2008. Proceedings of the Indiana Academy of Science 117(1):7–15

SOIL BACTERIAL COMMUNITY STRUCTURE UNDER EXOTIC VERSUS NATIVE UNDERSTORY FORBS IN A WOODLAND REMNANT IN INDIANA

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ABSTRACT. Invasions of exotic plants have strong effects on aboveground plant community structure, competitively excluding native plant species and driving down community diversity. Using terminal restriction fragment length polymorphism (TRFLP) analysis of PCR-amplified 16S-rDNA sequences, we characterized eubacterial community structure in soil collected on the Bloomington campus of Indiana University beneath native Canadian wildginger (*Asarum canadense* L.) and exotic invasive winter creeper (*Euonymus fortunei* (Turcz.) Hand.-Maz.). Overall indices of eubacterial diversity, richness, and evenness were similar beneath the two plant species. However, multivariate analysis of TRFLP profiles revealed significant differences among plant species in soil eubacterial community composition. Soil pH and water content also varied significantly by plant species. These results are consistent with the hypothesis that invasive exotics can influence belowground community structure, although microbial community composition may be more highly influenced than other aspects of microbial diversity.

Keywords: Microbial composition, eubacterial diversity, TRFLP, *Euonymus fortunei*, *Asarum canadense*

The invasion of non-native plant species into native ecosystems is a global-scale phenomenon with strong effects on above-ground plant communities, including displacement of native species and altered community composition and diversity (Vitousek et al. 1997; Mack et al. 2000). These changes to above-ground plant communities are often associated with altered ecosystem-level processes, including changes in the pools and fluxes of carbon, nitrogen, and other elements (Ehrenfeld 2003; Rothstein et al. 2004; Mayer et al. 2005). Soil biota are key drivers of biogeochemical processes and interactions between microbes and plants are important in structuring both above-ground and below-ground communities, yet we know relatively little about the consequences of plant invasions for the composition and diversity of soil communities.

Plant traits may affect soil biota in a number of ways. Plant matter provides the major carbon source for soil microbes, and differences in the quality or quantity of root exudates and litter deposits are thought to drive differences in soil microbial composition under different plant species (Grayston & Campbell 1996; Kourtev et al. 2002). Furthermore, plant chemistry, root architecture and canopy or litter characteristics can influence soil biota through alteration of the soil environment (e.g., pH, water, temperature; (Hobbie 1992)). Exotic invasive species can differ strikingly from native plant species in one or more of these traits, and associated changes in the composition and diversity of soil communities is therefore a predicted result of invasions (Wolfe & Klironomos 2005).

Whole-community methods for characterizing soil microbiota (reviewed in Sinsabaugh et al. 1999; Wolfe & Klironomos 2005; Rösch et al. 2006) are beginning to demonstrate that exotic plant invasions can indeed alter the composition and structure of soil biota. For example, phospholipid fatty acid (PLFA) analysis has been used to detect changes in the ratio of bacteria:fungi (Kourtev et al. 2002; Kourtev et al. 2003; Callaway 2004a) and in the abundance of specific bacterial or fungal functional groups (Batten et al. 2006) following the invasion of exotic plant species. More functionally-based analyses, such as substrate utilization profiles (e.g., BIOLOG, Duda et al. 2003) and enzyme activity assays (Kourtev et al. 2002), have likewise demonstrated differences in the functional diversity of soil collected under exotic invasive versus native species.

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A newer and rapidly developing way to characterize soil microbial communities is through the use of nucleic acid techniques such as terminal restriction fragment length polymorphism (TRFLP) analysis. Nucleic acid analyses use polymerase chain reactions (PCR) and specifically-chosen primers to amplify desired taxonomic groups from soil DNA extracts, followed by processing to yield community "fingerprints" of the metabolically active microbes within the amplified target group of interest (Hill et al. 2000). For TRFLP analysis, PCR amplicons are fluorescently tagged at the 5′ end and cut with restriction enzymes, and the terminal restriction fragments can then be quantified by electrophoresis to yield an index of microbial composition, relative abundance, and overall diversity (Blackwood et al. 2003; LaMontagne 2003). Nucleic acid analyses permit a high level of taxonomic and phylogenetic resolution, either through standard cloning and sequencing techniques, or in the case of TRFLP analysis, via web-based research tools that compare terminal fragment lengths to those in web-based libraries of known fragment taxonomy (Marsh et al. 2000; Kent et al. 2003; Rösch et al. 2006). TRFLP analysis has been used to characterize whole soil microbial communities in a range of different environments (e.g., Clement et al. 1998; Liu et al. 1997) and is beginning to be applied to the characterization of soil communities from different plant species. For example, TRFLP analysis has revealed consistent differences in the bacterial community composition of soil associated with native and exotic invasive plant species of an arid grassland (Kuske et al. 2002).

In this study, we used TRFLP analysis and the web-based phylogenetic assignment tool (PAT) to investigate the structure and composition of bacterial communities beneath two forest floor species common in Indiana. Canadian wild ginger (*Asarum canadense* L., Aristolochiaceae) is a perennial understory herb native to deciduous forests throughout the eastern United States and Canada (Gleason & Cronquist 1991). Winter creeper (*Euonymus fortunei* Turcz. Hand.-Maz., Celastraceae) is an introduced perennial creeping or climbing vine, invasive in eastern forests (USDA, NRCS 2007). Used as an ornamental lawn cover, winter creeper often escapes confinement and invades native areas, where it forms a dense groundcover, competitively excluding adjacent species (Hutchinson 1992). We hypothesized that *Euonymus* invasion alters soil communities and predict that microbial community structure and taxonomic composition will differ in soil collected from adjacent patches of *Asarum* and *Euonymus*.

METHODS

Site description and soil sampling.—Our study was conducted at Dunn's Woods, an approximately 120-year old, 4-ha wooded area on the Indiana University campus in Bloomington, Indiana, USA (N 39°09,′ W 86°31′). The site has a deciduous forest canopy and is laced with brick paths but otherwise maintained with a minimum of disturbance (Mike Schrader pers. comm.). Commonly occurring canopy trees include American Beech (*Fagus grandifolia* Ehrh., Fagaceae), Sugar Maple (*Acer saccharum* Marsh., Aceraceae), Tuliptree (*Liriodendron tulipifera* L., Magnoliaceae) and White Oak (*Quercus alba* L., Fagaceae). *Euonymus* is spreading throughout the site and dominates much of the forest floor, although there are areas dominated by dense patches of *Asarum* in the southeast corner of the site. Where *Euonymus* and *Asarum* are absent, the ground layer vegetation includes spring ephemerals (e.g., Spring Beauty (*Claytonia virginica* L., Portulacaceae), Squirrel Corn (*Dicentra canadensis* (Goldie) Walp., Fumariaceae), Trout Lily (*Erythronium americanum* Ker-Gawl., Liliaceae), Mayapple (*Podophyllum peltatum* L., Berberidaceae), and Prairie Trillium (*Trillium recurvatum* Beck, Liliaceae)) and abundant *Acer* seedlings. Soils at Dunn's Woods are part of the Crider-Urban complex, a well-drained silt loam layered over glacial paleosols with an underlying limestone residuum (Thomas 1981).

Asarum is clonal, spreading by rhizomes located just beneath the leaf-litter layer (Cain & Damman 1997) and capable of forming large forest floor colonies (Brundrett & Kendrick 1990). Adventitious roots extend from the rhizomes to a depth of approximately 10–30 cm (Brundrett & Kendrick 1990) and are heavily colonized by vesicular arbuscular mycorrhizal fungi (Brundrett & Kendrick 1988). *Euonymus* is stoloniferous, has a rooting depth of at least 12–18 cm, and is capable of rapid vegetative spread (USDA, NRCS 2007). We

found no information on the mycorrhizal status of *Euonymus* in the literature.

Soils of each species were collected from an approximately 400 m^2 area at the southeast corner of the site, where *Asarum* and *Euonymus* co-occur. Soils were collected 1 September 2004, within the active growth period of both species (Brundrett & Kendrick 1990; USDA, NRCS 2007). To reduce the effects of uncontrolled environmental heterogeneity, soil was collected beneath paired patches of *Asarum* and *Euonymus*, yielding seven replicate pairs scattered throughout the site. To obtain representative soil samples from each of the 14 sample points, five 1 cm diameter, 10 cm deep soil cores were taken within an approximately 15 cm × 15 cm area within each patch and bulked. Soil from each of the 14 patches were sealed within Whirlpak® bags (NASCO Industries, Fort Atkinson, Wisconsin) and held on ice approximately 2 h until transport to the lab for storage at 7°C. Within 24 h of collection, soil samples were sieved through a 2 mm mesh to homogenize soil and remove debris. Sieved soil was divided into two sets of subsamples. One set was stored in Whirlpak® bags at −80°C for TRFLP analysis (as described below) and soil pH determination (1:1 water:-soil suspension, Thermo Orion Triode pH Electrode), and the other set was used for determination of gravimetric soil water content (Jarrell et al. 1999).

Nucleic acid extraction and TRFLP analysis.—Soil DNA was extracted from soil samples using the MOBio UltraClean Soil DNA extraction kit (Solana Beach, California) as recommended by the manufacturer. Our PCR, restriction digest, and electrophoresis protocols were adapted from Grüntzig et al. (2002) and Kuske et al. (2002) as follows. 16S rDNA from the extracted DNA samples was PCR -amplified using the universal eubacterial primers 8Fhex (5′AGAGTTTGATCCTGGCTCAG) and 1392R (3′ACGGGCGGTGTGTRC) (Invitrogen Life Technologies, Carlsbad, California). Each 100 μL PCR reaction was performed with a reaction mixture of 10 μL HiFi buffer, 8 μL dNTP, 1 μL taq (Eppendorf Triplemaster, Hamburg, Germany), 5 μL DMSO, 1 μL each of primers 8Fhex and 1392R, and 69 μL sterile mili-Q distilled H_20. PCR was performed using a TTC-100 programmable thermal controller (MJ Research, Inc., Waltham, Massachusetts) at the following specifications: an initial denaturation step of 95°C for 3 min followed by 30 cycles of a program consisting of denaturation at 95°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 2.5 min. This was followed by a final extension at 72°C for 5 min. PCR products were examined for successful amplification using gel electrophoresis on 0.8% agarose gels. Three separate PCR reactions were performed with each soil DNA sample, and replicates were combined and purified using the QIAQuick PCR purification kit (Qiagen, Valencia, California). The concentration of purified PCR products was determined using plasmid DNA samples of known concentrations as markers and standardized as necessary to a concentration of 100–150 ng/μL.

Restriction digests were performed with the purified PCR product in a 40 μL reaction with approximately 1200 ng of DNA (24 μL), 2 μL of restriction enzyme, 4 μL of restriction enzyme 10× buffer, and 10 μL of sterile mili-Q distilled H_20. In complex soil microbial communities, phylogenetically similar species may produce identical restriction profiles (Yu et al. 2005). The use of multiple restriction enzymes helps to alleviate this problem (Kent et al. 2003). Therefore, to increase the resolution of subsequent community analyses, three separate restriction digests were performed for each sample using restriction enzymes *HhaI* (GCG/C), *MspI* (C/CGG), and *RsaI* (GT/AC) (New England Biosystems, Beverly, Massachusetts). Each reaction was incubated at 37°C for 3 h, then at 65°C for 15 min. Digested product was purified using a QIAQuick nucleotide removal kit (Qiagen, Valencia, California) to remove excess enzyme, salts, and impurities. The purified product was suspended in 45 uL of QIAQuick buffer EB diluted 1:100 with sterile mili-Q distilled water at pH 7–8. The length and abundance of the hex-labeled DNA fragments were determined using the Applied Biosystems 3730 model automated DNA analyzer with a 500 bp LIZ size standard (Applied Biosystems, Foster City, California). GeneMapper software (Applied Biosystems Instruments, Foster City, California) was used to measure terminal restriction fragments up to 600 bp in length with peak heights above 200 fluorescent units.

Resulting terminal restriction fragment (TRF) profiles were standardized for analysis using procedures similar to those described by

Dunbar et al. (2001) and Fierer et al. (2003). First, data were normalized to account for any potential variability in total DNA quantity among samples on the capillary gel. Fragments were therefore rescaled to reflect their proportional abundance in each sample, and fragments representing less than 0.5% of the total sample DNA were discarded. TRF profiles were then manually aligned to prevent similar peaks from being grouped separately. While time-consuming, this procedure was necessary to avoid errors associated with automated rounding algorithms (GeneMapper software calculates DNA fragment sizes to 0.01 bp, but sequencing errors can be up to 0.5 bp (Dunbar et al. 2001)). Errors introduced by manual reassignment are likely to deemphasize unique operational taxonomic units (OTU) (Fierer et al. 2003), leading to conservative estimates of total sample diversity and distinctiveness.

We used both fragment length and abundance (peak height) in comparing TRF profiles among samples. Although the use of multiple enzyme digests increases the specificity (Dunbar et al. 2001) of data generated by TRFLP analysis, different organisms can still produce TR fragments of similar lengths. Therefore, each fragment is assumed to represent a different OTU, but not necessarily a distinct species (Liu et al. 1997). Moreover, although preferential fragment amplification during PCR can lead to biases, peak height still provides a reproducible measure of relative OTU abundance within a given sample (Clement et al. 1998; Osborn et al. 2000; Dunbar et al. 2001).

Statistical analyses.—*Univariate analyses:* OTU richness was determined for each sample as the total number of TR fragments identified in that sample. OTU diversity and evenness for each sample were calculated with the Shannon Diversity Index (H′) and Equitability Index ($E_{H'}$), respectively (Magurran 1988), using both the number of distinct TRFs in each sample and the associated height of each fragment. OTU richness, diversity, and evenness as well as soil pH and water content in *Euonymus* vs. *Asarum* soils were compared using randomized block analysis of variance (ANOVA, Systat v. 11, SPSS, Inc.). All data met assumptions of homogeneity of variance (Levene's Test) and normality (Two-sample Komolgorov-Smirnov test) (SPSS v. 14.0, SPSS, Inc.).

Multivariate and phylogenetic analyses: We generated a Bray-Curtis similarity matrix of TRF number and peak height data from *Euonymus* and *Asarum* samples, combining the data from all three restriction enzymes. Data were square root transformed to promote a balanced view of dominant versus rare OTUs (Clark & Warwick 2001). With this similarity matrix, we performed a one-way analysis of similarity (ANOSIM, PRIMER Software, PRIMER-E Ltd, Plymouth, UK; Clarke & Warwick 2001) to test for a significant difference in the eubacterial community composition of *Euonymus* versus *Asarum* soils. To visualize community patterns, we conducted an ordination of the Bray-Curtis similarity matrix using non-metric multi-dimensional scaling (NMDS, PRIMER Software, PRIMER-E Ltd, Plymouth, UK; Clarke & Warwick 2001). We used the program SIMPER (PRIMER Software) to investigate the average percent contribution made by each bacterial OTU to the average dissimilarity between *Euonymus* and *Asarum* samples. Phylogenetic assignments of the OTUs contributing most to the difference in bacterial composition between *Euonymus* and *Asarum* soils were inferred using the web-based phylogenetic assignment tool (PAT), developed for multiple restriction digests by Kent et al. (2003) and available at http://trflp.limnology.wisc.edu. We follow Kent et al. (2003) and Pett-Ridge et al. (2005) in making phylogenetic assignments at the level of phylum-class, since errors in assignment increase with level of taxonomic resolution (Pett-Ridge et al. 2005) and the use of even three restriction enzymes may not be adequate for identification to the species level (Matsumoto et al. 2005).

RESULTS

Univariate analyses.—Total observed OTU richness, evenness (E_H), and diversity (H′) were not significantly different for bacterial communities associated with *Euonymus* versus *Asarum* (Table 1). Physical soil properties did vary significantly by plant species, with *Euonymus* associated with significantly drier and more acidic soils compared to *Asarum* (Table 1).

Multivariate and phylogenetic analyses.—There were 697, 769, and 728 peaks identified with the *Hha*I, *Msp*I, and *Rsa*I restriction enzymes, respectively. Multivariate analysis of TRFLP peak length and height profiles demonstrated that bacterial community composition was significantly different in soil collected underneath *Euonymus* versus *Asarum* ground-

Table 1.—Properties of soil samples (mean ± SE) associated with *Euonymus* or *Asarum*, with results of randomized block ANOVAs. Statistically significant *P*-values ($P < 0.05$) are indicated with an asterisk.

	Euonymus	*Asarum*	df	MS	*F*-ratio	*P*-value
Microbial structure						
Richness	161.43 ± 4.10	152.43 ± 6.38	1, 6	283.500	1.214	0.313
Diversity (H)	0.940 ± 0.006	0.93 ± 0.009	1, 6	0.057	1.772	0.232
Evenness (E_H)	4.78 ± 0.056	4.65 ± 0.077	1, 6	0.001	1.738	0.235
Physical properties						
Soil pH	6.34 ± 0.25	7.12 ± 0.14	1, 6	2.161	14.497	0.009*
Soil water (%)	30.81 ± 0.016	40.03 ± 0.029	1, 6	0.030	34.876	0.001*

cover (ANOSIM, $R = 0.544$, $P = 0.005$, Fig. 1). SIMPER analysis revealed that 10% of the OTUs accounted for over 26% of the total difference between the two plant species. *In silico* phylogenetic assignment of these OTUs to the phylum-class level (Fig. 2) shows that, compared to *Asarum*, *Euonymus*-associated soil communities had a higher relative abundance of *Bacterioidetes* and *Bacilli* and a lower abundance of *Sphingobacteria*. In addition, *Asarum*-associated soils supported a number of bacterial classes absent in soils collected under *Euonymus*, including α-, δ-, and γ-*Proteobacteria*, and *Mollicutes*. Thirteen percent of significant OTUs were found to belong to unnamed clones, strains, and symbionts whose 16S rDNA sequences are known, but are otherwise unclassifiable by PAT.

DISCUSSION

In this study, by comparing TRFLP profiles, we were able to detect differences in the bacterial communities associated with *Euonymus*- and *Asarum*-inhabited soils. These results agree with our overall prediction that distinct eubacterial communities would be present in the soils associated with native versus exotic species. Moreover, they are supported by a growing body of literature reporting structural changes to soil communities in association with non-native plant invasions (Belnap & Phillips 2001; Kourtev et al. 2002; Kuske et al. 2002; Batten et al. 2006).

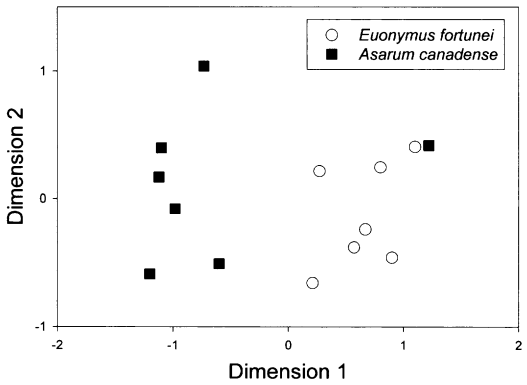

Figure 1.—Biplot generated from a Bray-Curtis similarity matrix of TRFLP profiles collected from soil beneath *Euonymus* and *Asarum* (stress = 0.11). Significant clustering of microbial community profiles can be observed by plant species.

While differences among soil communities from *Asarum* versus *Euonymus* were readily apparent with multivariate analyses of TRFLP profiles, univariate measures of community structure (OTU richness, diversity and evenness) failed to detect significant differences among our two soil types. A number of other studies have similarly found that multivariate but not univariate analyses of TRFLP data are capable of differentiating microbial communities (e.g., Dunbar et al. 2000; Kuske et al. 2002; Fierer et al. 2003), although this can depend on the particular univariate index used (Hackl et al. 2004). Dunbar et al. (2000) suggest that relatively low phylogenetic resolution (i.e., multiple OTUs possible for a single fragment size) and variation in restriction enzyme efficacy may make the TRFLP technique less effective for use with gross measures of community structure such as diversity and richness. Such univariate measures may work best when there are strong differences among soils. For example, studies comparing deep versus surface soils (LaMontagne et al. 2003) or soils along a transect of different grassland types (Brodie et al. 2002) have found concordance between uni- and multivariate TRFLP analyses.

Plant traits such as root exudates, litter chemistry, and root architecture have been

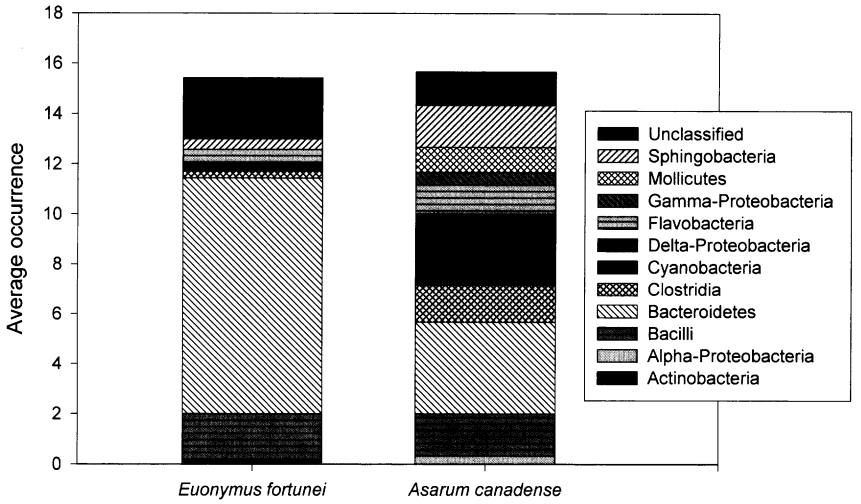

Figure 2.—Bacterial classes detected by TRFLP analysis under *Euonymus* and *Asarum*. The plots indicate the average occurrence of significant fragments that could be assigned to each indicated phylogenetic class.

proposed as possible drivers of plant-microbe associations (Wolfe & Klironomos 2005) and may explain the differences in soil microbial community structure beneath *Euonymus* and *Asarum* observed in our study. Particular attention has been paid to species-specific plant root exudates, which are thought to promote the development of distinct eubacterial communities (Grayston 2000). The roots of some invasive species are known to release compounds inhibitory to other plants (Hierro & Callaway 2003), and such allelochemicals may also have the potential to influence soil microbial communities (Wolfe & Klironomos 2005). For example, allelochemicals with antimicrobial properties have been identified from the roots of the invasive knapweed *Centaurea diffusa* (Vivanco et al. 2004), a species that has been shown to alter soil microbial community composition (Callaway et al. 2004a, b). As another example, the production of glucosinolates by the invasive forest herb *Alliaria petiolata* (garlic mustard) has been found to decrease spore germination, root colonization and/or inoculum potential of arbuscular mycorrhizal fungi (AMF) (Roberts & Anderson 2001; Stinson et al. 2006), and to impair the growth of native, AMF-dependent canopy species in eastern forests (Stinson et al. 2006).

Of course, the potential of plant inhibitory compounds to influence soil microbial community structure need not be limited to invasive exotics. We found no reports on the exudate chemistry of *Euonymus* and this species is not a known allelopath (USDA, NRCS 2007). However, *Asarum* has a long history of medicinal use (Lewis & Elvin-Lewis 1977; Bergeron et al. 1996) and a number of studies have identified antimicrobial properties associated with its shoots or roots. For example, such antimicrobial agents as methyleugenol and aristolochic acids have been isolated from *Asarum* root or rhizome tissues (Motto & Secord 1985; Shaneberg et al. 2002). Furthermore, screens of *Asarum* shoot or root extracts have shown antifungal and antibacterial properties (Cavallito & Bailey 1946; McCutcheon et al. 1994; Bergeron et al. 1996) as well as larvicidal and molluscicidal activity (Bergeron et al. 1996). Extracts may affect only certain microbial species (McCutcheon et al. 1992; Bergeron et al. 1996), providing one mechanism for producing shifts in microbial community structure. Inhibitory compounds might also provide unique resource niches, a possibility consistent with our findings that a number of bacterial classes were present only in soil collected from *Asarum* patches.

Soil microbial community structure can also be influenced by soil pH and water content (Fierer et al. 2003; Drenovsky et al. 2004; Kennedy et al. 2004; Marschner et al. 2004), factors that differed significantly in *Asarum* versus *Euonymus* patches. Differences in root exudate chemistry, nutrient uptake, or other plant species traits may have driven the

observed differences in soil pH and water content and the associated changes in microbial community structure under the two study plant species. Although our paired sampling design helped to factor out background variation in soils, it is also possible that our study plant species sorted into soil microsites with pre-existing differences in soil abiotic or biotic variables. Given the aggressive spread of *Euonymus* in Dunn's Woods, however, it seems more likely that this species is at least partially driving, rather than responding to, soil microbial communities. Future studies are needed to detangle the relative influences of biotic and abiotic controls and to clarify the role of plant inhibitory compounds in structuring soil communities beneath native and invasive species, and studies that measure additional soil properties and include multiple native and exotic species are needed. Nevertheless, the differences observed here between *Euonymus* versus *Asarum* suggest that invasion of *Euonymus* into forest understory areas will contribute to changes in soil community structure.

ACKNOWLEDGMENTS

We thank Noah Fierer for advice and protocols on TRFLP peak standardization and alignment and two anonymous reviewers for their feedback and assistance in improving this manuscript. Financial support was provided by an Indiana University Howard Hughes Medical Institute Capstone Grant and an Indiana University Undergraduate Research and Creative Activity Grant to Clare Glinka, with matching funds provided by Indiana University Departmental Biology funds awarded to Heather L. Reynolds.

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Manuscript received 30 November 2007, revised 21 April 2008.