) live within healthy leaves and stems without causing disease. They form highly localized infections and generally are transmitted horizontally among hosts, usually colonizing newly flushed leaves or entering through wounds in leaf tissue [20]. In addition to encompassing very high species richness and producing a rich array of secondary metabolites (e.g., [41,42]), some endophytes benefit the plants they inhabit by enhancing drought resistance, thermotolerance, and protection against pathogens and herbivores (see [20,35,40,43,44]). However, effects of fire on endophyte diversity and composition in foliage, and the capacity of endophyte communities to regenerate locally from regional pools, have not been evaluated.

The goal of this study was to examine the effects of severe wildfire on endophyte communities in ecologically important genera of forest trees, with a focus on traditionally fire-dominated, montane ecosystems in the southwestern USA. Specifically we examined the abundance, diversity, and composition of endophytic fungi in healthy foliage collected contemporaneously from trees in areas affected by recent, severe wildfire (i.e., trees that burned and resprouted new foliage following major fire events within the past 1-18 y) and paired areas not affected by recent fire (i.e., trees in proximate areas in which severe fires have not occurred within >50-100 y) in each of four mountain ranges in central and southern

Arizona. We focused on trees that often survive and regenerate from basal shoots after crown-killing fires in this region: *Juniperus deppeana* (Cupressaceae) and *Quercus* spp. (Fagaceae). Using a culture-based approach we tested the broad hypotheses that (a) recent, severe fires would alter the abundance, diversity, and composition of endophyte communities in regenerating trees relative to those in proximate, unburned trees; and (b) such differences would decrease as time since fire increases.

Using >1100 endophyte isolates from healthy foliage we tested five focal predictions relevant to these hypotheses: (1) Endophyte abundance will decrease in foliage of trees that were affected by recent, severe fires relative to nearby conspecifics not directly affected by such fires. (2) Endophyte diversity will decrease as a function of fire, as above. (3) These effects of fire will be similar in direction and scope for each tree species, consistent with a community response in these traditionally fire-dominated ecosystems. (4) Community composition (i.e., fungal species- and taxonomic composition at higher levels) will be altered significantly by fire, as above. (5) Fungal endophytes recruited after severe fires will represent distinctive subsets of the regional mycoflora. We evaluated the context of our findings by assessing the relationship of endophyte abundance and diversity to leaf nutrient content, herbivory, and pathogen damage, all of which are sensitive to severe wildfires and may strongly influence the structure and composition of endophyte assemblages.

Methods

Study sites

This study was conducted in four mountain ranges in central and southern Arizona, USA: the Santa Catalina Mountains, Santa Rita Mountains, and Chiricahua Mountains (Coronado National Forest (NF)), and the Sierra Prieta (Prescott NF) (Fig. 1, Table 1). The study areas in each mountain range are generally similar in terms of elevation, temperature, rainfall, and forest structure (Table 1). The canopy in each site generally consists of scattered to dense *Pinus arizonica* (Coronado NF) or *P. ponderosa* (Prescott NF), with an understory of *Juniperus deppeana* and *Quercus* spp. [45-48].

Field collections were conducted in 2011-2014 (Table 1). Forests in each mountain range experienced severe crown-killing wildfires 1-18 y before our sampling: the 1994 Rattlesnake Fire and 2011 Horseshoe II Fire (Chiricahua Mountains), 2003 Aspen Fire (Santa Catalina Mountains), 2005 Florida Fire (Santa Rita Mountains), and 2013 Doce Fire (Sierra Prieta) (Table 1, Fig. 2). Fire data were obtained from published records in which fire intensity, duration, and other characteristics are described in detail (Table 1).

Sampling structure

In Coronado NF we focused on *J. deppeana* (alligator juniper) and *Q. hypoleucoides* (silverleaf oak), which co-occur and were common and accessible in all sites. In Prescott NF we focused on *J. deppeana* and *Q. turbinella* (shrub live oak), as this area lies beyond the northern range limit of *Q. hypoleucoides*. All three focal species are evergreen and resprout from mature underground biomass following crown-killing fires in Arizona (e.g., [49-51]; Fig. 2).

Within each mountain range, we identified areas that were severely burned in each focal fire, and paired each burned area with an unburned area of similar slope, elevation, and aspect (Fig. 2). Unburned sites were located < 2km from burned sites in each mountain range. Examination of fire records and fire history studies in the sample sites [52] coupled with a lack of recent fire scarring, an abundant growth of lichens, and dense understory conditions (Fig. 2), indicated that these ‘unburned’ areas had not experienced crown-killing wildfires in at least 50 y, and in several cases, in at least 100 y (e.g., Prescott NF). Here we conservatively refer to these sites as having not experienced severe fire in > 50 y.

In burned areas of each mountain range, we identified three microsites approximately 15-20 m in diameter along a ca. 500 m transect (Table 1). In each microsite, we collected living, mature foliage from two trees of each species that had burned severely and had resprouted (Fig. 2), and two trees of each species that were located on the edge of the burned area and showed evidence of severe fire scarring consistent with the timing of the focal fire. These four individuals/species/microsite were treated as ‘affected by fire,’ and data on their fungal communities were combined after preliminary analyses indicated that they did not differ significantly in endophyte abundance, diversity, and composition as a function of occurring within vs. on the edge of the same burned areas. Where necessary we validated this observation by extending the number of microsites within burned areas of a focal range (Santa Rita Mountains), which allowed us to rule out local effects of slope, aspect, or exposure as major determinants of endophyte community traits (data not shown). We also repeated sampling within the Santa Rita Mountains across multiple seasons to examine whether the season in which sampling occurred would alter our inferences (addressed below).

This sampling was paired in each mountain range by collections from each focal tree species in three microsites along a similar transect in areas not affected by wildfire within the last 50 y, as described above (Fig. 2). In all cases, trees in areas affected by recent fire were similar in age to trees sampled in the unburned areas.

Tissue processing

From each tree we collected healthy, mature foliage from three branches chosen haphazardly ca. 1-2 m above ground. Leaves were of similar ages regardless of fire history. In total, 80 individual trees were sampled (Table 1, Supplementary Table 1).

Plant material was refrigerated within 24 h and processed within 72 h of collection. Samples were washed with tap water (30 s), cut into 2 mm² pieces, and surface-sterilized by sequential immersion in 95% EtOH (10 s), 0.5% NaOCl⁻ (2 min; diluted Clorox bleach), and 70% EtOH (2 min) [34]. Forty-eight (Santa Rita collections in 2011 and 2013) or 96 (all other collections) tissue segments per individual were then plated on 2% malt extract agar (MEA). We found no evidence for differences in isolation frequency or diversity as a function of the number of tissue segments processed per individual (multiple regression with fire age class set as a random factor, and 48 vs. 96 tissue segments serving as the explanatory variable, log-transformed isolation frequency: $F_{1, 55.3} = 0.0483$, $P = 0.8269$; fire age class and host species set as random factors, log-transformed diversity: $F_{1, 12.1} = 0.70$, $P = 0.4184$); therefore, data for the two sampling approaches were combined for analysis. Host

taxon was not considered in that analysis because subsequent analyses revealed that these host taxa did not differ in isolation frequency (below).

Tubes were sealed with Parafilm and incubated at room temperature with 12 h light/dark cycles for up to 6 mo. Emergent hyphae were isolated into pure culture on 2% MEA, vouchered in sterile water, and deposited at the Robert L. Gilbertson Mycological Herbarium (MYCO-ARIZ; accession nos. FF001-FF210, YLH0001-YLH0131, BC0001-BC0643, CM0001-CM0093, SR0001-SR0209, DF0001-DF0058).

Molecular analysis

Total genomic DNA was extracted from each isolate following Arnold and Lutzoni [32]. The nuclear ribosomal internal transcribed spacers, 5.8S gene, and D1-D2 region of the nuclear ribosomal large subunit were amplified as a single fragment (ca. 1000-1200 basepairs; ITSrDNA-partial LSU rDNA) with primers ITS1F and LR3 following Hoffman et al. [36]. If initial amplification failed, PCR was repeated with ITS5 and LR3, followed by ITS1F or ITS5 and ITS4 [39]. PCR products were visualized using SYBR green following electrophoresis on a 1% agarose gel in TAE [53] and submitted to the University of Arizona Genetics Core for cleanup, normalization, and bidirectional Sanger sequencing.

Sequences were assembled automatically and bases called using *phred* and *phrap* [54,55] with orchestration by Mesquite [56], followed by manual editing in Sequencher 4.5 (GeneCodes Corp.).

Sequences were compared against the NCBI non-redundant database using BLASTn to estimate taxonomic placement (Supplementary Table 2). Overall, bidirectional sequences of high quality were obtained for 1289 of 1337 isolates (96.4%), and unidirectional, high-quality sequences representing either the ITSrDNA or partial LSUrDNA were obtained for 9 additional isolates. Sequences have been submitted to GenBank (accession nos. KP990806-KP992103).

Sequences representing these 1298 isolates were analyzed using the *sanger_otu_clustering_workflow* implemented in the Moby SNAPP workbench [57,58]. The assembly pipeline, developed by [59], uses UCHIME [60] to detect chimeric sequences by checking a reference database of all fungal ITSrDNA sequences in GenBank that are named to the species level. It then uses the Fungal ITS Extractor [61] to automate the extraction of the ITSrDNA (i.e., ITS1-5.8S-ITS2) to assemble operational taxonomic units (OTU). After one potentially chimeric sequence was removed and filtering was complete, 1184 sequences containing both ITS1 and ITS2 were aligned in a pairwise fashion in ESPRIT [62,63]. The resulting distance matrix was used to place sequences into OTU using the furthest neighbor method in mothur [64,65]. For statistical analyses, OTU were based on 95% ITSrDNA sequence similarity [66]. More stringent groupings yielded qualitatively similar conclusions but were more limited in inference because of the high proportion of singletons, which precluded robust community analyses.

Structure of analyses

Units of analysis were defined as individual trees sampled under particular burn-status conditions in a given season and year. Time since fire was calculated based on fire data from published references or public records [49,67-70]. Endophyte isolation frequency was defined as the percent of tissue segments yielding an endophyte in culture. Diversity was measured using Fisher's alpha, which is robust to variation in sample size [66]. Isolation frequency and diversity were evaluated using multiple regression and analysis of variance (ANOVA) implemented in JMP 11.0 (SAS Institute, Cary, NC). Community structure at the OTU level was evaluated by analyses of similarity (ANOSIM) conducted on nonsingleton OTU, and results were visualized using non-metric multidimensional scaling (NMDS) in PAST V2.17 [71]. Analyses were conducted using both Jaccard's index (based on presence or absence) and the Morisita index (based on abundance). Taxonomic assignments were limited to the class level and were inferred by first querying BLASTn [72] (queries limited to Fungi in the database, taxid: 4751; results included 50 aligned sequences). BLAST files were imported to MEGAN5 [73,74] for parsing taxonomy (LCA parameter: max. matches = 50, min. score = 400, max. expected = 0.01, top percent = 10, min. support = 1, min. complexity = 0.3, use identity filter).

Preliminary analyses

Because isolation frequency per tree was often low (Supplementary Table 1), we explored ways to pool data for analysis of fire effects. In this region, historical fire intervals have been determined by fire-scar data and tree-ring records [5,7,75-80]. Fire-return intervals averaging 6 y were estimated for the period ca. 200 y before 1900 [75,81], leading us to use 6 y since fire as the division between very recent fires (i.e., those occurring 6 y before our sampling events) and recent fires (occurring 7-18 y before our sampling events). We observed marked changes in repeated sampling of the same burned area in the Santa Rita Mountains at points ranging from 6 y to 9 y since fire, providing confidence in the meaningfulness of the split between the 6 y and 7-18 y timeframes (Supplementary Fig. 1).

Trees in the > 50 y fire age class were sampled in each mountain range, but not all mountains contained areas burned in both the < 6 y and 7-18 y fire age classes. As a result, geographic differences in endophyte communities could have been misinterpreted as effects of time since fire. Therefore, we evaluated whether endophyte communities differed intrinsically among study sites (see Fig. 1, 2; Table 1). Specifically, we tested the null hypothesis that isolation frequency, diversity, community structure and taxonomic composition of endophytes are consistent among trees in the oldest fire age class (i.e., > 50 y since fire), independent of geographic location. We found no differences in these aspects of endophyte community structure as a function of geographic location (Fig. 3), allowing us to decouple location and fire for further analyses.

Tests of focal predictions

Because fire can negatively impact microbial biomass and abundance [82], we predicted endophyte abundance would be lower in trees affected by recent fires than in the nearby conspecifics not affected by these fires (prediction 1). We collected leaves of similar ages

from trees of similar ages in each fire age class, ruling out effects of tissue- or host age in influencing endophyte abundance. To address this prediction, isolation frequency values were log-transformed and evaluated using multiple regression to assess the effects of host taxon, fire age class, and the host taxon $\times$ fire age class interaction, with sampling season set as a random effect. Sampling season was included because exploration of the data revealed a significant relationship with isolation frequency (ANOVA, $F_{2, 78} = 6.95$, $P = 0.0017$), but it was treated as a random effect because sampling season was not central to our evaluation of fire effects. Host taxa were defined as *J. deppeana* and *Quercus* spp. in this and subsequent analyses.

As above, we predicted that endophyte diversity in trees that were affected by recent fires would decrease relative to that in the trees not affected by such fires (prediction 2). To address this prediction, Fisher's alpha values were log-transformed and evaluated using multiple regression to assess the effects of host taxon, fire age class, and the host taxon $\times$ fire age class interaction. Individual trees with fewer than eight sequenced cultures were excluded from analyses. Season was not included as a random effect in these analyses because exploration of the data indicated a nonsignificant relationship of sampling season to endophyte diversity in these evergreen trees (ANOVA, $F_{2, 20} = 3.23$, $P = 0.0610$). We found no relationship of sampling season to the variables described in the remaining predictions, such that it was not included in further analyses.

Because trees in these forests experienced periodic fires for centuries, we predicted that fire effects on endophytes would be similar in direction and scope for communities in each host taxon (prediction 3). This was evaluated using the interaction terms (i.e., host taxon $\times$ fire age class) in the analyses of isolation frequency and diversity, above.

Because severe fire can markedly alter microbial communities, we anticipated a shift in community structure as a function of fire. Specifically we predicted that fungal species (OTU) composition, and taxonomic composition at higher levels, would be altered significantly by recent fire (prediction 4). We first used ANOSIM with visualization by NMDS to examine the effects of fire age class on endophyte communities at the OTU level. Only non-singleton OTU were included in these analyses, and we excluded individual trees from which fewer than three OTU were obtained. We then inferred taxonomic placement at the class level for 1298 sequences as described above, and used chi-square analysis to test whether taxonomic composition differed as a function of fire age class for each host taxon.

Finally, the endophytes studied here are horizontally transmitted and thus accumulate in foliage primarily from air spora. If fire alters host plants such that distinctive communities are recruited from the available air spora, we predicted that post-fire endophyte communities would represent distinctive subsets of the regional mycoflora (prediction 5). We compared data from trees affected by recent fire against endophyte collections obtained from additional sites across southern Arizona in which fires had not occurred in at least 50 y. These regional collections comprise 161 additional endophyte isolates from healthy, mature foliage of *J. deppeana*, *Q. hypoleucoides*, and *Quercus* spp. collected in 2006-2010 from unburned areas of the Santa Catalina Mountains [36], Chiricahua Mountains [39], and Madera Canyon (an unburned riparian area at the base of the Santa Rita Mountains;

N31.7250°, W110.8794°; 1497 m.a.s.l.; [36]). These sites comprise mixed forests that are taxonomically and structurally similar to the forest at our primary study sites, and none of the individuals surveyed showed signs of recent fire damage. These data were combined with our data from trees in > 50 y fire class (total N = 406 isolates) and evaluated with ANOSIM to test the null hypothesis that endophytes in trees affected by very recent fire (i.e., < 6 y since fire; N = 400 isolates) or somewhat recent fire (i.e., 7-18 y since fire; N = 753 isolates) do not differ significantly from the regional mycoflora.

Measurements of damage by herbivores and pathogens

Because severe wildfires can influence co-occurring organisms such as insects and pathogenic fungi [83] that may, in turn, influence endophyte community structure, we scored focal branches for damage by insect herbivores and lesions consistent with damage by fungal pathogens. We focused on *Quercus* spp. because leaf damage was difficult to quantify on the distinctive foliage of *J. deppeana*. Branches collected for endophyte isolation were scored for each damage type on a scale from 0 (healthy branch without evidence of damage by herbivores or pathogens) to 5 (all leaves on the branch showed evidence of damage), with scores from 1 to 4 representing 25% increments of damage severity.

Nutrient analysis

Severe fire is often associated with changes in leaf nutrients [84-87]. Because we found that several measures of endophyte community structure were sensitive to fire, we evaluated whether systematic differences in leaf nutrient status could explain observed differences in endophyte assemblages. We focused on leaf Ca, K, Mg and N because these macronutrients can be influenced by fire and may be important for endophyte growth [84,86].

Leaves from three branches of 56 individual trees (Supplementary Table 3) were washed vigorously in running tap water, rinsed with sterile distilled water, and dried for 72 h at 60°C prior to grinding. Total Ca, K, Mg, and N were assessed for each sample by NCS analysis on a NA 1500 Elemental Analyzer (Carlo Ebra, Milan, Italy; N) or acid digestion and evaluation with a Model 5300 optical emission spectrometer (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA, USA; Ca, Mg, K).

Data for each nutrient were log-transformed for normality and then compared between host species using t-tests. We used simple linear regression to determine whether endophyte isolation frequency and diversity were significantly associated with each measure of leaf chemistry for each species. To examine effects of fire age class on each aspect of leaf chemistry, we accounted for different sampling seasons and potential variation as a function of location by saving residuals from simple linear regressions of each of those explanatory variables in sequence. These residuals were then used as response variables in ANOVA that separately compared Ca, K, Mg, and N as a function of fire age class for each host species.

The functional importance of leaf chemistry was assayed by evaluating fungal growth on leaf extract media *in vitro*. Media were prepared by blending 5 g of washed *J. deppeana* or *Q. hypoleucoides* foliage from the Santa Catalina and Chiricahua Mountains with 50 mL of

distilled water. Blended mixtures were vacuum filtered on Whatman filter paper and autoclaved to yield media containing 1% plant extracts and 1.5% agar. Four fungal strains were evaluated for growth on six 100 mm plates per medium type and mountain range of origin. Colony radius was measured every two days until fungal growth reached the edge of the plate. Student's t-tests were used to compare colony radius 2 d prior to total coverage of the plate. Because no differences were observed according to the mountain range from which the foliage was obtained (data not shown), data from the two mountain ranges were combined for analysis to quantify fungal responses to leaf chemistry.

Results

Endophytic fungi were isolated from asymptomatic foliage of *J. deppeana* and *Quercus* spp. in four focal mountain ranges in central and southern Arizona, in all study seasons, and in areas affected by significant wildfires over very recent (< 6 y), recent (7-18 y), and historical timeframes (> 50 y; Table 1, Supplementary Table 1, Fig. 1-3). Overall, 8832 tissue segments were processed, from which 1337 isolates were obtained (overall isolation frequency, 15.1%). Overall, 1184 sequences were segregated into 95 OTU (overall Fisher's alpha = 24.3; Supplementary Table 4).

Isolation frequency, diversity, community structure, and taxonomic composition of endophytes did not differ as a function of location (i.e., mountain range; assessed using trees of each species that had not experienced severe fire in > 50 y) (Fig. 3). Thus we evaluated responses of these community parameters to fire effects across four mountain ranges.

In preliminary analyses, we observed differences in endophyte communities as a function of host taxon (Fig. 4), and no interaction was detected between fire age class and host taxon (Supplementary Table 5). Many OTU occurred in both *Juniperus* and *Quercus*, albeit at different frequencies (Supplementary Table 4). Therefore, we considered host taxonomy in tests of our predictions, below.

Isolation frequency (prediction 1)

Isolation frequency differed as a function of fire age class, but not as a function of host taxon or as a function of the fire age class $\times$ host taxon interaction (Table 2). Least-squares means contrasts revealed significant differences in isolation frequency between trees in the recent fire age classes vs. those that have not experienced fire in > 50 y (Table 2, Fig. 5). However, isolation frequency did not differ significantly for the < 6 y vs. 7-18 y fire age classes (Table 2, Fig. 5).

Endophyte diversity (prediction 2)

Endophyte diversity differed as a function of fire age class and between host taxa, but not as a function of the fire age class $\times$ host taxon interaction (Table 3). Least-squares means contrasts revealed significant differences in diversity among all fire age classes (Table 3, Fig. 5). Diversity was greatest in trees affected by the most recent wildfire (< 6 y since fire). Overall, endophyte diversity in *J. deppeana* (mean Fisher's alpha per tree = 5.9, 95% confidence interval: 4.1-7.7) was higher than in *Quercus* spp. (Fisher's alpha = 1.8; 95% CI = 0.1-3.4) ($t_{30} = -3.45$, $P = 0.0017$).

Fire age class and host taxon interaction (prediction 3)

Overall, the lack of significance for the fire age class $\times$ host taxon interaction term in analyses of isolation frequency and diversity suggests broadly similar responses by endophyte communities in *J. deppeana* and *Quercus* spp. to severe fire (Tables 2, 3, Fig. 5).

Community structure and taxonomic composition (prediction 4)

At the OTU level, endophyte communities differed as a function of fire age class (data from *J. deppeana* only; Morisita index, $R = 0.1751$, $P = 0.0069$; Fig. 6). A similar trend was evident when communities were evaluated using Jaccard's index ($R = 0.0962$, $P = 0.0857$). Pairwise comparisons among fire age classes are shown in Fig. 6.

Among 1298 sequenced isolates, 1276 were defined as Fungi by MEGAN. Of these, one was assigned to the Basidiomycota (Agaricomycetes); all others were Ascomycota. In the Ascomycota, 37 sequences matched fungal endophytes from other studies that were not classified at the class level.

The remaining 1238 sequences were placed into five classes of Pezizomycotina: Dothideomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes and Pezizomycetes.

The relative abundance of each class differed significantly as a function of fire age class for both *J. deppeana* ($\chi^2_{10} = 153.08$, $P < 0.0001$) and *Quercus* spp. ($\chi^2_{10} = 238.12$, $P < 0.0001$; Fig. 7). In both host taxa, Pezizomycetes were only found in trees that experienced fires in the last 18 y (Fig. 7). Sordariomycetes and Dothideomycetes were especially prevalent in trees with a very recent history of fire (< 6 y; Fig. 7). In contrast, Leotiomycetes were rare (*J. deppeana*) or absent (*Quercus* spp.) in very recently burned trees, and became more frequent with increasing time since fire (Fig. 7).

Regional recruitment (prediction 5)

When compared against additional samples from conspecific hosts in unburned sites, communities in the foliage of trees affected by recent fires did not differ significantly from the regional mycoflora that occurs in unburned, conspecific trees across southern Arizona (Fig. 8).

Damage by herbivores and pathogens

We found no relationship between leaf damage scores and endophyte isolation frequency or diversity (Supplementary Fig. 2). ANOVA revealed that herbivory damage scores were significantly lower for trees in the < 6 y fire age class than in the 7-18 y or > 50 y age class, but pathogen damage scores did not differ significantly as a function of fire age class (Supplementary Fig. 2).

Leaf nutrients

Ca, Mg, and N content differed significantly between foliage of *J. deppeana* and *Q. hypoleucoides*, but K did not (Fig. 9). These differences were independent of season or geographic location (Fig. 9). *In vitro* analyses demonstrated significant responses of fungal growth to leaf extracts as a function of host taxon (Supplementary Fig. 3).

Foliar Ca, K, Mg, and N were not correlated with endophyte isolation frequency or diversity in *J. deppeana* (Supplementary Fig. 4). Foliar Mg and N were negatively correlated with isolation frequency in *Q. hypoleuroides*, and K was positively correlated with diversity in that species (Supplementary Fig. 5).

When effects of season and geographic location were taken into account, we found no relationship of these foliar nutrients to fire age class (Supplementary Fig. 6). Thus, factors other than fire history may be important in shaping leaf nutrient profiles in these tree species, and changes in leaf chemistry as a function of fire cannot explain fire effects on endophyte abundance or diversity.

Discussion

Fire is an important ecological and evolutionary force that shapes plant traits, plant community dynamics, and the associations of plants with other organisms [1,2,88-90]. Severe fires affect vegetation and soil directly [12-15] while also influencing the diverse microorganisms that shape plant productivity [16-19]. Foliar fungal endophytes are common inhabitants of plants in all terrestrial biomes, including those with historic patterns of periodic fire [59]. The goal of our study was to examine the effects of severe, crown-killing fires on foliar endophytic symbionts of forest trees, with a focus on historically fire-dominated, mixed-coniferous forests in the southwestern USA.

We compared cultivable foliar endophytes among trees that differed in fire exposure. Focal trees occurred within and at the edge of areas that burned severely in major wildfires over the past 1-18 y, and in paired areas within 2 km in which fire had not occurred in at least 50 y (Table 1, Fig. 2). Consistent with our overarching hypothesis, we recorded significant effects of wildfire on endophyte communities. In particular, our surveys in four mountain ranges revealed significant differences in isolation frequency, diversity, community composition, and taxonomic composition of endophytes in trees that experienced severe fire in the past 18 y vs. those that have not experienced fire in > 50 y. These aspects of endophyte community structure could not be explained by geographic location alone. Communities in each focal tree species generally responded similarly to severe fire, prompting the hypothesis that endophytic symbionts in other fire-influenced biomes would have similar responses in terms of isolation frequency, diversity, and composition.

Community reorganization after fire

Although Ascomycota were common as endophytes in trees regardless of fire age class, the relative abundance of particular fungal classes differed markedly: some classes (e.g., Pezizomycetes) were found only in trees that experienced recent fire; others (e.g., Leotiomycetes) were rare to absent in such hosts, yet present in trees with a long history of no fire exposure. Pezizomycetes include several well-known pyrophilous species [91,92], but it is not yet clear whether the isolates observed here are truly pyrophilic or fire-tolerant, have a phenology that corresponds to altered timing of leaf emergence after fire, or are early colonizers after disturbances (including fire).

Differences in diversity, community structure, and taxonomic composition as a function of fire have been reported for other functional groups of fungi in forest biomes. For example, fungal species richness increased after fire among ectomycorrhizal fungi in Mediterranean forests [93], consistent with our observations of highest endophyte diversity in the most recent fire age class (Fig. 5). These findings contrast with decreases in diversity and abundance of arbuscular mycorrhizal fungi observed by [94], but are similar to other studies (e.g., [23]). In turn, diversity of ectomycorrhizal fungi in *Pinus* forests in the Alps was significantly lower in sites that experienced severe fire in the past 5 y vs. in unburned sites [95]. These communities began to resemble those in burned areas by ca. 15-18 y after fire [95], similar to the timeframe observed in the present study. Similarly, species richness and community composition of mycorrhizal, lichenized, saprobic, and biotrophic fungi differed from those in unburned areas of *Pinus* plantations in northern Europe [96,97]. Crown-killing fires in those sites were associated with major changes in sporocarp production, including a high prevalence of pyrophilous species, for ca. 3 years after fire [96,97].

In a meta-analysis of microbial and fungal biomass after fire, Dooley and Treseder [82] reported that fire effects differ markedly among biomes. The pace of changes observed in our study sites may reflect relatively arid conditions and long generation times for endophytes, which typically reproduce when leaves senesce [20,37]. As described by [25,27], traits such as heat tolerance of propagules may explain the community-level reorganization observed here and in studies of other fungal guilds (see [23-31,82,93,95-97]). Our study suggests evaluating the relative importance of fire tolerance, pyrophilous life history, and/or post-disturbance colonization by endophytes will be insightful with regard to broader perspectives on fungal ecology in fire-driven ecosystems.

Effects of time since fire

Especially striking differences in endophyte assemblages were observed between trees affected most recently by severe fires (i.e., 6 y since fire) and trees without a recent fire history (> 50 y since fire): isolation frequency, diversity, community structure, and taxonomic composition all differed significantly across these fire age classes. In particular, diversity was 3.2 times greater in trees that experienced very recent wildfire relative to trees that have not burned in > 50 y, even though we focused on trees and leaves of a similar age in each fire age class. Colonization of newly flushed foliage after fire may include a broad sample of the available regional mycoflora, which then may be influenced by factors such as host-driven selectivity or competition among endophytes [32,37,98,99]. Leaf damage (e.g., by herbivores and pathogens) may alter endophyte colonization [20], but our data do not suggest that fire-induced changes in leaf damage strongly shapes endophyte isolation frequency or diversity in these hosts. Instead, particular fungi from the regional pool may colonize new foliage that emerges in a distinct timeframe after fire relative to standard seasonal patterns of leaf flush. Here we found that seasonality may influence endophyte isolation frequency but not diversity or composition, broadly consistent with other studies in temperate systems [100-102].

Notably, endophyte communities in trees that resprouted after burning up to 18 y ago did not always resemble those in trees without a recent fire history: isolation frequency, diversity,

and community composition differed significantly between the 7-18 y and > 50 y age classes. Thus, differences in endophyte communities may be detectable in several measures for ca. two decades after crown-killing fire events. The pace at which endophyte communities re-establish to match those in trees without a recent fire history may vary between tree species: in *Quercus* spp., communities in plants affected by fire 7-18 y ago resembled the taxonomic distributions found in congeners without a recent history of fire, but in *J. deppeana* significant differences remain between these two fire age classes. Notably, measures of isolation frequency and diversity were insufficient to reveal these more subtle aspects of endophyte community structure. More generally, it is possible that major wildfires will alter communities in a manner that precludes a 'return' to conditions similar to those in proximate trees without a history of recent fire, in part reflecting natural variation in available propagules (see [103]). However, our data suggest that in some ways (e.g., taxonomic composition of endophytes at the class level in *Quercus* spp.; similar community structure based on presence/absence data in *J. deppeana* for the 7-18 and > 50 y fire age classes), resemblance to proximate trees that have not experienced fire is possible. This is especially plausible if trees that re-sprout after fire are colonized by the regionally available mycoflora, as was observed here. Our observation that community differences were more difficult to detect when based on abundance, vs. presence/absence data alone, also is consistent with a lack of strict-sense specificity to host or fire age class.

Host affinity

Because wildfires often alter plant nutrient content [84-87], we examined whether leaf chemistry could explain observed differences in endophyte isolation frequency, diversity, and community composition as a function of fire. We found that leaf Ca, K, Mg, and N did not differ systematically as a function of time since fire. It is possible that effects of fire would be more detectable in the first leaf flush after fire.

Our analyses reveal strong differences in leaf chemistry as a function of host species. Differences between hosts correspond with significant differences in endophyte diversity and community composition between *J. deppeana* and *Quercus* spp. Host specificity has been described in endophytes in several settings [37,99,104,105], and previous studies suggest that host specificity reflects leaf chemistry [106-109]. However, it is premature to infer that aspects of leaf chemistry studied here select for particular endophyte communities, in part because many fungal OTU were present in both host taxa (albeit at different frequencies; Supplementary Table 4). As was shown previously for endophytes of forest trees in the tropics [106], leaf extracts from these tree species can influence fungal growth *in vitro*. However, further work is needed to determine whether the nutrients measured here – or other aspects of leaf chemistry, including defenses [110-112] – are important for endophyte growth in general, and under natural conditions.

Culture-free perspectives

The present work focused only on cultivable endophytes; similar sampling using culture-free methods is likely to reveal endophytes not observed here, and may provide complementary perspectives on community structure and diversity (e.g., [113-116]). For example, estimates of class-level taxonomic placement of endophytes in the Santa Catalina Mountains differed

significantly when assessed using culture-based surveys vs. a next-generation, high-throughput, culture-free approach (i.e., 454 pyrosequencing [58,117]). Species richness and diversity typically are higher when estimated using culture-free vs. culture-based methods [58,116-119], but may not necessarily provide different perspectives on host affinity or other ecological features of endophyte communities (U'Ren unpubl.). In general, future exploration of fire effects on endophyte communities, and endophyte studies in general, will benefit from the use of both culture-based and culture-free methods.

Conclusions

Using a culture-based approach we found that isolation frequency, diversity, and composition of endophyte communities differed significantly in trees affected by fires in the last 18 y vs. trees in proximate sites that have not experienced fire in at least 50 y. Data from >1100 isolates revealed that endophyte isolation frequencies were highest in trees without a recent history of fire (>50 y), but that endophyte diversity was highest in trees in the most recent fire age class (< 6y). Our analyses were consistent with predictions regarding the similar directionality and scope of endophyte responses in different but co-occurring host taxa, and revealed shifts in endophyte community composition and taxonomic composition as a function of fire. Although taxonomic composition differed as a function of fire at a local scale, fungal endophytes in hosts affected by fires in the last 18 y represented subsets of the regional mycoflora that appear to circulate at a regional scale among these traditionally fire-affected forests. We found little evidence for an effect of leaf nutrients in shaping communities as a function of fire, but observed marked differences in endophyte communities among host taxa, corresponding to differences in chemistry and observed differences in fungal growth *in vitro*. Together these findings contribute to an emerging perspective on the responses of diverse communities to severe fire, and highlight the importance of considering fire history when estimating endophyte diversity and community structure for particular biomes and individual plants. Future work will examine the functional importance of these endophyte communities, both with regard to plant regeneration and resiliency after major disturbance events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Sample sites in central and southern Arizona (bold font, black circles): Santa Catalina Mountains, Santa Rita Mountains and Chiricahua Mountains in Coronado National Forest; Sierra Prieta, in Prescott National Forest. Elevation is indicated on a scale from low (white) to high (darkest grey). Major cities are shown with open circles for reference. Inter-site distances were calculated using Google Maps

Fig. 2.

Examples of trees and sample sites. **a** Site ca. 1 y after the Doce Fire in Sierra Prieta. **b** *Q. turbinella* resprouted in that site. **c** Site ca. 3 y after Horseshoe II Fire in the Chiricahua Mountains. **d** *J. deppeana* regrowth in that site. **e** Site ca. 11 y after the Aspen Fire in Santa Catalina Mountains. **f** *Q. hypoleuroides* regrowth in that site. **g** Site and *Q. hypoleuroides* in Santa Rita Mountains after > 50 y without a severe wildfire. **h** *J. deppeana* in Santa Catalina Mountains after > 50 y without a severe wildfire. Image credits: a, b, g: AEA; c, d, e, f and h: YLH

Fig. 3.

Isolation frequency, diversity, community structure and taxonomic composition did not differ significantly among sample sites. Data were analyzed for trees that have not experienced wildfire in > 50 y. **a** Isolation frequency (ANOVA, log-transformed, $F_{2, 11} = 1.4777$, $P = 0.2701$). **b** Diversity (ANOVA, log-transformed, $F_{2, 5} = 0.4714$, $P = 0.6493$). **c** Community structure in *J. deppeana*. Results based on the Morisita index resemble those for the Jaccard index (data not shown). **d** Taxonomic composition in *J. deppeana* ($\chi^2_{10} = 14.43$, $P = 0.1078$)

Fig. 4. Endophyte communities differed as a function of host taxon. Non-metric multidimensional scaling (NMDS) based on the Morisita index (nonsingletons only) illustrating community structure at the OTU level, quantified statistically using ANOSIM. NMDS and ANOSIM based on Jaccard's index show a similar result (data not shown)

Fig. 5. Isolation frequency and diversity differed as a function of fire age class. Within each panel, different letters represent significant differences based on least-squares means contrasts for each host. **a** Isolation frequency, represented by least-squares means with season as a random factor. **b** Diversity, measured as Fisher's alpha

Fig. 6. NMDS (Morisita index) illustrating differences in endophyte community structure as a function of fire age class in *J. deppeana*, evaluated statistically using ANOSIM. Community structure differed significantly **a** among three fire age classes, **b** between the ≤ 6 y and > 50 y fire age classes, and **c** between the 7-18 y and > 50 y fire age classes, but **d** not between the ≤ 6 y and 7-18 y fire age classes. Complementary analyses based on presence/absence data (Jaccard index) detected differences between the ≤ 6 y and > 50 y fire age classes ($R = 0.4027$, $P = 0.0005$), but not between the 7-18 y and > 50 y fire classes ($R = 0.0097$, $P = 0.4297$) or the ≤ 6 y and 7-18 y fire age classes ($R = 0.0735$, $P = 0.1798$)

Fig. 7.

Taxonomic composition of endophytic Ascomycota differed among fire age classes for **a** *Juniperus* and **b** *Quercus*. Sequences that were not assigned to major groups or that matched lichenized fungi and mitosporic Ascomycota of uncertain placement were excluded.

Unclassified sequences were assigned only to the phylum level (Ascomycota). Numbers above bars indicate the total number of sequenced isolates in each fire age class and the number of OTU among OTU-assigned sequences only

Fig. 8. NMDS and ANOSIM (Morisita index, non-singletons only) reveals continuity of endophyte communities after recent fire with the broader regional mycoflora. Results resemble those based on Jaccard's index ($R = 0.0223$, $P = 0.1614$)

Fig. 9.

Leaf nutrients as a function of host taxon. Each nutrient was centered by season and location. Different letters represent significant differences between host taxa (Ca: $t_{53} = -7.89$, $P < 0.0001$; Mg: $t_{54} = -7.43$, $P < 0.0001$; N: $t_{54} = 3.54$, $P = 0.0008$; but K, $t_{51} = -0.88$, $P = 0.1916$)

Table 1

Location, elevation, climate, plant community, fire information, and collection information.

Site	Latitude Longitude	Elevation (m.a.s.l.)	Mean Annual Temperature (°C) ^a	Mean Annual Precipitation (mm) ^a	Plant Community	Fire Name	Ignition Date	Fire Size (ha)	Fire Age Class	Collected Trees ^j Q / J	Sampling Date ^k
Santa Catalina Mts.	N32.373° W110.691°	1797	9.2	464	Oak-pine woodland ^b	Aspen Fire ^f	Jun, 2003	34,297	7-18 y > 50 y	2 (6) / 2 (6) 1 (3) / 1 (3)	Sp 2014 Su 2014
Santa Rita Mts.	N31.713° W110.874°	2032	14.1	539	Oak-pine forest ^c	Florida Fire ^g	Jul, 2005	9,382	6 y 7-18 y 7-18 y 7-18 y > 50 y	2 (6) / 2 (6) 2 (6) / 2 (6) 2 (12) / 2 (12) 2 (6) / 2 (6) 1 (3) / 1 (3)	W 2011 W 2013 Sp 2013 Sp 2014 Su 2014
Chiricahua Mts.	N31.929° W109.259°	2300	12.6	537	Oak-pine forest ^d	Horseshoe II Fire ^h	May, 2011	90,226	6 y > 50 y	2 (5) / 2 (6) 0 (0) / 1 (1)	Su 2014 Su 2014
Sierra Prieta	N34.593° W112.601°	1703	11.9	387	Oak-juniper woodland ^e	Rattlesnake Fire ^d Doce Fire ⁱ	Jun, 1994 Jun, 2013	10,330 2,739	7-18 y 6 y > 50 y	1 (1) / 1 (1) 2 (6) / 2 (6) 1 (3) / 1 (3)	Su 2014 Su 2014 Su 2014

Latitude, longitude and elevation represent the first microsite in each sample site. Fire dates are the month and year of ignition; all fires ended in the same year as ignition. References for plant community and fire data are listed in footnotes. J= *J. deppeana* (all sites); Q= *Q. hypoleucoides* (Santa Catalina Mountains, Santa Rita Mountains, and Chiricahua Mountains) or *Q. turbinella* (Sierra Prieta)

^aData from closest weather station: Santa Catalina Mts. from Palisades; Santa Rita Mts. from Hopkins; Chiricahua Mts. from Portal 4 SW; Sierra Prieta from Walnut Creek: <http://www.wrcc.dri.edu/>.

^bNiering & Lowe, 1984 [46]

^cDavison et al., 2011 [48]

^dBarton, 2002 [49]

^eBaker, 2011 [47]

^fDesilet et al., 2007 [67]

^gUSDA Service, 2005 [68]

^hYouberg, 2013 [69]

ⁱInciWeb 2013 [70]

^jNumber of host species collected in each microsite; total number of individuals per species shown in parenthesis.

^kSp = spring, su = summer, w = winter

Table 2

Statistical tests of isolation frequency in the multiple regression model ($R^2 = 0.19$, $P < 0.0001$).

a. Explanatory variables	DF	F	P
Fire age class	2, 77.01	3.48	0.0357*
Host taxon	1, 87.92	2.33	0.1305
Fire age class $\times$ host taxon	2, 88	1.0710	0.3471

b. Fire age class	DF	F	P
6 y vs. 7-18 y	1, 70.99	2.01	0.1608
7-18 y vs. >50 y	1, 66.54	6.62	0.0123*
6 y vs. >50 y	1, 89.99	4.78	0.0314*

a Effect test of fire age class, host taxon, and fire age class $\times$ host taxon on isolation frequency. **b** Least-squares means contrasts pairwise comparison among fire age classes.

* indicates significant results

Table 3

Statistical tests of diversity in the multiple regression model ($R^2 = 0.68$, $P < 0.0001$).

a. Explanatory variables	DF	F	P
Fire age class	2, 2	7.36	0.0029 *
Host taxon	1, 1	30.12	< 0.0001 *
Fire age class $\times$ host taxon	2, 2	2.61	0.0930

b. Fire age class	DF	F	P
6 y vs. 7-18 y	1, 26	4.41	0.0456 *
7-18 y vs. >50 y	1, 26	4.52	0.0432 *
6 y vs. >50 y	1, 26	14.71	0.0007 *

a Effect test of fire age class, host taxon, and fire age class $\times$ host taxon on diversity. **b** Least-squares means contrasts pairwise comparison among fire age classes.

* indicates significant results