Introduced Canopy Tree Species Effect on the Soil Microbial Community in a Montane Tropical Forest

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Abstract: Within a single site in the Kohala Forest Reserve, Hawai'i, we examined composition and diversity of soil microbial communities under four introduced (*Cryptomeria japonica*, *Casuarina equisetifolia*, *Araucaria columnaris*, and *Eucalyptus* sp.) and one native (*Metrosideros polymorpha*) canopy tree species, as well as pasture. Terminal restriction fragment length polymorphism (T-RFLP) analysis of soil bacteria, fungi, and archaea indicated that soil under the native *M. polymorpha* had the highest richness and greatest number of unique terminal restriction fragments, whereas soil under *Eucalyptus* and in pasture sites had the lowest richness. The soil microbial community differed significantly between *Eucalyptus* and *M. polymorpha* but not between the other three introduced species and *M. polymorpha*. The *Eucalyptus* microbial community was more similar to that of an adjacent deforested pasture site than to those of other forested stands. Soil pH was the environmental variable that best correlated with the variation in soil microbial community composition between tree species.

Plant species can influence the composition of underlying soil microbial communities (Grayston et al. 2001, Kourtev et al. 2002, Garbeva et al. 2006). These influences can be due to differences in canopy cover, rooting depth, and litter quality and quantity (Gregory 2006) or to secondary effects on soil pH, moisture, and nutrient levels (Lynch and Whipps 1990, Wardle 1992). These observations suggest that invasive plants differing in their ecological traits from native plants have the potential to alter soil microbial communities, which may in turn affect biogeochemical cycling and ecosystem functioning. However, although some studies have examined the effects of introduced plants on soil microbial communities (Kourtev et al. 2002, Lejon et al. 2005, Carney and Matson 2006), more have evaluated the effects of soil biota on introduced plants (Reinhart and Calloway 2006, van der Putten et al. 2007).

The abundance of invasive plant species is especially acute in oceanic islands (Vitousek et al. 1997). Although the effects of certain invaders on the native flora, on primary succession, and on ecosystem structure and biogeochemistry of Hawai'i and other oceanic islands have been documented (Vitousek and Walker 1989, Rothstein et al. 2004, Hughes and Denslow 2005), there is little literature on their effects on soil microbial communities, although soil microbes could contribute to the community and ecosystem alterations that have been described. One study conducted in Hawai'i examined the effects of experimental fertilization, of duplicate sites, and of two plant species (the invasive understory plant *Hedychium gardnerianum* and the native tree *Metrosideros polymorpha*) on soil microbial communities (Kao-Kniffin and Balser 2008). The authors found that *H. gardnerianum* had little effect relative to site location and fertilization (Kao-Kniffin and Balser 2008).

In this paper, we evaluate the effects of different canopy tree species on soil microbial communities. Soil properties and microbial

community composition were examined under four introduced canopy tree species (*Araucaria columnaris*, *Cryptomeria japonica*, *Eucalyptus* [probably *robusta*], and *Casuarina equisetifolia*), the dominant native tree species (*Metrosideros polymorpha* [hereafter referred to by its common name, 'ōhi'a]), and adjacent pasture. We tested the hypothesis that the soil properties and microbial community composition under replicate stands of each canopy tree species would be more similar to each other than to those under stands of other tree species. Further, we hypothesized that *C. equisetifolia* would have a greater effect than other tree species due to its N-fixing ability. Of the four introduced trees studied, only *C. equisetifolia* is considered highly invasive (Little and Skolmen 1989). We also expected that pasture microbial communities would differ substantially from those under all trees given that numerous studies have found changes in soil microbial communities after deforestation (Nusslein and Tiedje 1999, Steenwerth et al. 2002).

MATERIALS AND METHODS

Field Site

The field site is located in the Kohala Forest Reserve on the island of Hawai'i, near a long-term research site on a substrate age gradient across the archipelago (Crews et al. 1995). The site is a tropical, wet, montane forest that receives an average precipitation of 2,500 mm per year and is at 1,122 m elevation (Crews et al. 1995). The underlying substrate is about 150,000 yr old. The site has relatively high levels of foliar N and P (Porder et al. 2005) and available soil nutrients (Crews et al. 1995) compared with other Hawaiian sites on the substrate age gradient. The site has also been investigated by high-resolution remote sensing, which found relatively high levels of foliar N, especially in plantations of introduced tree species (Vitousek et al. 2009).

Samples were taken from 10 tree-dominated stands and two pasture sites. All 12 sample areas were located on the same substrate and experienced approximately the same climate; differences in microbial communities among stands should reflect differences in canopy species or distance between sites, rather than climatic or substrate differences among sites. The stands were selected by dominance of the target tree species, with preference to stands that were more than 80% composed of the desired canopy species. Within each stand a 2 m by 2 m quadrat was established along the downhill plot edge. Three soil cores were taken at a depth of 0–10 cm at random locations within the quadrat. These soil samples were then used to determine soil chemical properties, including C and N concentrations and pH. Another five soil cores to the same depth were also taken at random locations and were used to analyze the microbial community. Leaf litter was collected from the forest floor within each quadrat from a 25 cm by 25 cm area; three random samples from each quadrat were used to determine litter biomass, cation concentrations, and total C and N.

Determination of Microbial Community Structure

The multiplex terminal restriction fragment length polymorphism (M-TRFLP) protocol described by Singh and Thomas (2006) was used to analyze soil bacterial, archaeal, and fungal communities. DNA was extracted from 0.5 g soil using an extraction kit (PowerSoil, MoBio Laboratories, Carlsbad, California) according to the manufacturer's instructions. The quantity and quality of extraction were checked using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, Massachusetts) to determine both concentration and the DNA/protein ratio. Three individual polymerase chain reactions (PCR) were then performed using the three different primer sets (Table 1). Each PCR was carried out in a total volume of 25 μl, with 0.08 μl each of the forward and reverse primer, 2 μl sample DNA, 12.5 μl of AmpliTaq Gold Master Mix (Applied Biosystems), and 10.34 μl PCR grade H_2O. This represented a primer concentration of 0.3 μM and 0.03–0.09 μg DNA per reaction. The reaction was carried out on a thermocycler (Gradient Vapo-Protect, Eppendorf AG, Hamburg, Germany).

Primer	Fluorescent Label	Sequence (5′ to 3′)	Target Region	Specificity	Reference
63f	VIC (green)	AGGCCTAACACATGCAAGTC	16S rRNA	Eubacteria	Marchesi et al. (1998)
1087r	None	CTCGTTGCGGGACTTACCCC	16S rRNA	Eubacteria	Hauben et al. (1997)
Ar3f	NED (yellow)	TTCCGGTTGATCCTGCCGGA	16S rRNA	Archaea	Giovannoni et al. (1988)
AR927r	None	CCCGCCAATTCCTTTAAGTTTC	16S rRNA	Archaea	Jurgens et al. (1997)
ITS4r	PET (red)	TCCTCCGCTTATTGATATGC	ITS	All fungi	White et al. (1990)
ITS1f	None	CTTGGTCATTTAGAGGAAGTAA	ITS	All fungi	Gardes and Bruns (1993)

The program was activation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min. The last cycle was followed by extension at 72°C for 10 min. The quality of PCR was determined by gel electrophoresis in 1% agarose stained with ethidium bromide and visualized with ultraviolet light on a gel imager (S-1500, Fotodyne, Hartland, Wisconsin). Ten μl of PCR product from each reaction were then combined and purified using a PCR clean-up kit (GenElute, MoBio Laboratories, Carlsbad, California) according to the manufacturer's instructions. A restriction digest was carried out in 30 total μl with 26.2 μl of PCR grade H_2O, approximately 500 ng PCR product, 3 μl 10× buffer C, 0.3 μl BSA at a concentration of 10 μg/μl, and 0.25 μl each of MspI and HhaI (all reagents from Promega, Madison, Wisconsin). After the digest, 1 μl of product was combined with 0.25 μl of LIZ-500 size standard and 9.75 μl of formamide buffer and then analyzed (on an ABI 3100 GeneScan, Applied Biosystems, Foster City, California) at the Stanford University Protein and Nucleic Acid Facility.

Determination of Soil and Litter Properties

Soil ammonium and nitrate concentrations were analyzed on a discrete analyzer (Smart-Chem 200, WestCo Scientific Instruments, Brookfield, Connecticut) after extraction with 2 M KCl (Keeney and Nelson 1982). Soil pH was measured in 0.01 M $CaCl_2$ with a soil to solution ratio of 1 : 5 (Hendershot et al. 2008). Soil and litter were oven dried at 65°C for 48 hr, then weighed to determine gravimetric water content and litter biomass, respectively. Litter cations were analyzed on a spectrometer (IRIS Advantage/1000 Radial ICAP, Thermo Jarrell Ash, Franklin, Massachusetts) after dry ashing at 550°C and dissolution in HCl (Miller 1998). The soil and litter total C and N were analyzed via combustion/gas chromatography (on a NA 1500 CHNS Analyzer, Carlo Erba, Milan, Italy). Soil extractable P was determined using the resin P method as in Crews et al. (1995). All equipment used was provided by the Environmental Measurement I: Gas-Solution Analytical Center at Stanford University.

Statistical Methods

M-TRFLP profiles were produced using Peak Scanner software (Applied Biosystems). A threshold of 50 fluorescence units was set to distinguish signal from noise. Peaks outside the 50 to 500 base pair range were excluded to avoid terminal restriction fragments (TRFs) caused by primer-dimers and to ensure that fragments did not exceed the linear range of

the LIZ-500 size standard. TRFs were aligned by rounding to the nearest integer in base pairs. True peaks were considered to be those whose height exceeded the standard deviation computed over all peaks (Abdo et al. 2006). TRFs that did not occur in both replicate amplifications were excluded. TRF profiles were expressed in relative abundance, using the respective peak height divided by the total peak height in a given sample as a proxy for abundance (Fierer and Jackson 2006).

The Shannon diversity index and Simpson's reciprocal diversity index were calculated for each group of taxa across sites, using the "vegan" package for R. Both indices were used because they respond differently to changes in rare and abundant species. The Shannon diversity index (H) has increased sensitivity to changes in rare species whereas Simpson's reciprocal diversity index (D) is more sensitive to changes in abundant species (Davis and Smith 2001).

The online program T-REX (Culman et al. 2009) was used to create a community data matrix that was then exported to R. To test for spatial autocorrelation, a Mantel correlogram was produced using the "ecodist" package for R (Goslee and Urban 2007). Nonmetric multidimensional scaling (NMDS) was performed to compare the microbial communities under each tree species using the "vegan" package for R (Oksanen et al. 2011). The "envfit" function in the "vegan" package for R then was used to test how well canopy tree species identity correlated with microbial community composition. This method calculates the centroid from the replicates of each factor and determines significance by random permutations. If randomly permuted data frequently produce equal or better r^2 values, then the data are nonsignificant (Oksanen et al. 2011). Environmental variables and diversity indices were then fit onto the ordination of the microbial community using the "envfit" function. This method produces vectors that point in the direction a variable is most rapidly changing and that have lengths proportional to the strength of the correlation between the ordination and a given variable (Oksanen et al. 2011); significance is calculated using permu-

tations. Soil and litter properties were all analyzed by analysis of variance (ANOVA).

<div align="center">RESULTS</div>

Microbial Community Composition

Stands differed in both their total TRF number and number of unique TRFs (Figure 1). Only 12 TRFs out of the 159 identified were common to all stands. The two ʻōhiʻa stands had the greatest number of unique TRFs, 12 and 10 for ʻōhiʻa stands 1 and 2, respectively. ʻŌhiʻa stand 1, *C. equisetifolia* stand 1, *C. japonica* stand 1, and ʻōhiʻa stand 2 all supported high numbers of total TRFs, whereas both of the *Eucalyptus* and pasture stands supported lower numbers of total TRFs. Bacterial TRFs were far more numerous (stand-level richness of 27–63) than were those of fungi (7–14) and archaea (7–14).

For both Shannon and Simpson's reciprocal diversity indices, the two ʻōhiʻa stands had the highest diversity, whereas *Eucalyptus* and pasture stands had the lowest diversity (illustrated for Shannon in Figure 2). These differences among all stands were significant (ANOVA, $F = 2.25$; df = 11, 48; $P < .05$). A post hoc test indicated that ʻōhiʻa stands 1 and 2 were significantly different from the *Eucalyptus* and pasture stands, but that all of the other values were overlapping and statistically indistinguishable.

Correlation with Environmental Variables

A Mantel correlogram between Bray-Curtis similarity and physical distance between plots showed no indication of spatial autocorrelation. The lowest P value ($P = .22$) occurred at a lag of 724 m, but it was well outside the bounds of significance. The NMDS analysis indicated that the stands grouped into three clusters: pasture, *Eucalyptus*, and all other species (Figure 3). The 95% confidence ellipses for standard error of the means overlapped for all ʻōhiʻa, *A. columnaris*, *C. japonica*, and *C. equisetifolia* stands, indicating that they cannot be separated into distinct groups (Figure 3). On the other hand, the 95% confidence ellipses for both pasture and *Eucalyptus*
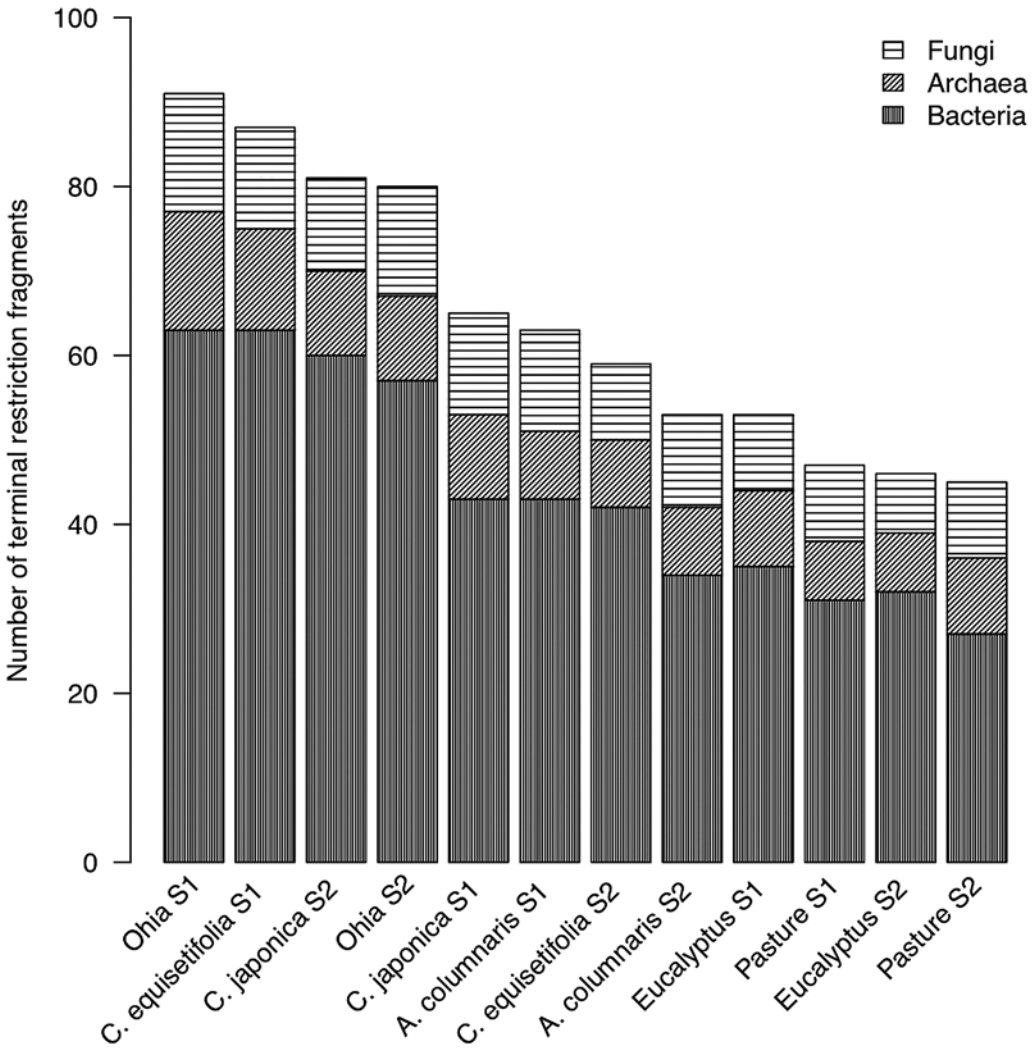
Figure 1. Total number of terminal restriction fragments found in each stand.

were distinct and did not overlap with any other species, indicating that they both had unique microbial communities. Canopy species identity explained a significant amount of the variation in the ordination ($r^2 = 0.73$, $P < .001$). Finally, we found that nearly all soil and litter variables were correlated with the microbial community (Figure 3). The strongest correlation was with soil pH ($r = 0.89$), followed by soil C ($r = 0.84$), litter C ($r = 0.81$), litter P ($r = 0.77$), and litter biomass ($r = 0.76$).

DISCUSSION

The native tree species had the highest microbial diversity as measured by both the Shannon and Simpson's reciprocal diversity indices. In addition, both 'ōhi'a stands had noticeably higher levels of unique TRFs, indicating that although their overall microbial community may be similar to that of *C. equisetifolia*, *C. japonica*, and *A. columnaris*, 'ōhi'a harbors and supports microbial species that are lost under the introduced species.
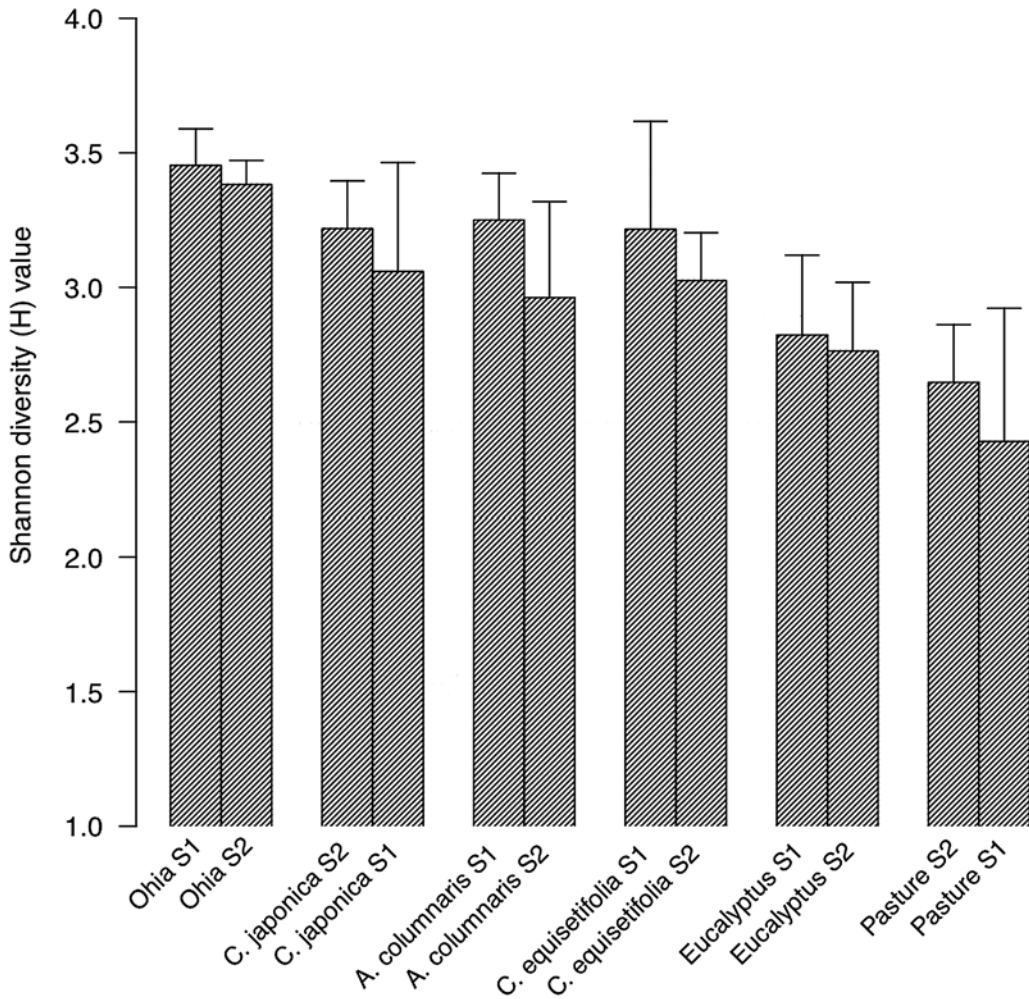
FIGURE 2. Shannon diversity index for all taxa using fluorescent intensity as a proxy for abundance. Paired bars are stands 1 and 2 for each group, with standard errors for each stand.

Both *Eucalyptus* stands had microbial communities more similar to each other than to those of other tree species, indicating that *Eucalyptus* had a strong influence on the soil microbial community. The soil and litter traits that were most significantly correlated to the microbial community composition were soil pH, soil C, litter C, litter P, and litter biomass. Soil within *Eucalyptus* stands was significantly more basic than the pH of soil near the other tree species, which was reflected in a soil microbial community diversity that was more similar to that of the pasture site, which also had a more basic pH, than to other forest sites. The strong effect of pH on the soil microbial community has been well studied (Fierer and Jackson 2006) and is substantiated here. We do not know why the pH under *Eucalyptus* was more basic than under other sites; other studies have found that several species of *Eucalyptus* acidify the soil (Bargali et al. 1993, Rhoades and Binkley 1996). However, the pH of the soils at the Kohala site was already lower than the soil pH reported in other studies, possibly accounting for the observation of the opposite
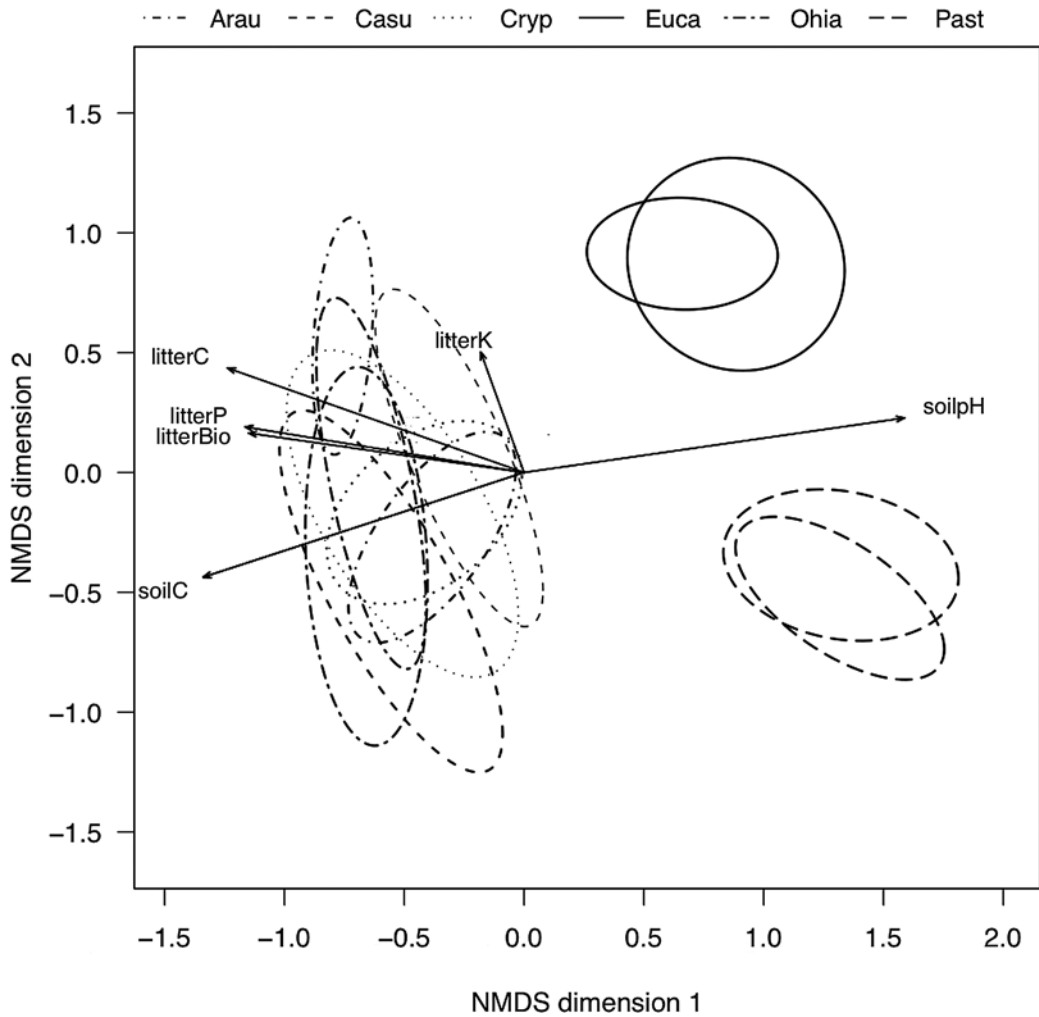
FIGURE 3. NMDS analysis of the microbial community by peak area as determined by M-TRFLP. Ellipses represent 95% confidence intervals for the standard error of the mean for each stand. Environmental variables that have a threshold correlation of $r = 0.71$ with the microbial community are overlaid onto the ordination. Although litter K has an r value below the threshold, it is also shown because it is the only environmental variable that changes with the second ordination axis. Vectors point in the direction that variables are changing most rapidly, and length of the vectors is scaled by the strength of the correlation. Abbreviations: Arau, *A. columnaris;* Casu, *C. equisetifolia;* Cryp, *C. japonica;* Euca, *Eucalyptus;* Past, pasture.

trend of reduced acidity observed in this study.

An additional explanation for the unique microbial community of *Eucalyptus* is that the microbes that can survive under *Eucalyptus* are adapted to its production of toxic terpenes and phenolic acids, including cineole, α-pinene, and caffeic and gallic acid (del Moral and Muller 1970). These compounds have been shown to inhibit the growth of understory plants (del Moral and Muller 1970). Although pH most likely explains the separation of *Eucalyptus* and pasture from the other stands along the first axis of the ordination, these unique biochemical properties of *Eucalyptus* may explain the separation of

Eucalyptus and pasture along the second axis of the ordination (Figure 3).

As expected, ordination also differentiated pasture sites. The reduction in diversity and abundance of the soil microbial community due to deforestation is well established (Borneman and Triplett 1997, Nusslein and Tiedje 1999, Cleveland et al. 2003). Here this difference correlated strongly with the more basic pH and low litter biomass of the pasture sites.

We anticipated that the two replicate stands of each tree species would be more similar to each other than to stands of other tree species, but this was not true for all species. Replicate stands of both *C. equisetifolia* and *A. columnaris* were more similar to stands of other tree species than to each other. These results suggest that for plant covers with distinct properties and effects (such as the more basic pH of *Eucalyptus* and pasture, the low biomass of pasture grasses, and the toxic phenolics and terpenes of *Eucalyptus*) plant species effect overrides environmental variation and produces a distinct soil microbial community. However, for other species with less distinctive properties the effects of environmental variation in microtopography, moisture, or other variables override the effect of tree species.

We anticipated that *C. equisetifolia* would have a more distinctive microbial community given its novel nitrogen-fixing capability. The levels of soil and litter N were indeed significantly higher in the two *C. equisetifolia* stands, but these variables were only weakly correlated with the ordination of the soil microbial community.

In this study all samples were taken within 1 km of each other on a single substrate, to control for environmental variation and to isolate the effect of the canopy tree species. Even within such a small area, only *Eucalyptus* and pasture had replicates that were more similar to each other than to those of other species. Would sampling at a finer scale have reduced the signal of environmental variation enough so that all canopy tree species had distinct microbial communities? If the spatial scale of sampling were increased, at what point would the *Eucalyptus* and pasture sites become more similar to the rest of the sites than to hypothetical sites farther away in geographic space?

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