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Fungi are a Persistent Legacy:
Drivers of fungal abundance and community composition over time

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Fungi are a Persistent Legacy:
Drivers of fungal abundance and community composition over time

by

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Abstract

Fungi are a Persistent Legacy:
Drivers of fungal abundance and community composition over time

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Plants are a major force shaping how ecosystems function, including non-native plants. The effects that non-native plants have on ecosystem properties and processes can be particularly important as invasive plants are associated with changes in nitrogen and carbon cycling Plants can also indirectly affect ecosystem processes through their influence on the soil microbial community and different plants are associated with different microbial communities. The legacies produced by invasive plants can be long-lasting and inhibit the restoration of damaged ecosystems. Because of the central role of soil fungi in ecosystem processes, I examined how fungal abundance and community composition were altered by non-native plants, and the persistence of these changes. Specifically, I examined how two different cases of invasion by non-native species affected soil fungi over three years compared to soil fungi in native, undisturbed sites. I further tested how the soil fungi responded to the removal of the non-native plants and to inoculation with local native microbial communities. Legacy effects of land use history on soil fungal abundance and community composition were found in these central Florida

communities. There were substantial differences in soil fungal abundance and community composition in disturbed and pasture sites compared to native scrub, and these differences persisted for three years after non-native grasses were removed. Not only did the grass-dominated pasture and disturbed sites differ from the undisturbed native shrub-dominated ecosystem, they differed significantly from each other, indicating that the different non-native grasses and other specific changes associated with each land use played a role in soil fungal communities. The combined results of this study have implications for restoration ecology. The current dependence of the fungal community on land use and the associated non-native species invasions (along with other analyses done in this system) suggest that a different approach to restoration is required here to overcome the observed legacy effects.

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INTRODUCTION

Plants are a major force shaping how ecosystems function. They are responsible for the majority of primary production in terrestrial ecosystems, and thus control resource availability for many trophic levels, both above- and belowground. Additionally, plants play a direct role shaping important ecosystem functions, such as nitrogen and carbon cycling. For example, the quantity and quality of plant litter can change the rate of decomposition (Coûteaux et al. 1995). When plant community composition is altered by non-native species invasions, the associated changes in plant traits can reverberate throughout an ecosystem (D'antonio & Vitousek 1992, Jules et al. 2007).

The effects that non-native plants have on ecosystem properties and processes can be particularly important to consider for a number of reasons. These changes can produce undesirable ecosystem properties, from the human point of view. In Indiana, waterway invasion by the aquatic plant Eurasian watermilfoil (*Myriophyllum spicatum*) is a nuisance and obstructs recreational use of those waterways (Indiana DNR, 2011). In other systems, invasive species can reduce or eliminate commercial activities, as in the case of the Dungeness crab (*Cancer magiste)* whose population is reduced by invasion of smooth cordgrass (*Spartina alterniflora)* into its habitat (Holsman et al. 2010). More importantly from an ecological point of view, invasive plants are able to, directly and indirectly, alter important ecosystem processes of the habitat they invade.

Invasive plants are associated with changes in nitrogen and carbon cycling. Liao et al. (2008) found that invasions were associated with increases in important ecosystem

fluxes like annual net primary production, litter decomposition, net nitrogen mineralization, and nitrification in the soil. Increases were also found in both plant carbon and nitrogen concentrations which carried over into litter. In part, this is likely due to invasive plants having traits that differ from the native plants, such as tissue quality. Peñuelas et al. (2010) found that invasive plants differ from natives in a suite of leaf traits that allow them to occupy unique biogeochemical niches. Thus, trait differences may lead to impacts on ecosystem process related to nutrient cycling.

Plants can also indirectly affect ecosystem processes through their influence on the soil microbial community (Klironomos 2002, Stinson et al. 2006). Microbes are responsible for a wide variety of nutrient transformations, and thus their feedback with the aboveground plant community is an important link to overall ecosystem function. Soil microbial community responses to invasive plants are likely to be a part of the changes associated with invasions. Microbes are responsible for most of the transformations in the nitrogen cycle, including nitrogen fixation, mineralization, nitrification, and denitrification (Newton 2007). Microbes also play a key role in overall nutrient cycling as decomposers, responsible for the chemical breakdown of plant litter and soil organic matter. Shifts in the decomposer community can change the rate of decomposition, and consequently, the rates of nutrient cycling.

Different plant species can be associated with different microbial communities, including both free-living and symbiotic microbial taxa. In bacteria, for example, different microbial communities can be found in the rhizosphere soil of different plant

species, but the strength of this specificity can vary widely (Hawkes et al. 2007). Some fungi show stronger patterns of association. For example, in upland forests in Alaska, different fungal taxa in the genus *Russula* showed distinct preferences for forests dominated by either black spruce or birch–aspen–white spruce community of trees (Geml et al. 2010). Furthermore, arbuscular mycorrhizal fungi, which are obligate plant symbionts, can preferentially colonize different plant species, forming unique communities in each plant's roots (Vandenkoornhuyse et al. 2003).

Of the plant groups that wield a strong influence over the soil microbial community, non-native species are of particular interest. Non-native plant species can change soil microbial communities as they invade and establish. The development of altered microbial communities has been observed along an invasion front in California grasslands, where soils from areas that were recently invaded by *Centaurea solstitialis* (yellow star- thistle) and *Aegilops triuncialis* (barb goatgrass) had microbial communities that remained similar to those of native grasslands, while those areas invaded for several years were associated with substantially different soil microbial communities (Batten et al. 2006). In other invasions, it is apparent that the entire structure of the belowground community can be altered. For example, *Bromus tectorum* invasion of semi-arid grasslands in Utah resulted in lower species richness and abundance of both soil fungi and invertebrates . Microbial communities altered by invasive species can also affect the microbial community of neighboring native plants. At two grassland sites, the community of arbuscular mycorrhizal fungi in the non-native plant roots was different from the

community in the native roots and, when the non-native and native species were grown together, the fungal community in the native roots changed to be more similar to that found in the non-natives (~80% overlap; Hawkes et al. 2006). These changes to microbial communities can make native plant recovery difficult if the new microbial community negatively affects the native plant species.

Plant effects on soil properties and soil microbial communities can persist after plant removal, in what is known as a 'legacy effect' (Corbin and D'Antonio, in press). Legacies can be critical when caused by non-native species, because they can inhibit restoration success if the legacy conditions are detrimental to native plants and moreso if there is positive feedback to the invasive plants that might preferentially facilitate their re-establishment (Kulmatiski & Beard 2011). The legacies and associated feedbacks of non-native species invasions may be further amplified when combined with disturbance. Thus, both positive and negative feedbacks can either inhibit re-colonization by native species or promote the re-colonization of the invasive species. For example, when grasses invade woody areas in the Western USA, the additional litter inputs can increase both the frequency and intensity of the fire, which can alter nutrient cycling to favor the invasive species, or inhibit the return of woody and other native species (Levine et al. 2003). In either case, restoration of the native plant community is much more difficult if not impossible without removing the legacy first.

Legacies, by definition, are persistent effects, but the rate of development and rate of decay can be species-specific (Figure 1). Some non-native plant species have short-lived

legacies. For example, soil nitrogen returned to pre-invasion levels four years post-removal of the nitrogen-fixing tree *Robinia pseudoacacia* after a multi-pronged restoration approach that included not only removal of the invasive species, but also mechanical disturbance of the soil and re-planting of desired native species (Malcolm et al. 2008). In other cases, the legacies of non-native plants on soil develop rapidly and are persistent. Grman and Suding (2010) found that exotic annual grasses in California created soil legacies within a single season that reduced biomass of native plants by 74%. Non-native plant communities can also create soil legacies that can last through entire growing season or longer (e.g., Kulmatiski & Beard 2011). For example, invasion by an N-fixing shrub left soils with elevated nitrogen that persisted up to 5 years after the removal of the shrub (Maron and Jefferies 2001). Extremely persistent legacies can create an alternative stable state for that ecosystem (Suding et al. 2004).

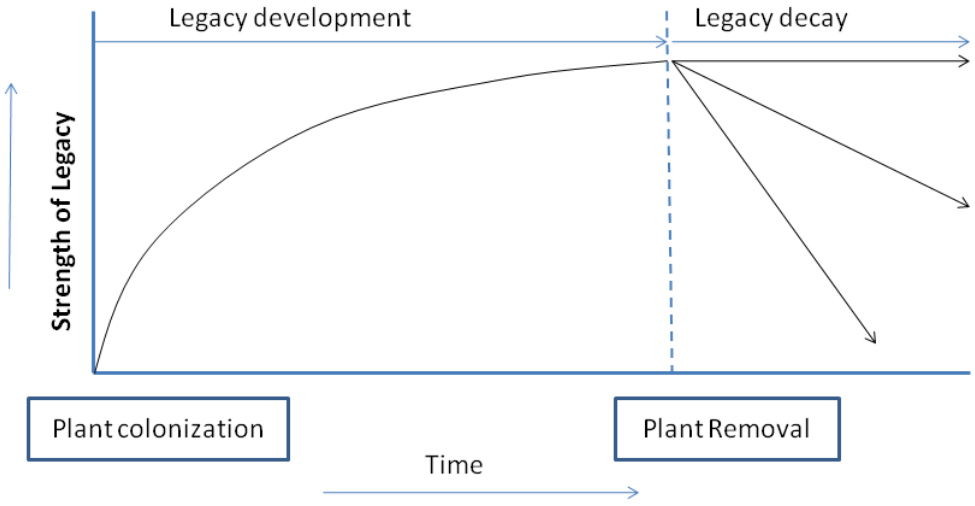


Figure 1. Soil legacy development and hypothetical rates of decay.

Fungal community composition and fungal abundance are often affected by non-native plant invasions and those changes represent important soil legacies that can in turn affect native plants. Symbiotic arbuscular mycorrhizal fungi rely on carbon from plants and are often altered by non-native plant invasions (Mummey, Rillig, and Holben 2005; Hawkes et al. 2006; Rillig and Mummey 2006; Hausmann and Hawkes 2009; Hausmann and Hawkes 2010), as can other fungi living inside plant roots (Kivlin & Hawkes 2010). Free-liging saphrotrophic fungi may not directly affected by non-native plants, but instead may respond to soil properties altered by the plants, such as resource levels and soil moisture (Waldrop et al. 2006; Kao-Kniffin and Balser 2008; Kivlin and Hawkes 2010). Waldrop et al. (2006) found that fungal diversity increased with higher resource availability, not higher plant diversity. Similarly, Kao-Kiffin et al (2008) found that the fungal community responded to nutrient status more than to plant identity. Thus, invasive plants are likely to change the soil fungal community via their effects on resource availability and nutrient status.

Because of the central role of soil fungi in ecosystem processes, I examined how fungal abundance and community composition were altered by non-native plants, and the persistence of these changes. Specifically, I examined how two different cases of invasion by non-native species affected soil fungi over three years compared to soil fungi in native, undisturbed sites. I further tested how the soil fungi responded to the removal of the non-native plants and to inoculation with local native microbial communities. Compared to native sites, the two cases of invasion differed in the dominant non-native

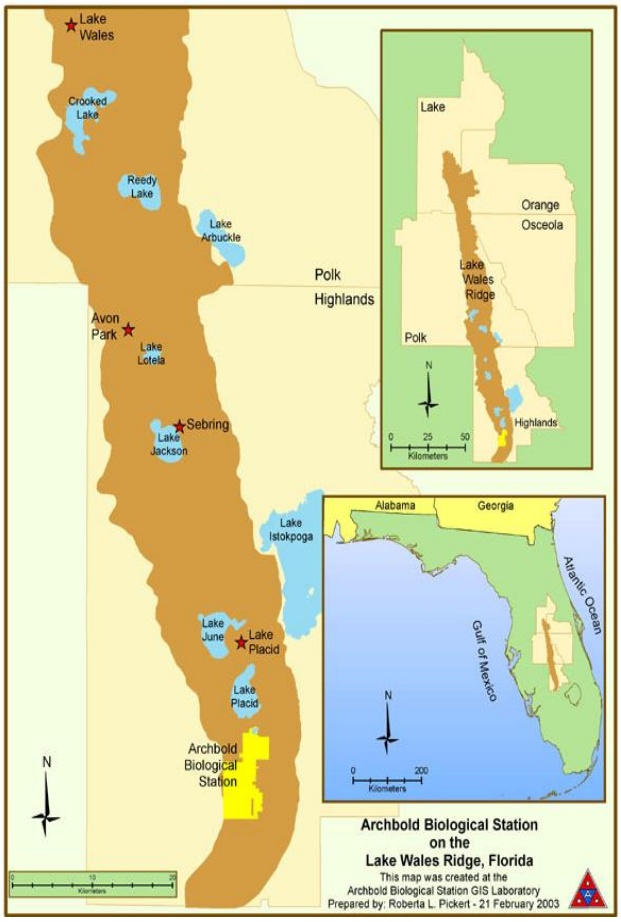
species, the degree of invasion, the degree of disturbance, and associated soil nutrient legacies. Specifically, I expected that the fungal community would be more strongly altered in the more severely invaded site because of the extensive changes. I further hypothesized that fungi in the less invaded site, because the site retained more native species, would remain more similar to fungi the native undisturbed sites. Finally, by tracking the fungal community seasonally over three years, I expected to be able to understand the relative importance of invasion history and non-native plant removals compared to background variation in climate.

METHODS

Study Site

This study was carried out at the Archbold Biological Station in central Florida (27°11’ N, 81°21’ W). The station is located in central Florida, at the southern tip of the Lake Wales ridge (Figure 2a). Average annual precipitation is 1324 ± 29.59 mm (1 SE) with hot, wet summers and mild, dry winters (Archbold Biological Station records, 1932-2001). The native vegetation is shrub-dominated and pyrogenic, with many endemic and several endangered species (Menges & Hawkes 1998; Quintana-Ascencio et al. 1998; Menges & Quintana-Ascencio 2004). Soils are sandy, rapidly permeable and well drained.

a)



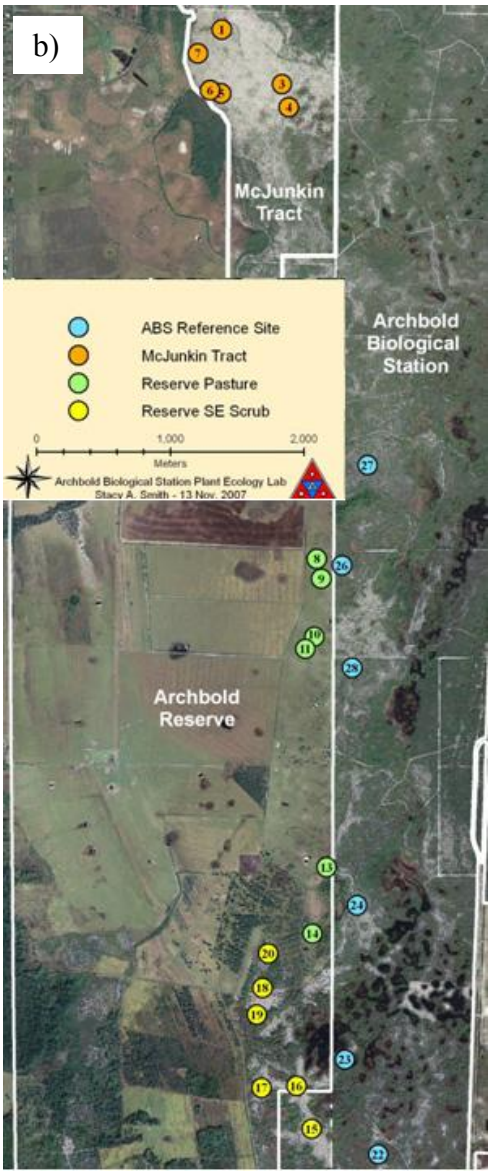


Figure 2. a) Location of Archbold Biological Station. b) Map of sites sampled. Sites 5, 6, 7, 9, 10, 11, 13, 14, 18, 20, 22, 24, 26, 27, and 28 were used in this study. Images courtesy of Archbold Biological Station.

To examine the effects of non-native species on soil fungi, I sampled sites that contained different populations of non-native grasses and had two different land use histories (Appendix Table 1, Figure 2b). Specifically, I selected 'disturbed' sites that had been invaded by *Melinis repens* (Willd.) Zizka (rose Natal grass) and 'pasture' sites that had been converted to pasture by the planting of *Paspalum notatum* Flueggé (Bahia grass). All sites were located on Satellite series soils (Archbold Biological Station soil data). In disturbed scrub sites, the native vegetation was disturbed by rollerchopping in 1995, which allowed *M. repens* to invade; the disturbed scrub sites were subsequently undisturbed, allowing some native plants to recover. In pasture sites, native vegetation was removed in ~1970 by rollerchopping, burning, and fertilizing, followed by seeding with *P. notatum* and continuous grazing until 2005. The two non-native grasses differ in their life history traits: *M. repens* is an annual or perennial, warm-season grass native to South Africa that reproduces primarily by seed; *P. notatum* is a perennial, rhizomatous, sod-forming, warm-season grass native to South America. In our experiments, we tracked the fungal community in these sites both in control plots and in areas where the non-native grasses were removed.

To provide a baseline for the effects of non-native species in the disturbed and pasture sites, I also sampled native, undisturbed sites (Appendix Table 1, Figure 2b). The native sites were 'undisturbed scrub' vegetation, dominated by Florida rosemary (*Ceratiola ericoides*) shrubs with intervening open spaces, and were also on Satellite series soils (Abrahamson et al. 1984). These native sites have abundant biological soil

crusts dominated by filamentous green algae, cyanobacteria, fungi, and bacteria, with mosses and lichens at older sites (Grman and Katharine N. Suding 2009). Crusts in rosemary scrub are sensitive to physical disturbance, recovering some structural integrity within a year, but requiring more than a decade to recover the ability to fix N (Hawkes 2003). Because of the presence of crusts at the native sites, we included a crust addition treatment to examine how native microbes might help overcome any legacy of the non-native grasses.

Experimental Design

To test the effects of land use, grass removal, and the combination of biological soil crust addition with grass removal on habitat characteristics, nitrogen cycling, and native plant recovery, I sampled a series of 4-m^2 plots established in each land use type (undisturbed native scrub, disturbed scrub, pasture) in May 2006 (Hamman and Hawkes, unpublished; Figure 3). In each disturbed and pasture site, there were three plots: one control and two in which the non-native grasses were removed. These last two plots were treated with 2% glyphosate (Roundup®, Monsanto, St. Louis, MO, USA) in August 2006 with follow-up spot treatments in December 2006. In one of each pair of removal plots, we applied native microbial crust inoculum in the form of homogenized soil crusts applied to a depth of ~1 cm in December 2006 and May 2007. In native scrub sites, we established two plots, one control and one crust addition (as there were no invasive species to be removed in the undisturbed native scrub, no herbicide plots were

established). Crusts for inoculum were collected from three native scrub sites (not involved in this study) with different fire histories to represent a wide range of native microbes.

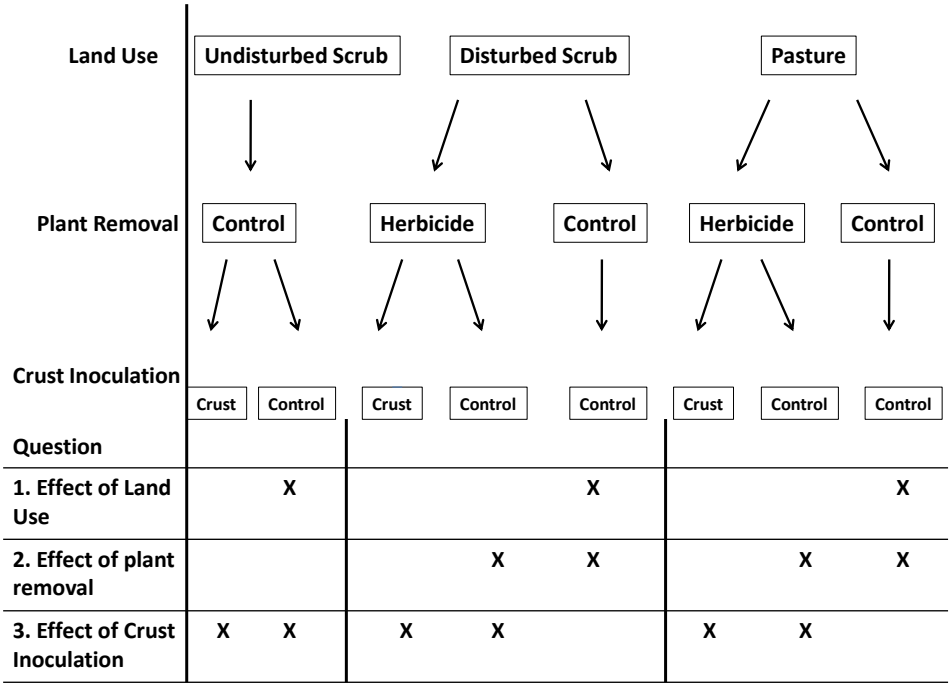


Figure 3. Experimental design and treatments included in each analysis. Note that the undisturbed scrub sites lacked non-native grasses and thus had no removal treatment. In addition, there was no crust addition treatment in the plant removal control for disturbed and pasture sites, because this is not a realistic restoration strategy. Each treatment combination had five replicates, for a total of 40 plots.

Soil Sampling

Soils from the subplots were sampled three times per year from 2006 to 2009 for a total of ten time points to measure fungal abundance and fungal community composition. For measures of hyphal abundance, we sampled the top 15 cm of soil using a 1-cm diameter soil corer. Two cores from each treatment were taken at each time point, pooled, and homogenized. Samples were placed on ice for transport to the lab and refrigerated for no more than two weeks before hyphal extractions and soil moisture measurements were taken. For fungal community analysis, we sampled the top 2-cm of soils because preliminary sampling suggested this depth of soil had sufficient DNA for PCR amplification in these sandy soils; the surface soil was collected by pressing a petri dish into soil adjacent to the core samples.

Fungal Abundance

Fungal hyphae were extracted from soil following Brundrett et al. (1994). Briefly, ~10 g of soil was mixed with 10% sodium hexametaphosphate for 5 min at high speed, 200 ml of this solution was mixed at high speed for another 2 min, and two, 5-ml subsamples were filtered through nitrocellulose membrane filters with 20-um pore size (Millipore, Billerica, MA, USA). Hyphae were stained with acid fuchsin and filters were mounted onto glass slides with Permount (Thermo Fisher Scientific Inc., Waltham, MA, USA). Gravimetric soil moisture was measured for each sample at the time of extraction. To quantify the length of hyphae in soil, the filters were viewed under 160X and quantified using grid-line intersect microscopy with 50 fields of view (Brundrett et al.1994).

Fungal Community Composition

To examine fungal community composition, DNA was extracted using a variation on the phenol-chloroform method (Kennedy et al. 2003). Approximately 1g of soil was combined with 500mL of 10%CTAB buffer and incubated at 70C for 10 minutes, 500mL of P/C/I was added to the samples, and they were shaken in a Mini Beadbeater (Biospec Products, Bartlesville, OK) for 30 seconds. Samples were placed on ice, then spun down at 16000xg for 10 min. The aqueous layer was combined with an equal volume of C/I in a clean tube, shaken well, and spun down at 16000xg for 10 minutes. The DNA was precipitated using 2 volumes of 100% EtOH and 10% NaAcc and incubated over night at 4C. Samples were spun down at 16000xg for 20 minutes and rinsed with 70% EtOH, dried, and re-suspended in 50uL dH2O. Sample concentration was measured with a Nanodrop MODELX and by gel. Samples were standardized to a 10 ng ul^{-1} concentration in a 96-well plate.

PCR

PCR amplification was performed using the fungal-specific primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (Rillig and Mummey 2006). The final PCR reaction contained 10 mM KCl, 50 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% Triton X-100, 0.25 mM dNTPs. The primers were added to a final concentration of 0.33pmol/ul. PCR reactions were run with an initial melting at 95°C for 5 min, followed by 30 cycles of 95°C for 30

s, 52°C for 60 s, 72°C for 60 s, and a final extension of 7 min at 72°C on a BioRad iCycler.

Clone Library

An experiment-wide clone library was created to generate a key for Terminal Restriction Fragment Length Polymorphism (T-RFLP) following Hausmann and Hawkes (2009). Briefly, PCR reactions from each sample in the experiment were pooled, purified using the Invitrogen PCR Cleanup Kit (Invitrogen, Carlsbad, CA, USA), and cloned with the Invitrogen Topo Cloning kit (TOPO-TA Kit, Invitrogen, Carlsbad, CA, USA) using a ratio of 0.5mol insert to 1mol vector (pCR2.1-TOPO plasmid). Transformed cells were grown on Ampicilin plate with X-gal. Colonies were picked and grown up in LB with Amp, and colony PCR was done using the M13 primers. Successful clones were sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Putative chimeric sequences (73) were eliminated using the Emerencia chimera-checker (Nilsson et al. 2005). I sequenced a total of 585 clones, resulting in ~500 usable clone sequences and 323 unique clone sequences.

To define initial phylotypes, the clone sequences from each phylum (Ascomycota, Basidiomycota, Glomeromycota, and basal fungal lineages) were aligned with their closest BLAST matches and ~1000 reference sequences using Simultaneous Alignment and Tree Estimation (SATé v2, Liu et al., 2009) with the default parameters. To confirm the accuracy of this approach, all unknowns were also confirmed using the blastn algorithm in GenBank.

Initial phylotypes were defined as monophyletic groups of sequences with less than 3% sequence divergence, the same level of similarity found in known monophyletic clades in each tree. This definition resulted in 220 initial phylotypes (133 Ascomycota, 21 Basal, 58 Basidiomycota, and 8 Glomeromycota). The initial phylotypes were collapsed into operational taxonomic units (OTUs) based on those that could be distinguished by T-RFLP (i.e., those distinguishable via fragment patterns in restriction enzyme digests, see details below).

T-RFLP

Each sample was amplified via PCR using primers ITS1F and ITS4 with HEX and FAM-5 labels, respectively. Two PCR reactions were run for each sample and the products were combined. PCR products were quantified via gel electrophoresis and with a Nanodrop (ND 1000, NanoDrop Technologies, Inc.) and brought the same concentration. Equal quantities of DNA were added to enzyme digests. The enzymes used were HinfI and HaeIII. Additional enzymes from the library of enzymes provided by GENEIOUS PRO v. 4.8.3 (Drummond et al., 2009) and suggested by Dickie & Fitzjohn (2007), including but not limited to TaqI, BslI, CviKI-1, Hpy8I, and MboI, were tested for their ability to further distinguish OTUs, but were unable to provide additional differentiation. Digested samples were quantified using a Nanodrop (ND 1000, NanoDrop Technologies, Inc.) and brought to a standard concentration. Fragments were quantified using a ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA,

USA). The resulting TRFLP profiles were analyzed using PeakScanner Software v.10 (Applied Biosystems) with a baseline threshold of 50 fluorescence units.

A total of 184 TRFLP OTUs were defined based on the fragment patterns (forward and reverse) generated by the two enzymes HaeIII and HinfI. The initial OTUs that were collapsed at this stage were all sister taxa at terminal nodes in their respective phylogenetic trees.

Sample profiles were matched to *in silico* digests of the TRFLP OTU sequences using TRAMPR (Fitzjohn and Dickie 2007). We considered an OTU present if at least two identifying fragments were found within 5 bp of the expected fragment length. In previous work, *in silico* digests were accurate within 5 bp of experimental digests (Hausmann and Hawkes 2009). We used the TRFLP data to detect only the presence or absence of an OTU in each sample, thus allowing us to estimate fungal richness but not relative abundance.

Statistics

Fungal abundance and community composition were examined to understand (1) the effect of land use history (disturbed, pasture) compared to native scrub, (2) the legacy of land use after non-native grasses were removed, and (3) the legacy of land use when non-native grass removals were combined with native biological soil crust addition. Because these were sampled over three years, the persistence of legacy effects on soil

fungi can also be addressed. Repeated measures ANOVAs were used for fungal hyphal abundance, but because the full design was incomplete (e.g., there were no non-native grasses in the undisturbed control sites and thus no removal treatment), separate analyses were performed on three subsets of the data (Shaw and Mitchell-Olds 1993). (1) To compare the effects of land use history type, only samples from the control plots (no herbicide, no crust addition) from each land use history were considered. Land use types were compared, with plot as the unit of replication. (2) For the second question regarding plant removals, only pasture and disturbed land use types were included, all crust addition subplots were excluded, and plots with and without herbicide were compared (3) Finally, to address the third question of combined crust addition and plant removal effects, only the plant-removal (herbicide-treated) plots were used from the pasture and disturbed sites because the plant cover in the untreated plots prevented the formation of a biological soil crust entirely and the addition of crust without the removal of the invasive species is not a viable restoration strategy. The analysis also included the plots from the undisturbed scrub, where no herbicide was used as there were no invasive plants present. Subplots with and without crust addition were compared. In all analyses, a factor was considered significant if $P < 0.05$. When sphericity was violated in repeated measures ANOVAs, we applied the Greenhouse-Geisser epsilon correction. When date or any interactions with date were significant factor in the repeated measures ANOVA, univariate ANOVAs were used for each date to examine the experimental treatments at individual dates.

To examine the changes in fungal community composition, multiple response permutation procedures (MRPPs) were used to address the three questions above. The MRPPs were run parallel to the ANOVAs used for hyphal abundance. When date was an important factor, the seasonal drought patterns were tested post hoc an explanation for date-to-date variability. Three values are reported for the MRPPs: T (the test statistic), A (effect size), and *P* (*P*-value). A is a comparison between within-group homogeneity, and the random expectation. The highest possible value is A=1, which means that there is no within group heterogeneity. When A = 0, the within group heterogeneity is equal to that expected by chance. If A < 0, the within group heterogeneity is greater than expected by chance. In ecological datasets, A values near 0.1 are common, and A>0.3 is considered quite high (McCune & Mefford, 2006). Pair wise MRPPs were run post-hoc and Bonferroni corrected to examine significant interactions or comparisons between multi-level variables. To visualize the significant differences identified by the MRPP tests, non-metric multidimensional scaling (NMS) analysis with Bray- Curtis dissimilarity matrices was implemented via PC-ORD v. 5.32 (McCune & Mefford, 2006). Two runs were performed, the first with the 'fast' default settings to provide a starting configuration, and the second with the 'medium' settings with the results from the first run as a starting point. The initial NMS was performed including all data in a single run; when the MRPP tests identified significant interactions, additional NMS analyses were run to visualize the breakdown of the interaction term as well.

Finally, because date was repeatedly a significant factor and from a cursory examination of seasonality appeared to be related to dry vs. wet conditions, I further analyzed the relationship between drought and fungal community composition. To do this, I classified each sampling date as either being under normal or drought conditions (as a binary variable) based on data from NOAA (REF). I then ran MRPP tests to examine whether drought explained the fungal community data associated with each of my three questions. Because this is a post-hoc comparison, I adjusted my *P*-values using the Bonferroni method.

RESULTS

Question 1: What is the effect of land use on hyphal abundance and fungal community composition?

Hyphal abundance varied with land use (Appendix Table 2), with pasture sites having the most soil hyphae, disturbed sites having fewer hyphae, and undisturbed native scrub sites having the fewest hyphae (Figure 4a). Hyphal abundance was also significantly different among dates and there was a significant date*land use interaction, indicating that the effects of dates differed among land uses (Appendix Table 2). There was a significant difference in hyphal abundance by land use for September 2006 ($P = < 0.001$) and December 2006 ($P = 0.001$). There was no difference in hyphal abundance between land uses during the first sampling point after treatment, and during the last two sampling points.

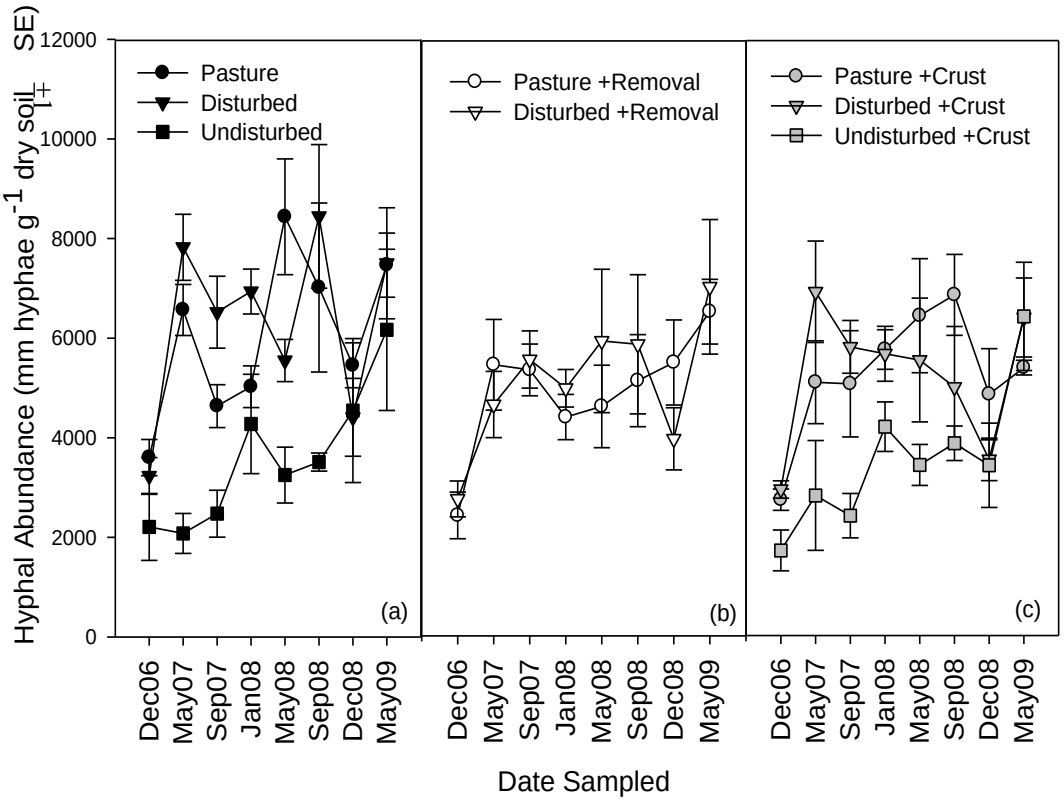


Figure 4. Soil fungal hyphal abundance over all sample dates by question. (a) the effect of land use on hyphal abundance. (b) the effect of plant removal on hyphal abundance. (c) The effect of biological soil crust inoculation on hyphal abundance (note that the pasture and disturbed values refer to the herbicide-treated values). Points are mean ± 1 SE (n = 5).

The soil fungal community was distinct in each land use (Appendix Table 3) and there was little overlap of taxa (Figure 5). Fungal community composition also varied among dates (Appendix Table 3); some of this variation may be accounted for by drought status (Appendix Table 3).The undisturbed community had greater variation within each

time point and varied less from date to date than the disturbed or pastures sites (Figure 6). The disturbed community showed an intermediate level of variation within each time point, and the pasture sites showed the least variation within each time point (Figure 6, 7). However, the interaction between date and land use had a larger effect than either land use or date alone (Appendix Table 3, Figure 6).

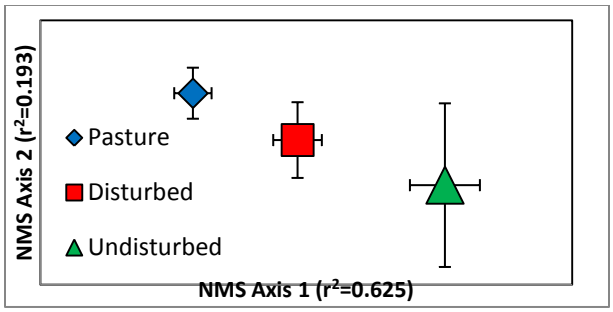


Figure 5. NMS ordination of fungal communities associated with native scrub, disturbed, and pasture sites. Each point reflects the average ± 1 SE of the NMS scores for each land use across all sample dates (Pasture, n = 128. Disturbed, n = 132. Undisturbed, n = 55). The stress value was 16.68.

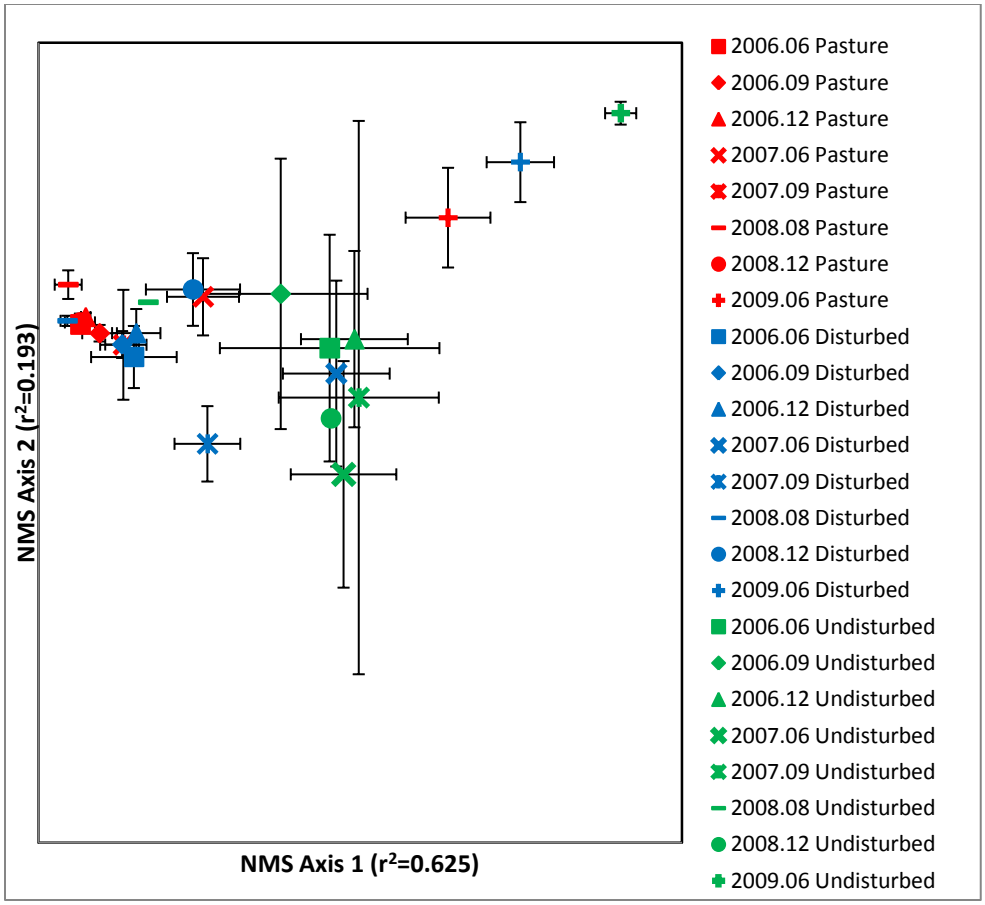


Figure 6. NMS ordination of fungal communities associated with each land use across ten sampling dates. Each point reflects the average ± 1 SE of the NMS scores for each land use on each date (Pasture, n = 10, 10, 20, 18, 15, 19, 20. Disturbed, n = 10, 9, 20, 15, 20, 19, 20. Undisturbed, n = 4, 5, 9, 9, 3, 10, 9, 6). The stress value was 16.68.

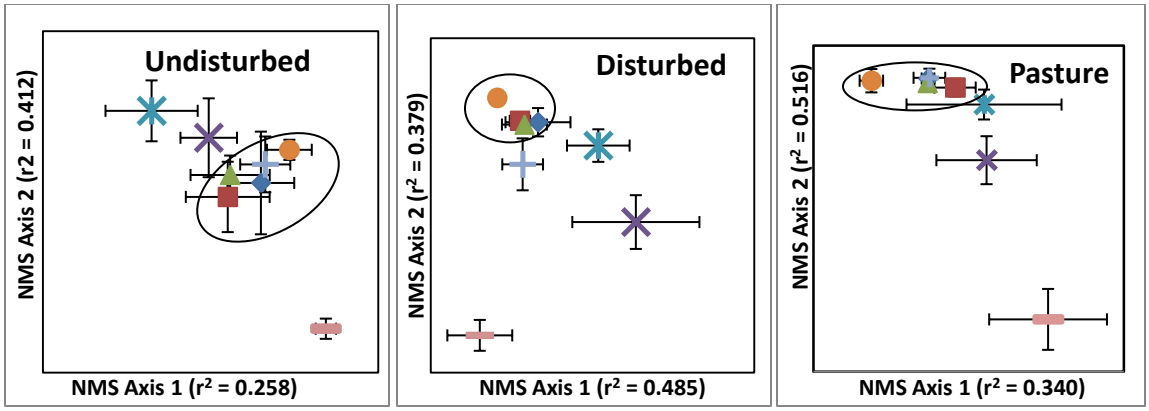


Figure 7. NMS of fungal community composition across sample dates in native scrub, disturbed scrub, and pasture sites. Each point reflects the average ± 1 SE for NMS scores on each date (n = 5). Circles indicate time points where drought conditions prevailed. NMS axes are scaled differently because of the overall differences in the degree of community change (see Figure 6): Undisturbed Native scrub spans 100 x 100 units. Disturbed spans 2 x 2 units, Pasture spans 20x100 units. Undisturbed scrub stress value = 13.46, Disturbed stress value = 16.58, Pasture stress value = 11.49.

When the interaction term was broken down to examine fungal community composition separately in each land use across dates (Appendix Table 8), it became clear that the fungal community was highly similar on some dates but not others in each land use (Figure 7). Some of this variation appears to have been due whether drought conditions prevailed on the sample dates. During times of drought, the fungal community

in each site repeatedly returned to a similar composition; during non-drought conditions, however, the fungal community was far less predictable (Figure 7). Pasture sites were the least variable in fungal community composition during drought (Figure 7c), while native scrub sites were the most variable (Figure 7a).

Question 2: Do changes in fungal community composition and abundance act as a persistent legacy effect after non-native grasses are removed?

Plant removal reduced hyphal abundance (Appendix Table 4) by a factor of X in pastures and by X in disturbed sites (Figure 4b). At some time points in disturbed sites (but not in pasture sites), there was a large difference in hyphal abundance between the control and plant removal plots (Figure 4b), possibly accounting for the three-way interaction between date, land use history, and plant removal (Appendix Table 4).

Plant removal had a significant effect on fungal community composition, although this varied with date and land use (Appendix Table 5). Based on the post-hoc pair wise comparisons of the interaction between land use and removal, non-native grass removal did not significantly change fungal community composition within land use (i.e., there was no difference between fungal communities in pasture control and removal plots) (Appendix Table 5). The three way interaction between date, land use, and plant removal was significant (Appendix Table 5). Looking at the post-hoc pair wise MRPP comparisons, the effect seems to be driven primarily by differences in date, followed by

land use, and then rarely by within year and land use and plant removal. The comparison between grass removal and controls in pastures was only significant at one date (May 2006, disturbed removal vs. disturbed Control, $P = 0.017$). More often, community composition is significantly different across dates and across land use in the (Appendix Table 5). The effects of date, land use history, and the interactions were consistent with the patterns observed in the control plots (Question 1).Similarly, drought conditions appear to be associated with more predictable fungal communities in disturbed scrub and pasture removal plots (NMS not shown), as they were across the control plots (Figure 7b, c).

Question 3: Does the combination of biological soil crust inoculation and non-native grass removal affect soil fungal legacies and their persistence?

The addition of biological soil crust to the non-native grass removal treatments did not affect hyphal abundance relative to control plots in any land use (Appendix Table 6, Figure 4c). Land use and the interaction of date with land use did significantly affect hyphal abundance (Appendix Table 6), following the patterns previously observed in Questions 1 and 2.

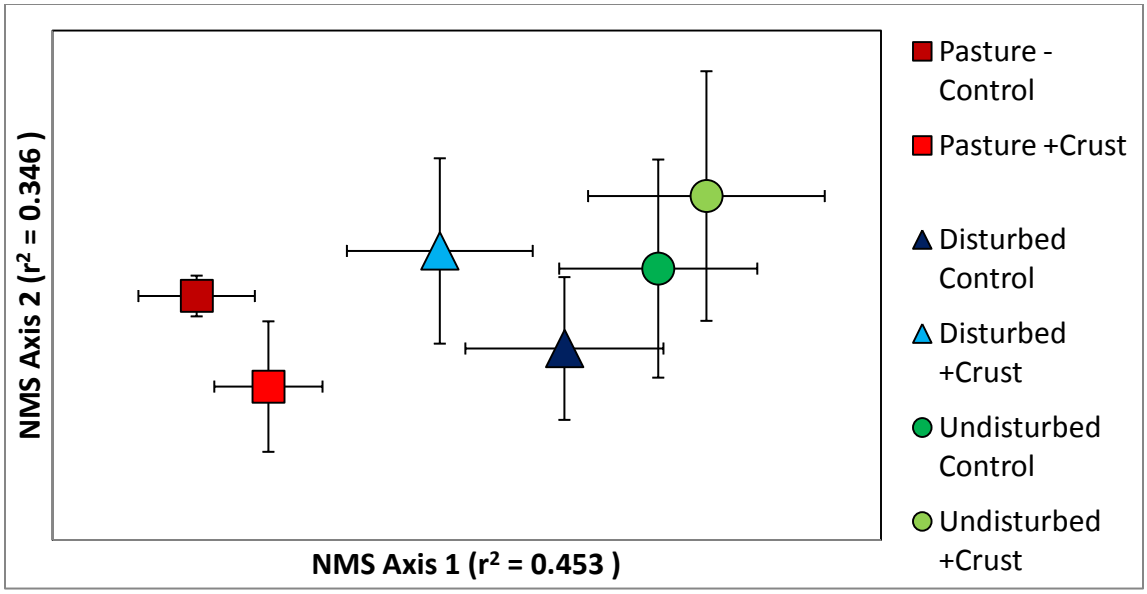


Figure 8. NMS visualizing the effect of crust addition onfungal community composition. Each point reflects the average ± 1 SE for NMS scores on each treatment combination (Pasture Control n = 27, Disturbed Control n = 28, Undisturbed control n = 24, Pasture +Crust n = 28, Disturbed +Crust n = 27, Undisturbed +Crust n = 22). The final stress was 17.66.

Fungal community composition, was affected by soil crust additions, but the effect was dependent on both date and land use (Appendix Table 7). The effect size for the three way interaction between date, land use, and crust inoculation was due to crust inoculation consistently causing a small change in fungal community composition, but the directionality of that shift differed in each land use (Figure 8). Fungal community composition remained most similar within land use after crust addition. The main effects of land use and date (Appendix Table 7), as well as their interaction, were consistent with

those observed for the control plots (Question 1). Finally, drought conditions appeared to be associated with fungal community composition in a manner consistent with that identified earlier (NMS not shown).

DISCUSSION

Legacy effects of land use history on soil fungal abundance and community composition were found in these central Florida communities. There were substantial differences in soil fungal abundance and community composition in disturbed and pasture sites compared to native scrub, and these differences persisted for three years after non-native grasses were removed. Not only did the grass-dominated pasture and disturbed sites differ from the undisturbed native shrub-dominated ecosystem, they differed significantly from each other, indicating that the different non-native grasses and other specific changes associated with each land use played a role in soil fungal communities. In a study separating the effect of land use history from those of the current aboveground plant community in and near ex-arable fields, Kulmatiski & Beard (2008) found that land use had a persistent legacy effect on the soil microbial community that was not overcome by the current plant community. Other studies have demonstrated similar persistent legacies of non-native plants on soil microbial communities (e.g., Maron and Jeffries 2001, Kulmatiski & Beard 2008, Corbin & D'Antonio in press).

Non-native grass removals caused a significant reduction in hyphal abundance, and also changed fungal community composition in the pasture sites. The reductions in abundance were not sufficient, however, to return abundance to the low levels found in the undisturbed native vegetation sites. These patterns persisted even three years after the removal of non-native grasses. It is likely that abundance of this largely saprotrophic community was driven more by soil resources than by the presence of non-native grasses

per se (Waldrop et al. 2006). Invasive species are well known for altering both carbon and nitrogen cycling (Liao et al. 2007) and the available soil carbon and nutrients will likely have been affected by the long-term presence of the non-native grasses.

Invasive species alter litter characteristics, changing both the quality and quantity of the litter. The most common patter is an increase in the nitrogen concentration of litter as a result of invasion, and a reduction in the carbon:nitrogen and lignin:nitrogen ratios (Kao-Kniffin and Balser 2008), making the litter of 'higher' quality as more nitrogen per unit carbon is acquired during its decomposition. Related to litter quality, invasive species often also increase the rate of decomposition (Liao et al. 2008). Invasive species also tend to increase the concentrations of both soil NH_4^+ and NO_3^- (Liao et al. 2008), which was also observed at the current study site (Hamman and Hawkes, unpublished).

Some invasive species effects on soils may thus be proportional to the amount of biomass and litter they contribute to the ecosystem. While biomass was not specifically measured in this experiment, based on total percent cover at the three different sites types, the pasture sites had much higher aboveground plant cover than the other two sites (Appendix Table 1). As with the majority of the patterns observed in this system, the disturbed sites were intermediate, with the undisturbed native vegetation sites having the lowest total percent cover. Consistent with the previous studies, the increase in cover was accompanied by greater nitrogen and carbon in the soils of the pasture and disturbed sites than in undisturbed control sites. This suggests that when dealing with plant legacies on fungi, altering resource availability between the desired and current systems would be

more effective for achieving restoration goals than only the removal or re-introduction of aboveground plant communities.

Inoculation with biological soil crust had no effect on soil hyphal abundance or fungal community composition, with inoculated plots remaining most similar to their untreated controls. Inoculation with native microbes is sometimes used as a method for restoring microbial community composition. In this system, inoculation with biological soil crusts was not an effective means to affect the fungal community, but it may be more effective for other members of the microbial community in the biological soil crust such as soil algae or cyanobacteria. Additionally, the inoculation was prepared by collecting soil crust from several native sites across a range of successional stages, mixing it, and applying it to the treatment sites; to alter the fungal community may require a more tailored approach, perhaps via inoculation with isolated spores or cultured fungal tissues from native sites. Fungal communities have been successfully added to sites where the fungi were symbiotic with the plant community that they were attempting to introduce (Smith & Read 2008). Given our understanding of drivers of fungal abundance (resource availability), it makes sense that the application of crust did not reduce hyphal abundance to undisturbed site levels.

One possible underlying driver for the changes we observed in both the abundance and composition of the fungal community may have been seasonal and annual changes in factors such as moisture availability (or potentially correlated factors such as dissolved soil nutrients). Soil moisture is likely to be particularly important in these soils,

which are xeric due to their sandy nature. Hyphal abundance in soil has been shown to respond to various measures of water availability. Across published studies, the relationships between hyphal abundance and soil moisture are inconsistent, including increased, decreased, and no change in hyphal abundance with increasing drought stress (Lutgen et al. 2003, Staddon et al. 2003, Clark et al. 2009, Querejeta et al. 2009, Hawkes et al. 2011). It is possible that hyphal responses differ based on the relative amount of water available for fungal metabolism (Raich and Potter 1995), which could be affected by soil type, plant community composition, and so forth.

Fungal community composition, unlike hyphal abundance, appeared to respond predictably to water availability in the current study. During drought, fungal community composition tended to return to a single, predictable set of taxa that was specific to the land use. In contrast, periods of water abundance were associated with different, less predictable shifts in fungal community composition. This is similar to what was observed in California grasslands in the only other study to track fungal communities for a similar period of time (four years; Hawkes et al. 2011). During low-water periods, competition might be high, resulting in the dominance of the few species that are best able to grow under drought conditions or best at acquiring water. During periods of water abundance, the competitive pressure may be lower, allowing fungi that are not so drought specialized to flourish as well, resulting in a different and more diverse fungal community. Alternatively, drought may moderate fungal competition (Chase & Leibold 2003), thereby predictably allowing more taxa to coexist.

When comparing fungal community composition across time in each land use, there was more variability in fungal community composition in native scrub sites than in either disturbed or pasture sites. This may be partly because the native scrub sites were chosen to include the natural range of successional stages, ranging from 5 to 23 years after fire. The narrower responses observed for fungal community composition in pastures and disturbed sites over time could also reflect differences in how these communities respond to seasonal or annual changes in environmental conditions compared to native scrub sites. For example, fungal communities in pastures may be somewhat more buffered against drought than native sites are due to the complete cover of grasses and increased soil organic matter, which could lead to sufficient moisture retention to maintain fungal communities. Alternatively, the higher levels of soil organic matter in pastures may maintain fungal taxa regardless of other environmental conditions, consistent with studies that have found resources as the primary driver of fungal community composition (e.g., Waldrop et al. 2006).

The combined results of this study have implications for restoration ecology. The current dependence of the fungal community on land use and the associated non-native species invasions (along with other analyses done in this system) suggest that a different approach to restoration is required here to overcome the observed legacy effects. While in some ecosystems, the removal of invasive plant species is all that is required to return a given ecosystem parameter to its original state (Malcolm et al. 2008), in many cases it is not nearly that simple (Eviner and Hawkes 2008). The persistence of plant legacy effects

in both fungal community composition and abundance indicates that, once the fungal community is established in this system, the plant community is not the proximate driver (if it ever was). In the Florida ecosystem, the altered fungal communities may be considered an alternative stable state (Suding et al. 2004). If, however, the fungal communities are maintained by resource availability, then direct removal of the soil nutrient legacy may be required for successful restoration. This may be achieved by topsoil scraping, which has been successful in some other ecosystems (Heneghan et al. 2008). More and more we are recognizing that fungi play an important role on multiple levels of ecological organization. When fungi are part of an invasive plant legacy, their restoration may be an important aspect of whole-ecosystem restoration.

APPENDIX

Table 1 Comparison of study site types. Values are means ± 1SE.				
	Undisturbed Scrub	**Disturbed Scrub**	**Pasture**	**Source**
Non-native vegetation cover (%)	0	16.8 ± 11% (*M. repens)*	68.0 ± 2.5% (*P. notatum*)	Hamman & Hawkes, unpublished
Native plant presence	Abundant native herbs and shrubs (dominated by *Certiola ericoides*)	Loss of *C. ericoides*, some native herbs persist	Few native shrubs, no native herbs	Glinka, personal obs.
Site Openness (% bare ground)	85.6 ± 5.3	57.2 ± 17.1	2.5 ± 0.8	Hamman & Hawkes, unpublished
Total inorganic nitrogen (ug g^{-1} soil)	1.0 ± 0.1	1.5 ± 0.2	2.5 ± 0.2	Hamman & Hawkes, unpublished
Soil organic matter (%)	0.95 ± 0.09 %	0.60 ± 0.09 %	1.15 ± 0.09 %	E.S. Menges, unpublished
Biological soil crust depth (mm)	14.6 ± 1.2	10.3 ± 1.3	3.5 ± 0.8	Hamman & Hawkes, unpublished

Table 2. Results from ANOVA on the abundance of hyphae in the untreated plots in each of the three land uses.

Between subjects Factors				
	df	MS	*F*	*P*
Land Use	2	6.55E+07	18.616	<0.001*
Error	12	3.52E+06		
Within subjects Factors				
Date	6	2.30E+07	7.655	<0.001*
Date*LandUse	12	8.47E+06	2.825	0.003*
Error	72	3.00E+06		

Table 3. Summary of MRPP testing the effect of land use on fungal community composition.

MRPP Summary			
	T	*A*	*P*
Drought	-12.0242	0.04151451	< 0.001
Date	-14.0338	0.13173259	< 0.001
Land Use	-12.3981	0.06077161	< 0.001
Datex Land Use	-11.7281	0.21914504	< 0.001

Table 4. Post-hoc MRPP analysis for Question 1 testing the effect of date on each land use individually.

	T	A	*P*
Undisturbed Scrub			
Date	-2.7903146	0.16710779	0.007
Drought	-1.6167483	0.03191567	0.072
Disturbed Scrub			
Date	-5.1147532	0.13447506	< 0.001
Drought	-5.276304	0.04817037	0.001
Pasture			
Date	-9.4805197	0.2347719	< 0.001
Drought	-6.3727583	0.0546358	< 0.001

Table 5. ANOVA testing the effect of plant removal on hyphal abundance. As sphericity was violated, the Greenhouse-Geisser correction (ε = 0.611) was applied.

Between subjects factors	df	MS	*F*	*P*	GG-*P*
Land Use	1	348643.9505	0.06302	0.805	
Removal	1	30028822.37	5.42755	0.033	
LandUse*Removal	1	7127.04	0.00129	0.972	
Error	16	5532667.905			
Within subjects factors					
Date	6	3.36E+07	15.729	<0.001	<0.001
Date*LandUse	6	4140420.675	1.941	0.082	
Date*Removal	6	2853537.446	1.33803	0.248	
Date*LandUse*Removal	6	5691382.924	2.66871	0.019	0.045
Error	96	2132635.443			

Table 6. Summary of MRPP testing the effect of plant removal (herbicide) on fungal community composition.

MRPP Summary			
	T	*A*	*P*
Drought	-20.9299	0.056271	<0.001
Date	-21.1351	0.153531	<0.001
Land Use	-5.18602	0.013943	0.002
Removal	-3.11801	0.008391	0.016
Date*Land Use	-16.2379	0.1779	<0.001
Date*Removal	-15.2802	0.155345	<0.001
Date*Land Use*Removal	-11.0089	0.171767	<0.001
Post-hoc Pair wise Comparison			
Pasture Control vs. Pasture Removal	-1.61048257	0.00844938	0.07369123
Pasture Control vs. Disturbed Control	-3.83431969	0.01619548	0.00716323
Pasture Control vs. Disturbed Removal	-7.68112229	0.03952999	0.0001582
Pasture Removal vs. Disturbed Control	0.00402924	-0.00002294	0.37613816
Pasture Removal vs. Disturbed Removal	-0.60915153	0.00484078	0.20618945
Disturbed Control vs. Disturbed Removal	-1.22611248	0.00713221	0.11032779

Table 7. ANOVA testing the effect of biological soil crust addition combined with grass removals on hyphal abundance. Sphericity was violated, and the Greenhouse-Geisser correction (ε = 0.592) was applied. Note that the 'land use' treatment compares the native scrub sites with the grass removal plots from the disturbed and pasture sites.

Between subjects factors	df	MS	*F*	*P*	GG-*P*
Land Use	2	5.68E+07	7.456	.003*	
Crust	1	295943.822	0.039	0.845	
LandUse*Crust	2	371055.942	0.049	0.953	
Error	24	7.62E+06			
Within subjects factors					
Date	6	3.88E+07	15.061	<0.001	<0.001
Date*LandUse	12	6.52E+06	2.533	.005*	.020*
Date*Crust	6	2.73E+06	1.059	0.39	
Date*LandUse*Crust	12	1.74E+06	0.676	0.772	
Error	144	2.58E+06			

Table 8. Summary of MRPP testing the effect of crust addition on fungal community composition.

MRPP Summary			
	T	*A*	*P*
Drought	-6.5299248	0.02023181	< 0.001
Date	-17.467701	0.14981771	< 0.001
LandUse	-8.2376897	0.03616674	< 0.001
Crust	1.0312111	-0.00319172	0.92088543
LandUse * Crust	-8.2376897	0.03616674	< 0.001
Date * LandUse	-15.119562	0.21885194	< 0.001
Date * Crust	-12.621228	0.14242641	< 0.001
LandUse * Crust	-4.8113344	0.03372422	< 0.001
Date * LandUse * Crust	-10.171913	0.21686455	< 0.001

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