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Factors influencing communities of foliar fungal endophytes in riparian woody plants

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ABSTRACT

Riparian areas within a given arid region frequently contain broadly similar plant communities despite substantive geographic separation. Whether they also harbor similar communities of fungal symbionts, or feature assemblages unique to each riparian zone, is unknown. We examined fungal endophytes in foliage of woody angiosperms in six riparian areas in Arizona. Abundance and diversity differed among host species according to leaf longevity and phytochemistry, and among sites as a function of rainfall. Community composition varied among sites and host species. Comparison with regional data revealed that riparian areas harbor different subsets of the regional mycota rather than a consistent group of riparian taxa. Overall a high species- and phylogenetic richness of endophytes was recovered, especially among *Mycosphaerella* and affiliated anamorphs. Variation in endophyte communities across sites despite the relative consistency of plant communities underscores the importance of riparian zones both singly and in combination for harboring fungal biodiversity.

Introduction

Fungal endophytes, defined functionally as fungi living within healthy, asymptomatic plant tissues, constitute an underexplored dimension of fungal diversity and plant ecology (e.g., Saikkonen et al., 1998; Arnold, 2007). Those that form localized infections via horizontal transmission in photosynthetic tissues (Class 3 endophytes, sensu Rodriguez et al., 2009; hereafter, foliar endophytes or FE) comprise one of earth's most prevalent symbioses, but the factors shaping their distributions are not well understood. Observed in all major lineages of land plants and in plant communities from the Arctic to the tropics, FE often are highly abundant and diverse in individual woody plants (e.g., Lodge et al., 1996; Arnold et al., 2000; Higgins et al., 2006; Herre et al., 2007; Hoffman and Arnold, 2008; U'Ren et al., 2010) and exhibit remarkable beta diversity across the geographic ranges of the plant taxa they inhabit (e.g., Arnold and Lutzoni, 2007; Hoffman and Arnold, 2008; U'Ren et al., 2012). Communities of FE typically are phylogenetically diverse, with members of multiple

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classes of Pezizomycotina frequently co-occurring in the same tissues (Lodge et al., 1996; Higgins et al., in press). Preliminary inferences regarding the evolution of endophytism suggest that the symbiosis has arisen multiple times, often from ancestors with pathogenic or endolichenic trophic modes (Arnold et al., 2009; see also U'Ren et al., 2009).

Although knowledge of the diversity, ecological roles, and potential uses of FE has grown rapidly, especially over the past decade (e.g., Arnold et al., 2003; Saikkonen et al., 2003; Arnold, 2007; Mejía et al., 2008; Pan et al., 2008; Arnold et al., 2009; Lee et al., 2009; Rodriguez et al., 2009; Van Bael et al., 2009; Rojas et al., 2011; Gazis et al., 2012; U'Ren et al., 2012), factors shaping their abundance and distribution in woody plants remain poorly known. Fidelity of FE to particular plant taxa has been reported frequently (see Petrini, 1996; Elamo et al., 1999; Ahlholm et al., 2002; Saikkonen et al., 2003), but several recent studies have detected remarkable host breadth both in surveys and experimental inoculations (e.g., Arnold et al., 2003; U'Ren et al., 2010; Higgins et al., 2011; U'Ren et al., 2012) and have suggested that host specificity varies positively with latitude (Arnold and Lutzoni, 2007). Studies at various spatial scales have highlighted turnover in FE assemblages over small- (Higgins et al., 2011) or large spatial scales (Higgins et al., 2006; Arnold and Lutzoni, 2007; Davis and Shaw, 2008), with mechanisms often ascribed broadly to habitat- or microsite characteristics (Higgins et al., in press; U'Ren et al., 2012). At small scales, wind, moisture and temperature can affect the deposition and survival of FE propagules on leaf surfaces (Juniper, 1991; Arnold and Herre, 2003), whereas broader patterns of biogeographic history, plant density, and climatic patters often are invoked at larger scales (U'Ren et al., 2012). However, studies rarely consider host- and geographic factors simultaneously or in a robust statistical framework, leaving the relative contribution of geographic location and host taxonomy in question.

In arid and semi-arid regions, riparian areas frequently harbor relatively species-rich communities that greatly enrich regional biodiversity (Naiman et al., 1993; see also Sabo et al., 2005). Riparian areas within a given biogeographic region often contain broadly similar plant communities, or are dominated by overstories of the same species or genera (e.g., *Populus* in much of the western US; Hultine et al., 2007; Merritt and Poff, 2010) despite sometimes substantive geographic separation (Patten, 1998). Whether they also harbor similar communities of fungal symbionts, or feature assemblages unique to each riparian zone, is unknown. Because endophytes are increasingly recognized as potentially important for plants' mitigation of abiotic and biotic stress (see Rodriguez et al., 2009), we are especially interested in understanding the factors that shape their distributions at regional and local scales, and the degree to which often vulnerable riparian areas act as potential hotspots of endophyte diversity.

Here we assess abundance, richness, diversity, and community composition of foliar endophytes in representative woody plants in six riparian areas of north-central Arizona, USA. Previous studies have examined FE associated with some riparian trees in this region (e.g., Wilson, 1995; Faeth and Hammon, 1996), but none has investigated communities in multiple host taxa, assessed the relative role of host and geography in shaping endophyte assemblages, nor placed these communities in a broader regional context.

Materials and methods

Mature, apparently healthy leaves were collected from representative and locally abundant woody plant species in six riparian communities in north-central Arizona, USA in Sep. 2007 (Table 1). Communities consisted primarily of Fremont cottonwood (*Populus fremontii*), with velvet ash (*Fraxinus velutina*) and Sonoran scrub oak (*Quercus turbinella*) occurring as co- or sub-dominants in three sites (OCC, RTD, and EVR; Table 2). Other native and non-native woody plants in some sites included Russian olive (*Elaeagnus angustifolia*), New Mexico olive (*Forestiera neomexicana*), Fremont's mahonia (*Mahonia fremontii*), point-leaf manzanita (*Arctostaphylos pungens*) and four-wing salt bush (*Atriplex canescens*) (Table 2).

Sixteen leaves were collected haphazardly from the canopy on the north and south sides of each of two to three individuals per focal species in each site (Table 2), each separated by ≥25 m. Leaves were processed within 24 hr of collection. A surface-sterilized hole-punch was used to cut two discs, each 0.5 cm in diameter, from the middle lamina of eight haphazardly selected leaves per individual. Discs were rinsed in tap water and surface-sterilized by sequential immersion in 70 % ethanol (2 min) and 10 % Clorox bleach (0.5 % NaOCl; 2 min) followed by three rinses with sterile,

Table 1 – Coordinates, elevation and climate information for six riparian forest sites in north-central Arizona: CIB = Cibola National Wildlife Refuge, La Paz County; JOC = Joseph City, Navajo County; CAV = Camp Verde, Yavapai County; RTD = Red Tank Draw, Yavapai County; OCC = Oak Creek Canyon, Yavapai County; and EVR = East Verde River, Gila County. Climate data were obtained from the Western Regional Climate Center, http://www.wrcc.dri.edu/, and in all cases reflect long-term averages for sites of comparable elevation within 20 km of our study areas.

Site code	CIB	JOC	CAV	RTD	OCC	EVR
Coordinates	33.312°N, 114.691°W	34.961°N, 110.392°W	34.579°N, 111.853°W	34.684°N, 111.721°W	34.884°N, 111.744°W	34.299°N, 111.358°W
Elevation	65 m	1 499 m	956 m	1 196 m	1 361 m	1 385 m
Precipitation, May–Oct. 2007	30 mm	155 mm	144 mm	255 mm	274 mm	294 mm
Mean annual precipitation	97 mm	210 mm	326 mm	322 mm	453 mm	540 mm
Mean annual high temperature	31.2 °C	22.2 °C	26.8 °C	26.0 °C	24.2 °C	22.5 °C
Mean annual low temperature	13.2 °C	3.2 °C	5.9 °C	8.0 °C	7.8 °C	3.9 °C

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Table 2 – Host species surveyed at six riparian forests in north-central Arizona in 2007, plant families, number of individuals surveyed, percent of leaves from which endophytes were isolated in culture, and number of isolates obtained for each host species/site combination. Site codes are defined in Table 1.

		Sites					
Host species	Family	CIB	JOC	CAV	RTD	OCC	EVR
Atriplex canescens[a]	Amaranthaceae	3/8.9/5					
Arctostaphylos pungens[b]	Ericaceae					1/40.0/8	3/64.4/31
Elaeagnus angustifolia[a]	Elaeagnaceae		3/0/0				
Forestiera neomexicana[a]	Oleaceae		3/0/0				
Mahonia fremontii[a]	Berberidaceae			3/8.9/4			
Prosopis glandulosa[a]	Fabaceae	3/0/0					
Fraxinus velutina[a]	Oleaceae			3/11.1/5	3/4.4/5	3/17.8/7	3/75.8/47
Populus fremontii[b]	Salicaceae	3/0/0	3/0/0	3/0/0	3/0/0	2/10.0/3	3/6.7/6
Quercus turbinella[b]	Fagaceae				3/60/28	3/53.3/24	3/97.8/51

a Deciduous.
b Evergreen.

deionized water (see Arnold et al., 2000). Overall, 456 leaves from 57 individuals were processed (Table 2), yielding 912 leaf discs.

Fifteen discs per individual (855 discs total) were chosen haphazardly and cultivated individually in Petri dishes (60 mm diameter) on 2 % malt extract agar (MEA; Fisher Scientific), which promotes growth by diverse endophytic fungi (Arnold et al., 2000). Plates were sealed with Parafilm™, incubated at room temperature under ambient light, and checked every 4 d over 8 weeks for fungal growth. Emergent fungi were transferred to new 2 % MEA plates and grouped into morphotypes after 2 months based on whole-colony and hyphal characteristics, including color, texture, size, shape and presence of secondary structures (Arnold, 2002).

Molecular analyses

Because few isolates produced reproductive structures in culture we used molecular techniques to assist in delimiting operational taxonomic units (OTU). Total genomic DNA was extracted from fresh mycelium using the Extract-N-Amp Plant PCR Kit (Sigma–Aldrich) or following Arnold et al. (2007). The nuclear internal transcribed spacers and 5.8S (nrITS), and ca. 600 base pairs of the nuclear ribosomal large subunit (LSU) were amplified as a single fragment following Higgins et al. (2011). Positive amplicons were cleaned, normalized, and sequenced bidirectionally as a single fragment following Higgins et al. (2011) at the Environmental Genetics and Genomics Facility (Northern Arizona University) or the Genomics Analysis and Technology Core (The University of Arizona).

Basecalls were edited and contigs assembled in SeqMan (SeqMan Software) or Geneious (Biomatters Development Team). Consensus sequences were grouped into OTU at 95 % sequence similarity using Sequencher v. 4.2.2 (GeneCodes Corp.), which employs a conservative grouping algorithm to cluster sequences into phylogenetically informative groups that approximate species boundaries in representative genera of foliar endophytes (Arnold et al., 2007; U'Ren et al., 2009, 2010; see also Higgins et al., 2011). BLAST searches of GenBank were used to estimate taxonomic placement, but because such matches are sensitive to database content, do not employ phylogenetic approaches, and may yield matches to erroneously named isolates, species- and genus-level matches are not considered indicative of true identities (Zhang et al., 2000; see also U'Ren et al., 2009; Gazis et al., 2012). Top BLAST matches for each isolate are listed in Supplementary Appendix 1, and were evaluated in phylogenetic analyses (below) for one focal genus. Sequence data have been submitted to GenBank under accession numbers JN120332–JN120442.

Ecological analyses of endophytes in riparian zones

Analyses of Variance (ANOVA) were carried out in *R* 2.15 (R Development Core Team, 2012) or JMP 9.0.2 (SAS Institute) to examine the relationship of host species, study site and climate variables to the percent of leaves colonized by cultivable endophytes, isolation frequency (defined here as the number of isolates mm^{-2} of leaf tissue), OTU richness, and diversity (Shannon's H; Shannon, 1948). For comparisons involving host species and site we focused on OCC, RTD and EVR, where the same three species were sampled (Table 2); however, analysis of the entire data set produced qualitatively similar results.

Sampling completeness was assessed using accumulation curves for the entire data set using EstimateS 8.2.0 (Colwell, 2011) and with respect to sites using the exact site addition method (Ugland et al., 2003). To investigate the relationship of host and site to community composition for OCC, RTD and EVR, we used a Permutation Multivariate Analysis of Variance (PerMANOVA) based on 99 999 permutations of the data (Oksanen et al., 2008), which avoids the inherent tendency of community data to violate distributional assumptions of standard MANOVA (Anderson, 2001). We used an adjusted Bray–Curtis dissimilarity metric to best represent the multivariate distances between observations and also include samples with low abundances (Clarke et al., 2006). Ordination and indicator species analyses for OCC, RTD, and EVR were conducted using the vegan and labdsv packages in *R* (Oksanen et al., 2008; Roberts, 2007; R Development Core Team, 2012) using only those OTU that were recovered more than three

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times (i.e., singletons and doubletons were excluded). Ordination results were visualized by non-metric multidimensional scaling (NMS; e.g., U'Ren et al., 2012). Ordination based on the adjusted Bray–Curtis dissimilarity metric was evaluated across a range of multi-dimensional configurations, with the chosen number of ordination axes representing the most parsimonious configuration based on the amount of stress, or residual error between the ordination and the original data, and the number of dimensions. One hundred random starting configurations and an upper limit of 1 000 iterations for the lowest stress solution were used to generate a scree-plot of stress by dimensionality, followed by an additional 100 configurations generated from random starting configurations at the optimal dimensionality given an upper stress threshold of 20 %. Given the small reduction in stress from the two to the three-axis solutions, the two-axis solution was selected, with a final stress of 18 %. For indicator species analysis, indicator value (a product of the relative frequency and abundance) $\geq$0.25 and $P \leq 0.05$ were considered significant (Dufrene and Legendre, 1997).

Regional comparisons of endophyte communities

Three approaches were used to place endophyte communities in these disparate riparian areas into a broader regional context. First, a Mantel test was used to test for the relationship between geographic distance and community dissimilarity between sites using the entire data set (Legendre and Legendre, 1998; singletons and doubletons excluded). Geographic distances were calculated based on the site coordinates (latitude and longitude) using the *fossil* package in *R* (Vavrek, 2011). Community dissimilarity was calculated as above, except that observations were pooled by individual plant to obtain an integrated community for each site. Statistical significance was assessed using 99 999 permutations, with three correlation coefficients (Pearson's r, Spearman's ρ and Kendall's τ) implemented to consider both linear and non-linear, monotonic relationships.

Second, endophytes isolated from the most productive host species (*Q. turbinella*, $N = 58$ isolates) were compared against a larger regional data set of oak-associated endophytes ($N = 158$ isolates obtained from seven species of *Quercus* in Arizona; Table 3; Hoffman et al., 2008; U'Ren et al., 2010; Devan et al., in revision) using cluster analysis and NMS using Jaccard's index (based on presence/absence of OTU) and the Morisita index (based on abundance of OTU). Similarity of endophyte communities in oaks throughout Arizona was evaluated further using pairwise comparisons of the Morisita index for each oak species/site combination, with tests structured to evaluate the importance of sampling region (southeastern vs. north-central Arizona), *Quercus* section (*Lobatae* vs. *Quercus*), leaf longevity (evergreen vs. deciduous), and environment (riparian vs. non-riparian). Similarity values did not differ significantly from a normal distribution (Shapiro–Wilk W, $P = 0.1235$). Thus, values were analyzed by *t*-tests to evaluate the importance of each explanatory variable individually and by multiple regression to evaluate their effects simultaneously. In addition, because taxonomy and environment were partially correlated (due to sampling of *Q. turbinella* only in north-central Arizona and its status as the only oak species sampled in riparian areas), an individual *t*-test of the residuals was applied after the effect of taxonomy (section) or environment (riparian vs. non-riparian) was taken into account.

Third, isolates representing the genus that contained the greatest number of OTU in our sample (*Mycosphaerella*, including 23 isolates in five OTU (based on 95 % sequence similarity) and six genotype groups (based on 99 % sequence similarity), with highest BLAST matches to *Cercospora*, *Cercosporella*, *Mycosphaerella*, *Pseudocercospora*, *Pseudocercosporella*, and *Septoria*; Supplementary Appendix 1) were evaluated phylogenetically in the context of all currently recognized species of *Mycosphaerella* and related anamorphs known from North America (Farr et al., 1989) and endophytes from other localities across North America (Higgins et al., 2006; U'Ren et al., 2012; Arnold unpubl. data). One representative isolate per genotype group obtained in our field surveys was chosen for phylogenetic analysis. nrITS sequences representing voucher specimens of all currently recognized species of *Mycosphaerella* in North America following Farr et al. (1989), including the affiliated anamorphic genera listed above, were retrieved from GenBank in spring 2012. Redundant sequences were identified and removed from the data set in MacClade v. 4.08 (Maddison and Maddison, 2009). The resulting data set comprised 102 sequences, including two outgroup sequences (*Teratosphaeria* spp.; see Crous et al., 2001) and six sequences

Table 3 – Isolates used for regional comparisons of FE from *Quercus* spp. in Arizona: species, section, region, leaf longevity (evergreen vs. deciduous), environment (riparian vs. non-riparian), and source of isolates and sequence data for 158 isolates from previous studies, and 58 isolates from the present study. Similarity was calculated for all pairwise comparisons below, grouped for analysis according to 'same or, different' for comparisons of section, region, longevity, and environment, and evaluated using *t*-tests.

Host species	Section	Region	Longevity	Environment	Source
Q. arizonica	*Quercus*	SE AZ	Evergreen	Non-riparian	Hoffman et al., 2008
Q. emoryi	*Lobatae*	SE AZ	Evergreen	Non-riparian	Hoffman et al., 2008, Arnold unpubl. data
Q. gambelii	*Quercus*	SE AZ	Deciduous	Non-riparian	Hoffman et al., 2008, Arnold unpubl. data
Q. grisea	*Quercus*	SE AZ	Deciduous	Non-riparian	Hoffman et al., 2008
Q. hypoleucoides	*Lobatae*	SE AZ	Evergreen	Non-riparian	Hoffman et al., 2008, Devan et al., in revision
Q. rugosa	*Quercus*	SE AZ	Deciduous	Non-riparian	Hoffman et al., 2008, U'Ren et al., 2010
Q. turbinella	*Quercus*	CEN AZ	Evergreen	Non-riparian	Hoffman et al., 2008
Q. turbinella	*Quercus*	CEN AZ	Evergreen	Riparian	Present study

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representing the diversity of riparian-endophytic *Mycosphaerella* and related anamorphs obtained in the present study. The data set was aligned automatically with default parameters in Clustal X v. 2.1 (Larkin et al., 2007) and manually adjusted in MacClade. Ambiguous regions were excluded. The model of evolution (GTR + I + G) was determined using the Akaike Information Criterion implemented through jModeltest v. 0.1.1 (Posada, 2008). Maximum likelihood analyses were carried out in GARLI v. 1.0 (Zwickl, 2006) on XSEDE, and Bayesian analyses in MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001) on GORDON, accessed through the CIPRES Science Gateway (www.phylo.org; Miller et al., 2010). The latter analysis comprised two runs of 50 million generations each, each with four chains and a sample frequency of 1 000. Support for branches was assessed using 100 maximum likelihood bootstrap replicates in GARLI, and Bayesian posterior probabilities calculated after removal of the burn-in based on assessment of −lnli values and standard deviations of split frequencies.

Examination of leaf material and effects of leaf extracts on fungal growth

Because we obtained very few endophytes from *P. fremontii*, we conducted four assays to distinguish a lack of colonization from the presence of unculturable FE. First, we examined leaf discs of *P. fremontii* for visual evidence of endophytic hyphae. Fifteen discs were surface-sterilized and cultured as above, except that they were cut to include portions of the midvein, which harbors a high incidence of FE in many species (e.g., Gore and Bucak, 2007). Cultures showed no signs of fungal growth after 21 d. Discs then were cleared by soaking in 10 % KOH for 3 d in tissue biopsy cassettes (Simport™, model M509), stained in 0.3 % Trypan Blue in 1:1:1 lactic acid:glycerol:water following Stone (1987), and destained by soaking for 12 hr in lactoglycerol. Discs were mounted on slides in polyvinyl-lactoglycerol (INVAM, 2008) and examined at 100–1000×.

Second, we tested the effects of *P. fremontii* leaf extracts on germination of propagules of fungi deposited in air spora. Leaf extracts (10 % w/v; Arnold and Herre, 2003) were prepared by homogenizing fresh, mature, apparently healthy leaves from *P. fremontii* at RTD with deionized water for 30 s in a commercial-grade blender. The resulting suspension was strained through 1 mm mesh and filtered through three layers of un-bleached coffee filters to remove solid leaf matter. Two percent MEA was prepared with deionized water (control) or leaf extract and autoclaved prior to allocation in 60 mm Petri plates. Ten plates/medium were exposed for 30 min to aerial spore rain beneath the canopies of mature *P. fremontii* (canopies ≥ 3 m diameter) at RTD in Sept. 2007. Plates were sealed with Parafilm and incubated for 3 d at room temperature. Colonies of filamentous fungi in the center 20 cm^2 of each plate were counted using 10–65× magnification following Arnold (2002).

Third, we tested the relative effects of leaf extracts on growth of epiphyllous fungi, relevant in studies of horizontally transmitted fungi because FE infections often result from fungal propagules on leaf surfaces (Arnold and Herre, 2003). Ten healthy, mature leaves of *P. fremontii* from RTD were cut in half along the midvein. The abaxial side of one half was pressed for 10 s onto the surface of 2 % MEA prepared with *P. fremontii* leaf extract, *Q. turbinella* leaf extract (prepared as above from individuals at RTD), or deionized water (control). Ten replicates per medium type were incubated for 3 d, with colonies counted as above.

Last, we tested relative effects of leaf extracts on growth of a representative endophyte. Three haphazardly selected isolates representing the most abundant genotype obtained in our surveys (Amphisphaeriaceae species from *Q. turbinella*; Supplementary Appendix 1) were inoculated onto 2 % MEA prepared with *P. fremontii* extract, *Q. turbinella* extract, or deionized water (control). Colony diameter was measured 1, 7 and 14 d after inoculation.

To test for the effects of leaf extracts on air spora and epiphyllous fungal growth we used parametric analyses (one- and two sample *t*-tests, respectively). However, the endophyte growth data violated normality and homogeneity of variance and could not be corrected by data transformation. To account for these issues, we used re-sampling techniques in *R* to test for the effects of leaf extracts on endophyte growth (Efron and Tibshirani, 1993; Gotelli and Ellison, 2004). Specifically, we used a two-factor bootstrap *F*-test to test for non-zero effects of leaf extract type, fungal isolate, and their interaction on endophyte growth rate. To examine the performance of the test, we estimated Monte Carlo error for *p*-values by repeated simulation ($n = 30$) of the test as the standard deviation of *p*-values divided by the number of simulations (Good, 2001).

Results

In sum, 224 foliar endophytes were obtained from 855 leaf discs. Endophytes were recovered from 14 of 20 host–site combinations. No endophytes were obtained in culture from leaves of three species (*E. angustifolia*, *F. mexicana*, or *Prosopis glandulosa*), nor from *P. fremontii* at four of six sites (Table 2).

Overall, the percent of leaves colonized was nearly seven times greater in evergreen vs. deciduous species (log-transformed data; $t_{11} = 4.07$, $P = 0.0019$), with the highest colonization percentage and diversity in the two evergreen species (*Q. turbinella* and *A. pungens*) (Table 2, Fig 1A and B). Average colonization percentage increased among sites as a function of annual rainfall ($F_{1,3} = 21.23$, $P = 0.0192$; Fig 2), which was positively associated with observed rainfall in May–Oct. 2007 ($F_{1,4} = 18.95$, $P = 0.0121$) but not with annual temperatures (data not shown).

Isolation frequency (isolates mm^{-2} leaf tissue) differed as a function of host species and study site (Fig 3A). For two of the three species of hosts evaluated in three locations, isolation frequency was greatest at EVR (*F. velutina* and *Q. turbinella*; Fig 3A).

Richness and diversity

The 224 isolates obtained from healthy foliage of *A. canescens*, *A. pungens*, *M. fremontii*, *F. velutina*, *P. fremontii*, and *Q. turbinella* in surveys of six riparian areas represented 29 morphotypes. Analysis of sequence data for 111 representative isolates, including 1–3 representatives per morphotype, revealed 31

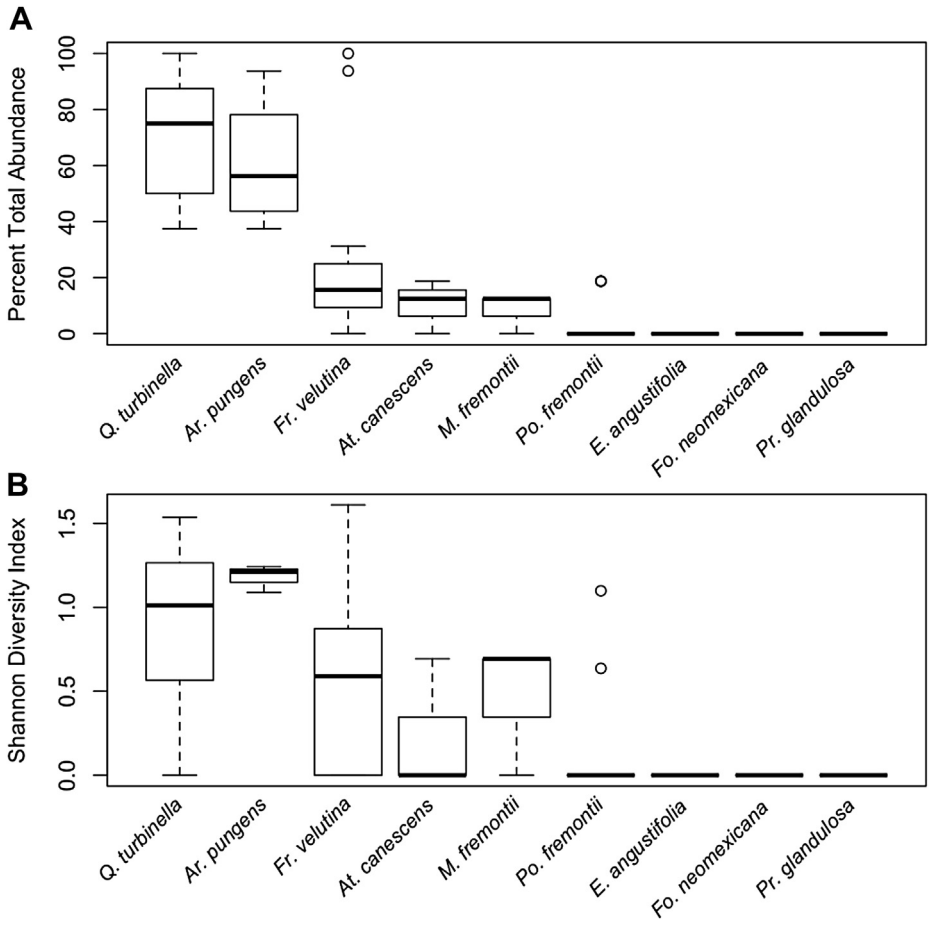

Fig 1 – Box and whisker plots showing (A) percent of leaves from which foliar endophytes (FE) were isolated (colonization percentage) and (B) diversity of FE for each host species. Bars represent the upper and lower quartiles; bold central lines indicate the median; whiskers represent the maximum and minimum values within the interquartile range; and dots represent values outside of 1.5 times the interquartile range.

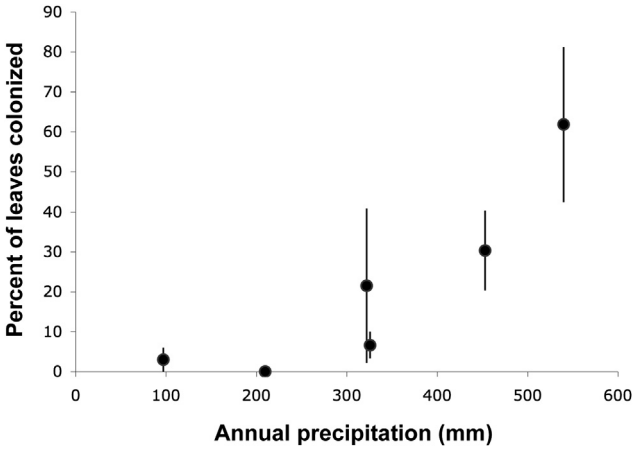

Fig 2 – Relationship of percent of leaves from which endophytes were isolated (colonization percentage) and annual precipitation. Study sites correspond to a precipitation gradient from driest to the wettest: CIB, JOC, RTD, CAV, OCC, EVR (Table 1). Points indicate means ± 1 SE.

OTU based on 95 % nrITS-partial LSU sequence similarity (Fisher's alpha = 14.3; Shannon index = 12.1). Singletons and doubletons represented 45 % and 12 % of OTU, respectively. Bootstrap estimates of total species richness fell within the 95 % confidence interval for observed richness across the full data set, indicating that >88 % of expected species richness was found (Fig 4). Richness and diversity of FE differed significantly among host species and sites (Table 4), with the greatest values in *Q. turbinella* and *A. pungens* at EVR (Fig 3B and C).

Community composition

Community composition of endophytes differed significantly among host species and sites (Table 4). Communities in *Q. turbinella* were separated in ordination analyses from those in *F. velutina* and *P. fremontii* across the three sites in which all were sampled (Fig 3D). Three OTU were significant indicators for *Q. turbinella* (ML3, ML4, and ML10: indicator values = 0.84, 0.60, and 0.47, $P = 0.0002$, 0.003, and 0.034). OTU ML10 also was an indicator of site (indicator value = 0.53, $P = 0.019$) (Supplementary Appendix 1).

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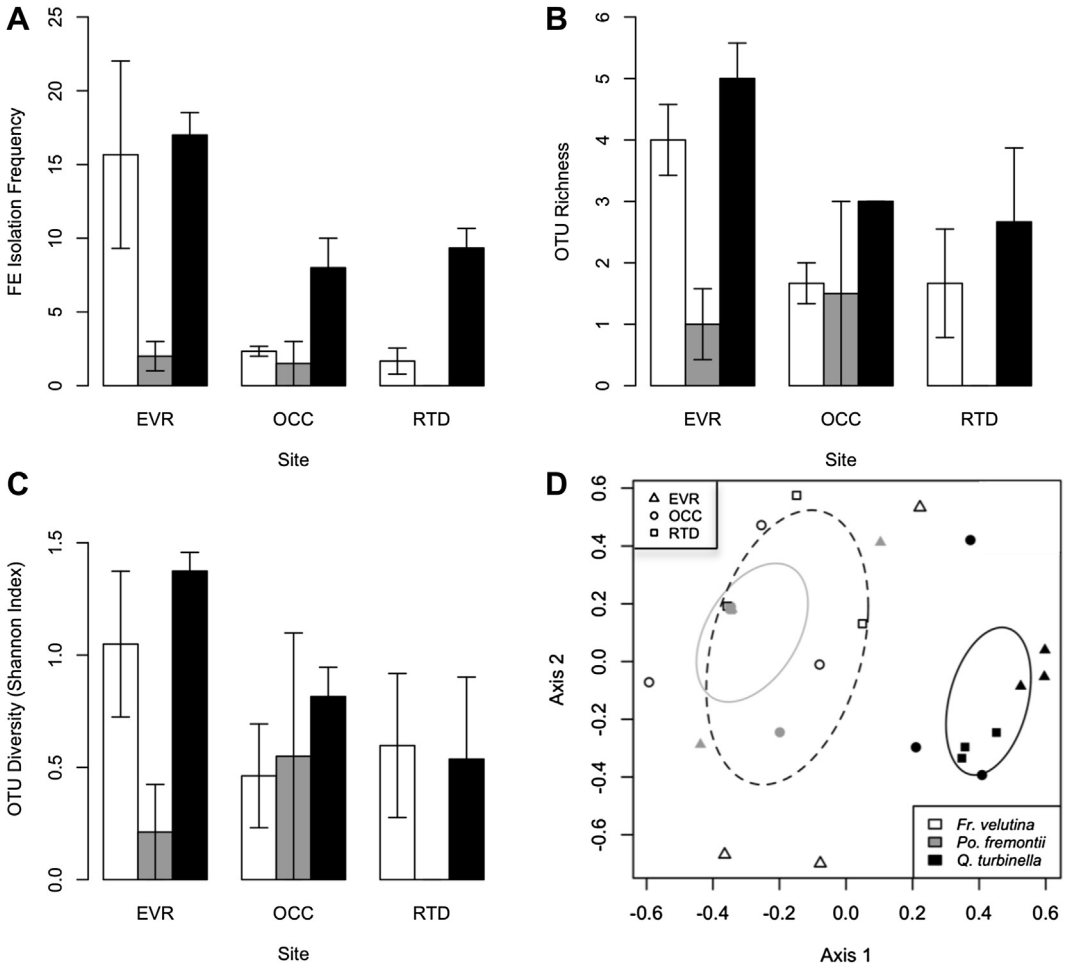

Fig 3 – Mean (±1 S.E.) isolation frequency (isolates mm^{-2}), panel (A); species richness (B); and diversity (Shannon's H) (C) of endophytes in three sites, and an NMS ordination plot for each host plant species that was fully replicated at each site (D) (EVR = circle, OCC = triangle, RTD = square). Ellipses represent 95% S.E. areas for FE communities of each host species. For all panels, white = *F. velutina*, gray = *P. fremontii*, black = *Q. turbinella*.

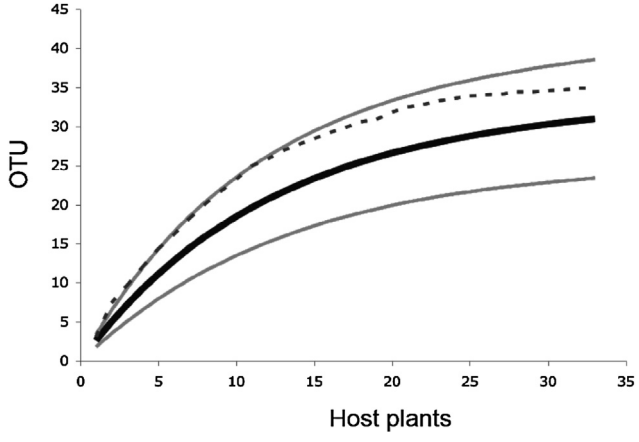

Fig 4 – OTU accumulation curve for sequenced isolates obtained from woody plants that yielded endophytes in culture, inferred using 50 randomizations of sample order (bold), 95 % confidence intervals (gray), and bootstrap estimate of total richness (dashed).

Regional and continental comparisons

Community structure was not correlated with geographic proximity of riparian areas (Mantel test; $r = 0.48$, $\rho = 0.55$ and $\tau = 0.39$; $P = 0.10$, 0.13, and 0.14, respectively).

Comparison of 58 isolates from *Q. turbinella* at RTD, OCC, and EVR with 158 endophytes from diverse *Quercus* species across Arizona (Table 3) revealed that communities in the riparian zones sampled here represent a subset of the larger regional mycota defined by endophytes of oaks in non-riparian areas (shown with polygon, Fig. 5A). Cluster analysis using both presence–absence and abundance revealed that communities in riparian *Q. turbinella* were most similar to those found in *Q. turbinella* in non-riparian areas of the same region (north-central Arizona), and less similar to those found in southeastern Arizona (Fig 5B and C). Community similarity was significantly greater between oaks from the same region than between oaks from different regions (Table 4, Fig 5B and C; $F_{1,26} = 4.9072$, $P = 0.0357$). Similarity was not influenced by subgeneric section (*Lobatae* vs. *Quercus*), leaf longevity

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Table 4 – ANOVA assessing effects of host species and site on isolation frequency (isolates mm^{-2}), species richness, diversity (Shannon's H), and PerMANOVA for community composition, including only host species and sites that were fully replicated (RTD, OCC, EVR: Table 1).

Response	Variation source	df	SS	MS	F	P	r^2
Isolation frequency	Host	2	451.00	225.51	12.48	<0.001	0.35
	Site	2	370.10	185.03	10.24	0.001	0.29
	Host × Site	4	152.10	38.02	2.10	0.125	0.12
	Residuals	17	307.20	18.07			
	Total	25	1280.40				
Richness	Host	2	35.59	16.80	11.66	0.001	0.41
	Site	2	17.19	8.60	5.97	0.011	0.21
	Host × Site	4	6.25	1.56	1.09	0.395	0.08
	Residuals	17	24.50	1.44			
	Total	25	81.54				
Diversity (H)	Host	2	2.11	1.06	5.30	0.016	0.28
	Site	2	1.15	0.57	2.88	0.084	0.15
	Host × Site	4	0.87	0.22	1.10	0.391	0.12
	Residuals	17	3.39	0.20			
	Total	25	7.52				
Composition	Host	2	2.43	1.21	8.76	<0.001	0.41
	Site	2	0.56	0.28	2.02	0.057	0.10
	Host × Site	4	0.54	0.14	0.98	0.466	0.09
	Residuals	17	2.35				
	Total	25	5.88				

(evergreen vs. deciduous), or environment (riparian vs. non-riparian; $F_{1,26} = 2.1538$, 0.1895, and 0.0018, respectively; $P = 0.1542$, 0.6670, and 0.9667, respectively). Results did not differ when the explanatory variables were analyzed simultaneously through multiple regression, nor when analyses of residuals were used to decouple correlated explanatory variables: in each case, only region was strongly significant ($P = 0.01$ and 0.03; data not shown).

All sequenced isolates were Ascomycota, including Sordariomycetes, Dothideomycetes, Eurotiomycetes, and Pezizomycetes (Supplementary Appendix 1). Overall, members of ca. 15 families were found. Amphisphaeriaceae (Xylariales) was especially common, with one OTU represented by 44 isolates from six sites and host species (Supplementary Appendix 1).

Mycosphaerellaceae (Capnodiales) was also common and species-rich, with 23 isolates comprising five OTU and six genotype groups (Supplementary Appendix 1). Phylogenetic analyses of all non-redundant, currently recognized species of *Mycosphaerella* and affiliated anamorphs from North America, supplemented with endophytes and seed-associated fungi from study sites in Arizona, Florida and North Carolina (USA), Québec (Canada) and central Panama, revealed the phylogenetic richness of the endophytes recovered here (Fig 6).

Mycosphaerella-type endophytes from riparian plants did not cluster together with one another, with other *Mycosphaerella*-type endophytes from other Arizona plants, nor with endophytic lineages found in other regions (Fig 6). With varying degrees of support, riparian-endophytic strains were affiliated with *Mycosphaerella populorum* (ML96, representing singleton OTU 22 from *P. fremontii* at EVR); *Mycosphaerella ulmi* (ML224, representing singleton OTU 12 from *F. velutina* at RTD); *Septoria artemisiae* and *Septoria obesa* (ML412 and ML140, representing two different genotypes of OTU 32, all of which were found in *F. velutina* at EVR); *Mycosphaerella musicola* (ML186, representing OTU 30, which was found in *F. velutina* and *A. pungens* at EVR and OCR); and an assortment of *Mycosphaerella* and *Pseudocercospora* strains (ML316, representing singleton OTU 15 from *M. fremontii* at EVR). These strains were not the top BLAST hits for these riparian endophytes except for a few isolates of OTU 32, for which the top BLAST match was *S. artemisiae* (Supplementary Appendix 1).

Leaf observation and chemistry assays

Cleared and stained leaf discs from *P. fremontii* that did not yield isolates in culture were transparent, and cellular and extracellular structures showed low amounts of residual staining. Cellular morphology was indicative of healthy, asymptomatic leaves. No inter- or intracellular fungal hyphae or other structures typical of FE were observed in the mesophyll or mid-veins.

Leaf extracts from *P. fremontii* reduced the number of colonies on nutrient media from aerial inoculum by 90.0 % ± 6.7 % (mean ± S.E.) compared to controls. Both leaf extract types reduced colony abundances of fungi from abaxial leaf surfaces of *P. fremontii*, with *Q. turbinella* extracts causing significantly greater reduction (85.0 % ± 3.8 % colony abundance reduction) than *P. fremontii* extracts (52.0 % ± 5.9 %; $P = 0.001$). Extract type and isolate identity significantly interacted to affect the growth rate of the most common genotype of FE from *Q. turbinella* (Table 5). *P. fremontii* extract inhibited the growth of all three FE strains, but *Q. turbinella* extracts stimulated the growth rate of two of the three strains (Fig 7).

Discussion

In arid regions, riparian and near-river areas frequently harbor a greater abundance and diversity of macroscopic

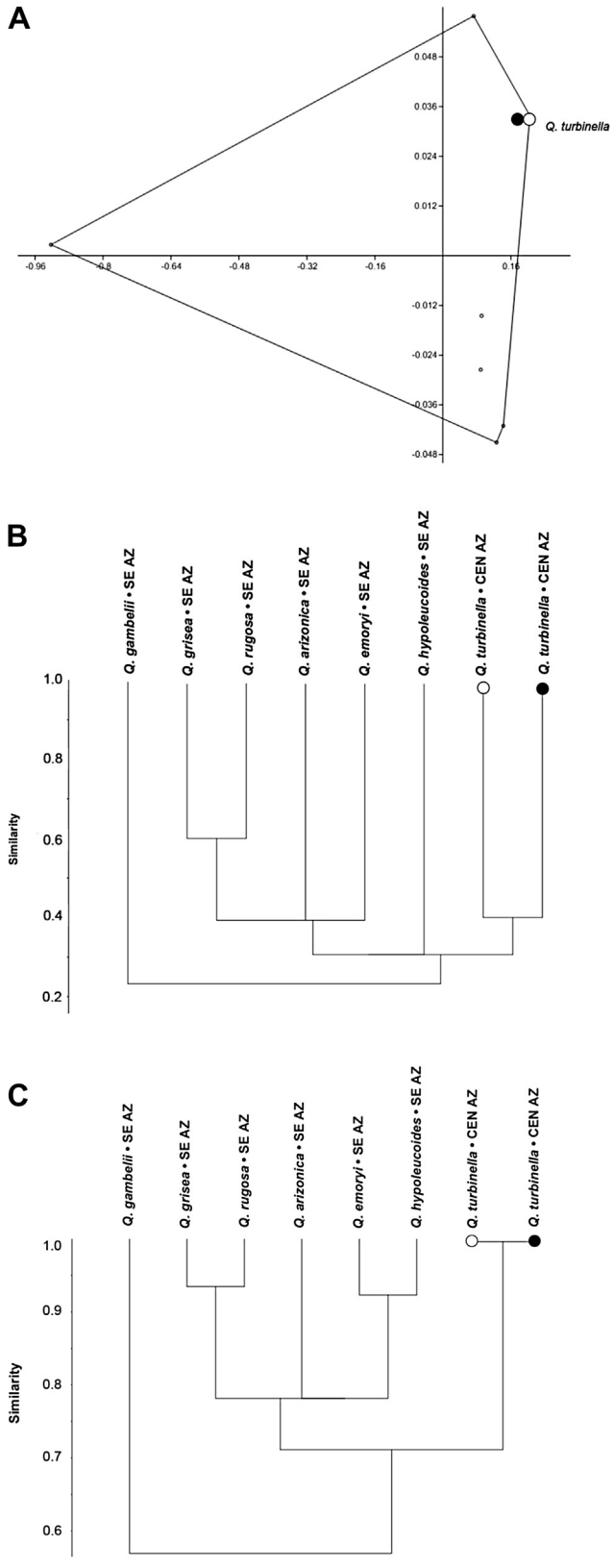

Fig 5 – Regional comparisons of endophyte communities in *Quercus* spp. in Arizona. (A) NMS based on Morisita similarity reveals that endophytes from *Q. turbinella* in riparian zones in north-central Arizona (large filled circle) represent a subset of those found in non-riparian *Quercus* spp. in Arizona, with greatest affinity for endophytes from *Q. turbinella* in north-central Arizona (large unfilled circle). Cluster analysis based on (B) Jaccard's index and (C) Morisita similarity to quantify the similarity of endophyte communities from riparian and non-riparian *Q. turbinella* in central Arizona, and oaks from other sites.

organisms than surrounding dryland communities, and in many cases support distinctive communities that greatly enrich regional biodiversity (e.g., Soykan et al., 2012). Recent surveys of soil microbes reveal the importance of riparian systems as hotspots of microbial biodiversity and as important reservoirs of both taxonomic and functional diversity in a rapidly changing climate (e.g., Rich and Myrold, 2004; Kim et al., 2008). Although several studies have begun to quantify alpha diversity of fungal endophytes in riparian systems (e.g., Nallini et al., 2005; Li et al., 2010), none to our knowledge has simultaneously evaluated the relative importance of host and site in shaping riparian endophyte communities, nor examined riparian endophyte communities in a regional context. Because endophytes are increasingly recognized as potentially important for plant defence against biotic and abiotic stress (reviewed in Arnold, 2007; Rodriguez et al., 2009), understanding their diversity and distributions across disjunct landscapes is of interest in the context of habitat loss and alteration in climate, through which riparian areas are particularly threatened (see Decamps, 1993; Palmer et al., 2008).

We assessed abundance, richness, diversity and community composition of foliar endophytes in representative woody plants in six riparian areas of north-central Arizona. We found that the incidence of endophytes, defined by the percent of leaves yielding endophytes in culture, was higher in evergreen vs. deciduous plants, varied among host species, and differed among sites as a function of annual rainfall. These observations are generally consistent with horizontal transmission, the general mode of dispersal and colonization by Class 3 endophytes (Rodriguez et al., 2009), and in this study were not significantly associated with differences in annual high and low temperatures. When isolation frequency (isolates mm^{-2}) was considered, host species explained more variation than did locality, revealing the relative interplay of host taxonomy and abiotic factors in shaping endophyte abundance.

Cultivable endophytes were rare to absent in several deciduous species sampled in sites with low annual rainfall. However, endophytes also were rare in *P. fremontii* in relatively wet sites in which co-occurring species had relatively abundant FE. This finding is consistent with previous studies in this region, which reported no evidence of FE in cottonwood leaves (Wilson, 1995). We found that hyphae were not visible in mature, healthy leaves of *P. fremontii* that did not yield isolates in culture, confirming their apparent absence from leaf tissue. Moreover, extracts from *P. fremontii* decreased the growth of cultures from air spora and leaf surfaces, and the most common endophyte OTU *in vitro* (Amphisphaeriaceae species from *Q. turbinella*). In turn, extracts of *Q. turbinella* enhanced growth of two strains of that OTU over controls. Leaf chemistry has been invoked previously as an important determinate of interspecific differences in endophyte communities, with

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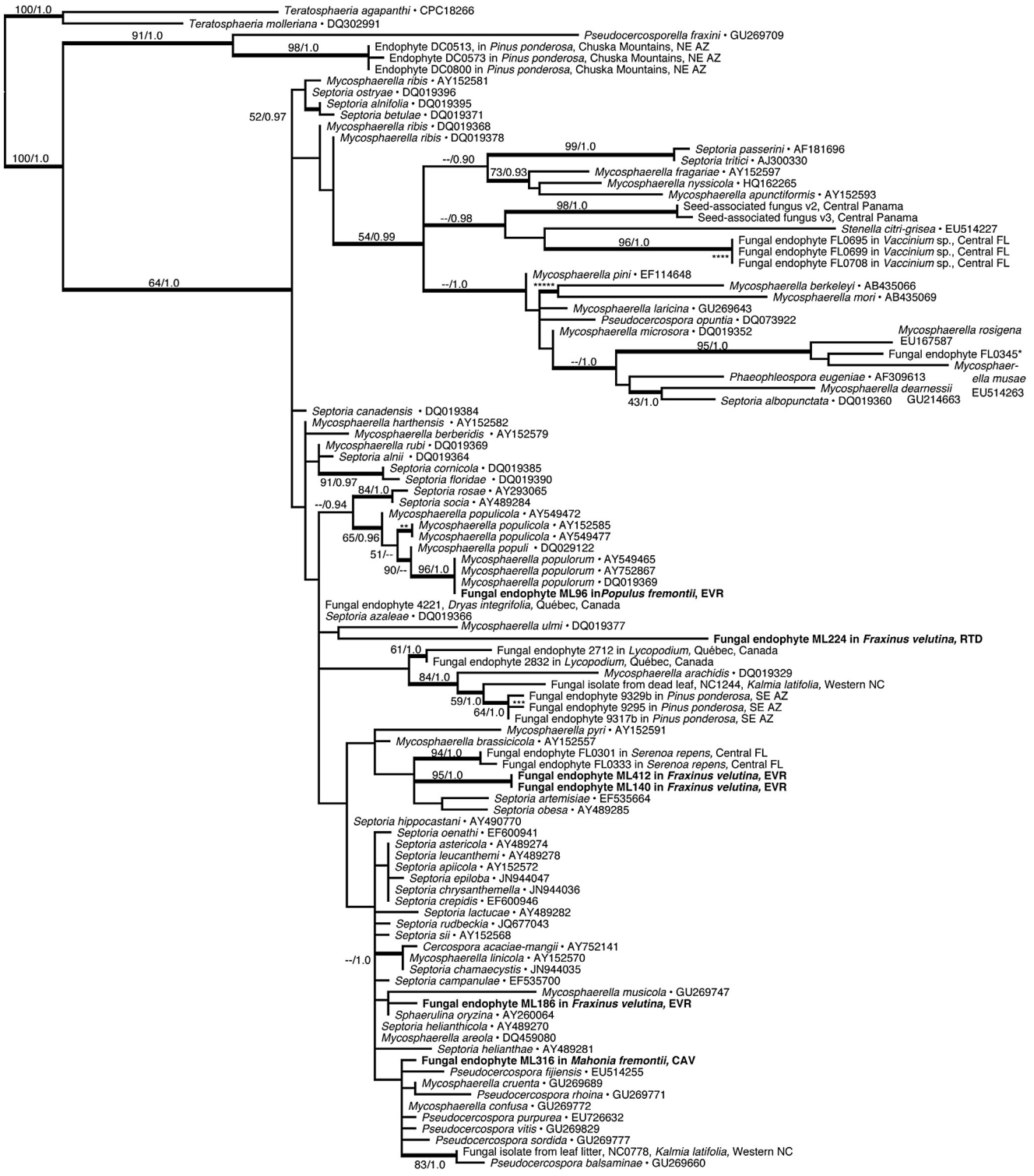

Fig 6 – Results of phylogenetic analyses of endophytes from riparian woody plants in north-central Arizona with affinity for *Mycosphaerella* and affiliated anamorphs (bold), in the context of all currently recognized non-redundant species of *Mycosphaerella* and anamorphs in North America (Farr et al., 1989) and endophytes and seed-associated fungi from surveys in other sites (Arizona, Florida, North Carolina, Québec, and Panama; Higgins et al., 2006; Hoffman et al., 2008; U'Ren et al., 2009, 2010, 2012; Arnold et al. unpubl. data). Topology resulted from maximum likelihood analyses; support values indicate maximum likelihood bootstrap (MLB, before slash; values ≥50 shown) and Bayesian posterior probability (BPP, after slash; values ≥0.90 are shown), with thickened branches indicating significant support by one or both measures (i.e., MLB≥70 and/or BPP≥0.95). *, FL0345 was isolated from foliage of a forest grass in central Florida (U'Ren et al., 2012). Multiple asterisks (, ***, ****) indicate short internodes with high support from MLB and/or BPP (values not shown).**

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Table 5 – Results of re-sampling tests assessing effects of leaf extracts from *P. fremontii* and *Q. turbinella* on growth of three different isolates of Amphisphaeriaceae species in culture. *P*-values and MC error estimates are based on 10 000 and 200 iterations, respectively.

Variation source	df	SS	MS	*F*	*P*	MC error
Extract type	1	659441	659441	406.95	<0.001	<0.001
Fungal isolate	2	1.00E+06	522984	322.74	<0.001	<0.001
Extract × isolate	2	1.00E+06	668227	412.37	<0.001	<0.001
Residuals	62	100467	1 620			
Total	67	3.00E+06				

inhibition or enhancement of growth in some strains anticipated as a possible mechanism (see Arnold and Herre, 2003; Saunders and Kohn, 2008). Bailey et al. (2005) found a strong negative correlation between condensed tannin concentration and twig endophytes of *P. fremontii*, *Populus angustifolia*, and *P. fremontii* × *angustifolia*, suggesting a potential link between phytochemistry and low FE abundances.

In turn, endophyte diversity and composition also differed among hosts and study sites. Diversity was greatest in evergreen species occurring in sites with higher annual precipitation. Previous studies in other systems have found effects of both host identity (i.e., species or genotype) and geographic variation on FE communities (Elamo et al., 1999; Ahlholm et al., 2002; Saikkonen et al., 2003; U'Ren et al., 2012), but their relative importance has not been reported previously. Here, we found that host species explained roughly twice the amount of variation in diversity as did study site, consistent with host-specific filtering of a regional pool of available endophytic symbionts (below). When analyses were restricted to three sites in which *F. velutina*, *P. fremontii*, and *Q. turbinella* were examined, communities differed markedly among host taxa.

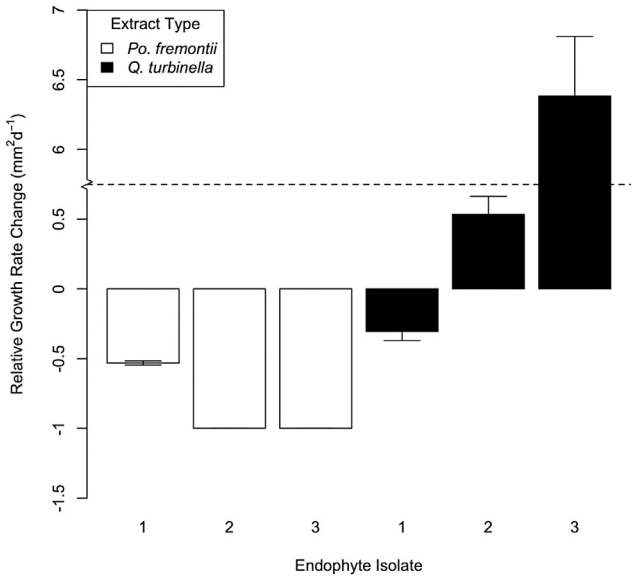

Fig 7 – Effect of leaf extracts from *P. fremontii* and *Q. turbinella* on the growth of three isolates of OTU ML33 (Amphisphaeriaceae species) isolated from *Q. turbinella* at RTD. Bars indicate relative change in extension rate for isolates on 2 % MEA prepared with leaf extracts relative to cultivation on 2 % MEA without leaf extracts. The dashed horizontal line indicates the mean value for the effect of *Q. turbinella* extract on isolate three. All replicates for isolates 2 and 3 on *P. fremontii* medium showed 100 % reduction, resulting in no variance. The Y-axis is broken to accommodate the extremely high growth enhancement of isolate 3 by *Q. turbinella* extract.

We placed these symbionts in a regional context by comparing endophytes of our most productive host, *Q. turbinella*, with endophytes of congeneric hosts from non-riparian sites. Within the broader community of endophytes associated with *Quercus* spp. in Arizona, we found a strong effect of sampling region that was independent of taxonomic relatedness (i.e., occurrence in the same or different subgeneric section), leaf longevity, or sampling environment. Our analyses suggest that congeneric plants harbor a diverse community of symbionts over their geographic ranges, with subsets of that mycota appearing in particular host–site combinations. This broader regional mycoflora may represent a scale that encompassed all of our study sites, thus explaining the lack of a significant relationship of community dissimilarity and intersite distance in Mantel tests.

Although our sampling sites crossed a broad geographic distance (435 km maximum distance) and differed markedly in elevation (1 300 m maximum difference) and precipitation (443 mm y^{-1} maximum difference), they were restricted to riparian forests and, thus, to a narrower spectrum of environmental variability than a truly random sample. Even so, a high richness of endophytes was recovered, especially among *Mycosphaerella* and its affiliated anamorphs. Topologies inferred by maximum likelihood and Bayesian methods are consistent with previous analyses in revealing that anamorphic genera such as *Septoria* are not monophyletic (e.g., Verkley et al., 2004). The analysis, which incorporates the known diversity of *Mycosphaerella* and affiliated taxa from North America, shows that endophytism occurs widely across the genus, rather than having a single origin or occurring specifically within a single clade; that isolates from our focal riparian areas were distinct from those isolated from other hosts (e.g., *Pinus ponderosa*) in other sites in Arizona, indicating phylogenetic richness at a regional scale and multiple independent occurrences in riparian zones; and that *Mycosphaerella* endophytes recovered among only 23 isolates in riparian areas of Arizona contribute substantively to the known richness of the genus. Because the data set was reduced to exclude redundant nrITS sequences, several known species from North America are not shown here. However, this sequence comparison revealed that none of the strains recovered here was 100 % identical to known and sequenced strains of *Mycosphaerella*, although in some cases phylogenetic affinity for known species was strong (e.g., short branch length for *M. populorum* and ML96 from *P. fremontii*).

Although all endophytes that had top BLAST hits to *Mycosphaerella* and affiliated anamorphs were reconstructed within the clade comprising those taxa, the finer-scale limitations of BLAST matches were illustrated by the frequent mismatch of phylogenetic placement and BLAST results (Fig 6;

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Supplementary Appendix 1). However, the conservative but effective nature of our OTU designation approach is illustrated by the tree: the five OTU represented here are distributed widely across the topology, and often are strongly supported in their placement. Discovery of multiple incidences of endophytism among lineages better known for pathogenicity is consistent with the dynamic evolutionary transitions between these trophic modes both across the Pezizomycotina (Arnold et al., 2009) and within *Mycosphaerella* (U'Ren et al., 2009).

Together these analyses provide a first in-depth examination of the richness, diversity and distributions of endophytic symbionts of riparian plants. This work complements our emerging understanding of ecological affiliations of fungal symbionts at regional and continental scales. We observed considerable variation in endophyte communities across host plant species and sites. These findings underscore the importance of systematic conservation of riparian zones, which singly and in combination harbor substantive and previously unexplored fungal biodiversity.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2013.06.003.

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