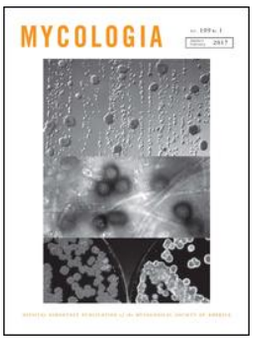

Mycologia

ISSN: 0027-5514 (Print) 1557-2536 (Online) Journal homepage: http://www.tandfonline.com/loi/umyc20

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To cite this article: Alina S. Campbell, Randy C. Ploetz, Tyler J. Dreaden, Paul E. Kendra & Wayne S. Montgomery (2016) Geographic variation in mycangial communities of Xyleborus glabratus, Mycologia, 108:4, 657-667, DOI: 10.3852/15-133

To link to this article: http://dx.doi.org/10.3852/15-133

Mycologia, 108(4), 2016, pp. 657–667. DOI: 10.3852/15-133

Geographic variation in mycangial communities of *Xyleborus glabratus*

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Abstract: Factors that influence fungal communities in ambrosia beetle mycangia are poorly understood. The beetle that is responsible for spreading laurel wilt in SE USA, *Xyleborus glabratrus*, was examined at three sites along a 500 km N–S transect in Florida, each populated by host trees in the Lauraceae. Fungal phenotypes were quantified in mycangia of individual females that were collected from a site in Miami-Dade County (MDC), 25.8N, with swamp bay (*Persea palustris*), one in Highlands County (HC), 27.9N, with silkbay (*P. humulis*) and swamp bay and another in Alachua County (AC), 29.8N, with redbay (*P. borbonia*). Based on combined *LSU*, *SSU* and beta-tubulin datasets the most prominent phenotypes were *Raffaelea lauricola* (cause of laurel wilt), *R. subalba*, *R. subfusca*, *R. fusca*, *R. arxii* and an undescribed *Raffaelea* sp. Mean numbers of colony forming units (CFUs) of *R. lauricola* varied by location ($P < 0.003$), and a multivariate analysis, which accounted for the presence and relative abundance of fungal species, indicated that there were significant variations in mycangial communities among the sites; thus climate and vegetation might have affected fungal diversity and the relative abundance of these fungi in the mycangia of *X. glabratus*. Statistically it was unlikely that any of the species influenced the presence and prevalence of another species.

Key words: ambrosia beetle, avocado, laurel wilt, mycangia, *Raffaelea lauricola*, symbiont, *Xyleborus glabratus*

Submitted 25 May 2015; accepted for publication 4 Mar 2016.
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Introduction

Laurel wilt affects many American members of the Lauraceae family (Fraedrich et al. 2008). Avocado (*Persea americana* Mill.) is the most important, susceptible crop in USA, but other hosts in the family are important in terms of cultural heritage, biodiversity and wildlife habitat and food (Snow and Stans 2001). Redbay (*Persea borbonia* [L.] Spreng.) and swamp bay (*Persea palustris* [Raf.] Sarg.), which were major components of forests in the southeastern Coastal Plain of USA, have experienced the highest mortality to date (Mayfield et al. 2011, Hughes et al. 2015).

Laurel wilt is caused by *Raffaelea lauricola* (T.C. Harr., Fraedrich & Aghayeva), which is a nutritional symbiont of an exotic ambrosia beetle, *Xyleborus glabratus* (Eichhoff) (Coleoptera: Curculionidae: Scolytinae). Although anthropogenic spread and root graft transmission of the pathogen play roles in the above epidemic, the efficiency of *X. glabratus* as a vector of *R. lauricola* is the primary reason this disease has spread so rapidly and extensively in the southeastern USA (Maner et al. 2013).

Adult female *X. glabratus* emerge from their natal trees and fly in search of new brood sites in the late afternoon and early evening (Kendra et al. 2012a). They carry spores of *R. lauricola* as well as other fungi and bacteria in their mandibular mycangia (Harrington and Fraedrich 2010, Hulcr et al. 2012). Host trees are inoculated with *R. lauricola* as beetles create galleries in host sapwood in which they lay eggs and cultivate gardens of the fungus (Kendra et al. 2013). After inoculation *R. lauricola* moves systemically in xylem vessels, causing vascular wilt.

Vertical transmission of ambrosia beetle symbionts occurs as progeny from natal galleries establish new gardens in new galleries. Although this supports the development of species-specific associations between the beetles and their fungi, two or more fungi may be cultivated by a given species (Biedermann et al. 2013). Six and Wingfield (2011) suggested that an association with more than one symbiont may be beneficial for bark beetles (close relatives of ambrosia beetles in the subfamily Scolytinae) because different symbionts may be suited to the different and changing environments that occur in attacked trees. They indicated that different symbionts may be better adapted to trees in the early, middle or late stages of decline, and that pathogens may be suited to the early stages whereas saprophytes would be adapted to the middle

and late stages when defensive compounds dissipate, tissues dry and the nutritional content of trees decline.

Although the factors that influence the mycangial composition of these insects are not known, responses to different hosts, environments, and other factors may determine their relative abundance. Since bark beetle communities are strongly influenced by environmental conditions (Six and Wingfield 2011), it is not unreasonable to assume that ambrosia beetle communities are also affected by these factors. For example, the bark beetle *Dendroctonus ponderosae* Hopkins is associated with two fungi that have different temperature optima (Six and Bentz 2007). *Grosmannia clavigera* dominates at lower temperatures, while *Ophiostoma montium* becomes more abundant at higher temperatures (Six and Bentz 2007). In general the compositions and dynamics of microbial communities in the mycangia of ambrosia beetles are poorly understood.

In cadavers of *X. glabratus* from Asia, Georgia and South Carolina, Harrington and Fraedrich (2010) and Harrington et al. (2011) reported variation in the quantities of *R. lauricola*, other *Raffaelea* spp. and other species of fungi. Based on this variation, they suggested that "Biological control of the pathogen may prove possible through manipulation of the mycangial microflora" (Harrington and Fraedrich 2010).

In the present study fungal communities in mycangia of *X. glabratus* were examined in three sites along a 500 km N–S transect in Florida. Each of the sites was populated by one or more host species in the Lauraceae. To minimize the recovery of secondary contaminants and maximize the recovery of symbionts, living beetles, instead of cadavers, were assayed soon after their recovery from the field or rearing facilities. In addition, when comparing locational differences fungi were quantitated from mycangia that were extracted from female beetles (males do not have mycangia) rather than from severed heads, thereby ensuring that mycangial communities were being assayed.

This is the first time that relationships between and among these communities, as well as the taxa that comprise these communities, have been examined in-depth (unlike this one, previous studies primarily used semiselective media). These data could inform future work on how and why *X. glabratus* and other ambrosia beetle species obtain and disseminate *R. lauricola* and might help understand the roles they play in the epidemiology of laurel wilt.

MATERIALS AND METHODS

Beetle collection.—Individual females of *X. glabratus* from three locations in Florida were collected either in flight or reared from logs of laurel wilt-affected trees. The Miami-Dade County site (25.8N) was dominated by swamp bay and the Alachua County site (29.8°N) by redbay. At the Highlands County site (27.9N) beetles were collected from an area where swamp bay predominated but silkbay (*P. humilis*) also was present in an adjacent dry scrub habitat. According to U.S. Climate Data (www.usclimatedata.com), the annual temperature is 14.2–26.6 C in Alachua County, with a mean of 20.4 C (based on data from Gainesville, Florida), 15.9–29.2 C in Highlands County, with a mean of 22.6 C (based on data from Avon Park, Florida), and 21.1–29.0 C in Miami-Dade County, with a mean of 25.1 C (based on data from Miami).

In-flight beetles were collected in the late afternoon, as described by Kendra et al. (2012b). Light-colored sheets were placed on the ground adjacent to symptomatic trees, and host wood bait (freshly cut branches and sawdust) was positioned in the center. As beetles landed (or were gently batted down), they were collected with a soft brush and placed in plastic containers with moist tissue paper. Reared beetles were collected from bolts of laurel wilt-affected trees that were placed in dark plastic containers with tightly sealed lids, as described by Carrillo et al. (2014). A 9 cm diam hole was cut into the side, near the bottom, and fitted with the metal screw band of a canning jar into which a clear glass jar filled with damp paper towels was secured. Beetles that emerged from logs were attracted to the light and collected in the jar.

Beetle processing.—Beetles were surface disinfested for 15 s in 70% ethanol and rinsed three times in sterile deionized water. Mycangia were removed by dissecting heads between the frons and the joints of mandibles (Hulcr et al. 2012), individually macerated in 1 mL sterile water (7 mL PYREX™ Tenbroeck Tissue Grinders, Fisher, 08-414-10B) and diluted 1 : 100 and 1 : 1000 in sterile water; 100 µL diluent was plated on malt agar amended with cycloheximide, streptomycin, ampicillin and rifampicin (CSMA+) (Harrington 1981, Ploetz et al. 2012), as well as on half-strength potato dextrose agar (one-half PDA). CSMA+ is somewhat selective for members of the Ophiostomatales, such as *Raffaelea* spp., because they tolerate cycloheximide (Harrington 1981) whereas one-half PDA enabled the recovery of a wider range of fungi.

Two plates were used for each dilution x media combination (1 : 100 one-half PDA, 1 : 1000 one-half PDA, 1 : 100 CSMA+, 1 : 1000 CSMA+), for a total of eight plates per beetle. In total, 4.4 µL original 1 mL maceration was assayed, 2.2 µL of which was plated on each medium. Members of the Ophiostomatales were detected on both one-half PDA and CSMA+, but non-ophiostomatoid fungi could not be detected on CSMA+. Thus minimum detection numbers for members of the Ophiostomatales were 228 colony forming units (CFUs) per beetle (1000/4.4), but 455 CFUs for non-ophiostomatoid fungi (1000/2.2).

In other beetles the entire head was macerated, rather than the extracted mycangia. To determine whether the body part that was used affected recoveries, comparisons were made between the proportion of *Raffaelea* spp. and CFUs of *R. lauricola* that were recovered from 84 mycangia and 21 heads of individuals from Highlands County that were caught in flight.

DNA extraction, PCR amplification and sequencing.—Colonies on CSMA+ and one-half PDA were grouped into operational

taxonomic units (OTUs) according to phenotype and counted. At least three isolates of each OTU were used for rDNA sequencing. Cultures were grown on one-half PDA 1–3 wk and DNA was extracted per Justesen et al. (2002). Sanger sequencing was performed at the Interdisciplinary Center for Biotechnology Research Genetic Analysis Laboratory at the University of Florida, Gainesville.

ITS (internal transcribed spacer region, ITS1-5.8s-ITS2) rDNA sequences, which are preferred for the identification of fungal species (Schoch et al. 2012), were used to identify the non-ophiostomatoid fungi that were recovered. They were amplified, sequenced using the ITS1 and ITS4 primers (White et al. 1990) and identified to species or genus based on 98–100% homology with sequences in GenBank database following a BLASTn query.

ITS sequences are difficult to amplify in many ophiostomatoid fungi and consequently are poorly represented in GenBank. Thus *LSU* (the ribosomal large subunit, 28S) rDNA sequences, amplified and directly sequenced with the LR0R and LR5F primers (Hopple and Vilgalys 1994, Tedersoo et al. 2008), were used to assess the accuracy of identifying *Raffaelea* spp. OTUs based on phenotype. OTUs were identified using phylogenetic analyses with portions of the *LSU*, *BT* (beta-tubulin) and *SSU* (small subunit, 18S) genes sequenced from two representatives of each OTU. The NS1/NS4 (Claasen et al. 1996) and Bt2a/Bt2b (Dupont et al. 1999) primer pairs were used to amplify and sequence the *SSU* and *BT* loci, respectively. Thermal-cycler programs were those used by Dreaden et al. (2014).

Consensus sequences were derived from forward and reverse sequences after aligning and editing with Geneious Pro 5.6.6 (Biomatters Ltd., Auckland, New Zealand). The ability to accurately group isolates based on morphology was verified by doing a maximum likelihood (ML) analysis in RAxML of *LSU* sequences of isolates from each of the OTUs (TreeBASE ID Tr86731 unpubl).

Phylogenetic analyses.—To determine the accuracy of phenotyping, maximum likelihood (ML) phylogenetic analyses of *LSU* data were conducted as by Dreaden et al. (2014). To assign taxonomy we chose to use three loci and conduct phylogenetic analyses rather than relying on a single locus, LSU and barcoding techniques. The *LSU*, *SSU* and *BT* combined dataset was used and analyses conducted as by Dreaden et al. (2014); however, 200 starting trees were used instead of 100. Clean *BT* sequences of *R. arxii* could not be generated and are missing from the analysis. Gene sequences that were missing from isolates were treated as missing data, then concatenated to form the combined dataset with a total of 1854 characters (Dreaden et al. 2014). DNA sequence alignments and phylogenetic trees were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S17415?x-access-code=c4333d71f24a545341efbceaab3101b9&format=html). Two to three isolates of each *Raffaelea* taxon recovered during this study have been submitted to the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, culture collection (SUPPLEMENTARY TABLE I).

Statistical analyses.—Variation in mycangial composition among the three locations was tested with multivariate and univariate analyses (SAS 9.3, SAS Institute, Cary, North Carolina). Multivariate methods detected overall patterns whereas univariate methods examined differences in individual variables.

Mean CFUs of each *Raffaelea* spp. that were recovered from the 122 *X. glabratus* that were assayed were compared with a Kruskal Wallis ANOVA of rank-transformed data (SAS 9.3). This nonparametric version of a one-way ANOVA was chosen to account for non-normal data distribution. The non-ophiostomatoid category was not included because it consists of several species.

Post-hoc Wilcoxon Mann-Whitney comparisons were used to determine which groups differed. This non-parametric test is analogous to a *t*-test for independent samples. Bonferroni's correction was applied to control the familywise error rate (Bonferroni 1950). In this case the critical value, $P = 0.05$, was divided by 5, which was the number of comparisons made; thus differences were significant at $P = 0.01$.

Differences in *R. lauricola* CFUs and the proportion of *Raffaelea* spp. among all species of fungi (CFUs of all *Raffaelea* spp./CFUs of all fungi) that were recovered from whole heads vs. excised mycangia of individual beetles captured in flight in Highlands County were analyzed with the Wilcoxon-Mann-Whitney test. This non-parametric test is analogous to a *t*-test for independent samples (UCLA Statistical Consulting Group) and was chosen to account for unequal variance in the data. The numbers of fungal species that simultaneously were present in beetle mycangia in the different locations were compared with a Pearson chi-square test.

The Wilcoxon-Mann-Whitney test also was used to compare the CFUs and the proportion of *R. lauricola* (CFUs *R. lauricola*/CFUs of all fungi) recovered from in-flight vs. reared beetles. Recovery from 13 individuals of *X. glabratus* reared from bolts of laurel wilt-affected redbay in 2013 was compared to recovery from 15 individuals caught in-flight at the same AC location in 2012.

Mean CFUs of *R. lauricola* carried by individuals of *X. glabratus* were analyzed with mixed models (Proc Mixed) to determine whether they differed among locations. A mean separation table was constructed using differences of least square means. Data were square-root transformed to normalize distribution and correct unequal variances, but only nontransformed means are provided (FIG. 4).

A discriminant analysis was run in JMP 11 (SAS Institute Inc., Cary, North Carolina 2013) to determine whether *X. glabratus* mycangial communities differed among the three locations. Significant variables (fungal taxa) were determined using forward stepwise variable selection, where variables with the smallest *p*-value were added one at a time. A 0.4 lambda value was chosen as a compromise between the linear and quadratic method. This was justified by unequal covariances and the large amount of data. All non-ophiostomatoid species were combined for the discriminant analysis.

A Spearman correlation was run to determine whether the abundance of any species was negatively or positively associated with other species. Spearman's correlation calculates Pearson's correlation on the ranked values of this data, where means with the lowest value were assigning a rank of

TABLE I. Symbiont recovery from heads and mycangia of *Xyleborus glabratus*[a]

	Raffaelea lauricola CFUs[b] (SE)	Proportion of *Raffaelea* spp.[c] (SE)
Mycangia (n = 84)	2400 (294) A	0.94 (0.03) A
Head (n = 21)	2726 (147) A	0.77 (0.05) B

[a] Differences in *R. lauricola* CFUs and the proportion of Raffaelea spp. among all species of fungi (CFUs of all *Raffaelea* spp./CFUs of all fungi) that were recovered from whole heads vs. excised mycangia of individual beetles captured in flight in Highlands County were analyzed with the Wilcoxon-Mann-Whitney test, and means followed by different letters are significantly different at $P < 0.05$.

[b] Recovery of *R. lauricola* colony-forming units (CFUs) did not differ between *X. glabratus* head and mycangia ($P < 0.19$), standard errors (SE) are in parentheses.

[c] The proportion of *Raffaelea* species among all species of fungi (CFUs of all *Raffaelea* spp./CFUs of all fungi) that was recovered per beetle was significantly greater in mycangia than in whole heads ($P < 0.0001$).

TABLE II. *Raffaelea lauricola* recovery from *Xyleborus glabratus* caught in-flight and reared[a]

	R. lauricola CFUs (SE)[b]	Proportion of *R. lauricola* (SE)[c]
In-flight (n = 15)	2220 (325) A	0.40 (0.08) A
Reared (n = 13)	1557 (349) A	0.33 (0.08) A

[a] Differences in CFUs and the proportion of *R. lauricola* recovered from *X. glabratus* caught in-flight vs. reared were analyzed with the Wilcoxon-Mann-Whitney test by comparing recovery from 13 individuals reared from bolts of laurel wilt-affected redbay in 2013 to recovery from 15 individuals caught in-flight at the same AC location in 2012. Means followed by different letters are significantly different at $P < 0.05$.

[b] Recovery of *R. lauricola* CFUs did not differ between *X. glabratus* caught in-flight vs. reared ($P < 0.25$).

[c] The proportion of *R. lauricola* CFUs among all species of fungi that were recovered per beetle (CFUs of *R. lauricola*/CFUs of all fungi) did not differ between *X. glabratus* caught in-flight vs. reared ($P < 0.52$).

1, the next lowest 2, and so on. All statistical analyses were done with SAS 9.3 (SAS Institute, Cary, North Carolina). Bonferroni's correction also was applied to correlations to control the familywise error rate (Bonferroni 1950). In this case the critical value, $P = 0.05$, was divided by 21, the number of comparisons made, and correlations were significant at $P = 0.0024$.

The percentages of assayed beetles from each location that carried each fungal species were calculated in Microsoft Excel 2010 but were not analyzed to detect statistical differences. All non-ophiostomatoid species were combined in a single category. If no fungi were recovered from an individual, it was excluded from the analyses.

RESULTS

Recovery from head vs. mycangia.—No differences were detected in mean *R. lauricola* CFUs that were recovered from excised mycangia compared to whole heads ($z = -1.31$, df = 1, $P = 0.19$); however, the overall proportion of *Raffaelea* spp. among all species of fungi that were recovered was significantly greater in mycangia than in whole heads ($z = -4.05$, df = 1, $P = 0.0001$) (TABLE I). Due to the importance of *Raffaelea* spp. as ambrosia beetle symbionts (Massoumi Alamouti et al. 2009), only data from mycangial assays were analyzed subsequently.

Before pooling data from in-flight and reared beetles the proportion and CFUs of *R. lauricola* recovered from *X. glabratus* individuals reared from bolts of laurel wilt-affected redbay was compared to that from individuals caught in flight. Because no differences were detected in the CFUs ($z = -1.11$, df = 1, $P = 0.25$) and the proportion of *R. lauricola* ($z = -0.6$, df = 1, $P = 0.52$) recovered between the two groups, the remaining analyses are of pooled data from both (TABLE II).

Mycangial diversity.—Ten species of fungi were recovered regularly from mycangia of *X. glabratus*, whereas individuals carried between one and five species. Six *Raffaelea* spp. were recovered: *R. lauricola*, *R. subalba*, *R. subfusca*, *R. fusca*, *R. arxii* and an undescribed species (FIG. 1). Non-ophiostomatoid fungi, including *Candida* sp., *Trichosporon dermatis*, *Verticillium leptobactrum* and *Cladosporium sphaerospermum*, were not detected as frequently, but when they were present were recovered at up to 59 500 CFUs per individual. Non-ophiostomatoid were most frequently recovered from AC beetles (ca. 75% of individuals carried at least one of these fungi), whereas HC beetles carried the least (less than 20%).

The number of fungal species that co-occurred in an individual's mycangia differed by location ($X^2 = 36.1$, df = 6, $P = 0.0001$) (FIG. 2). On average individuals from AC and MDC carried more species simultaneously in their mycangia, respectively 3.04 and 2.9, than those from HC, 2.2. Whereas 80% of individuals from AC and MDC carried ≥3 species only 31% of those from HC did so. Beetles from AC and MDC also carried a wider range of OTUs, despite smaller sample sizes from these locations (respectively 28 and 10 vs. 84 in HC).

Raffaelea arxii and the undescribed *Raffaelea* sp. were recovered only in MDC and *R. fusca* only in AC. In contrast, *R. subfusca* and *R. subalba* were recovered at all three locations but at different frequencies. *Raffaelea subfusca* was found in 50% of the beetles from AC and MDC, compared to 6% of those from HC, and

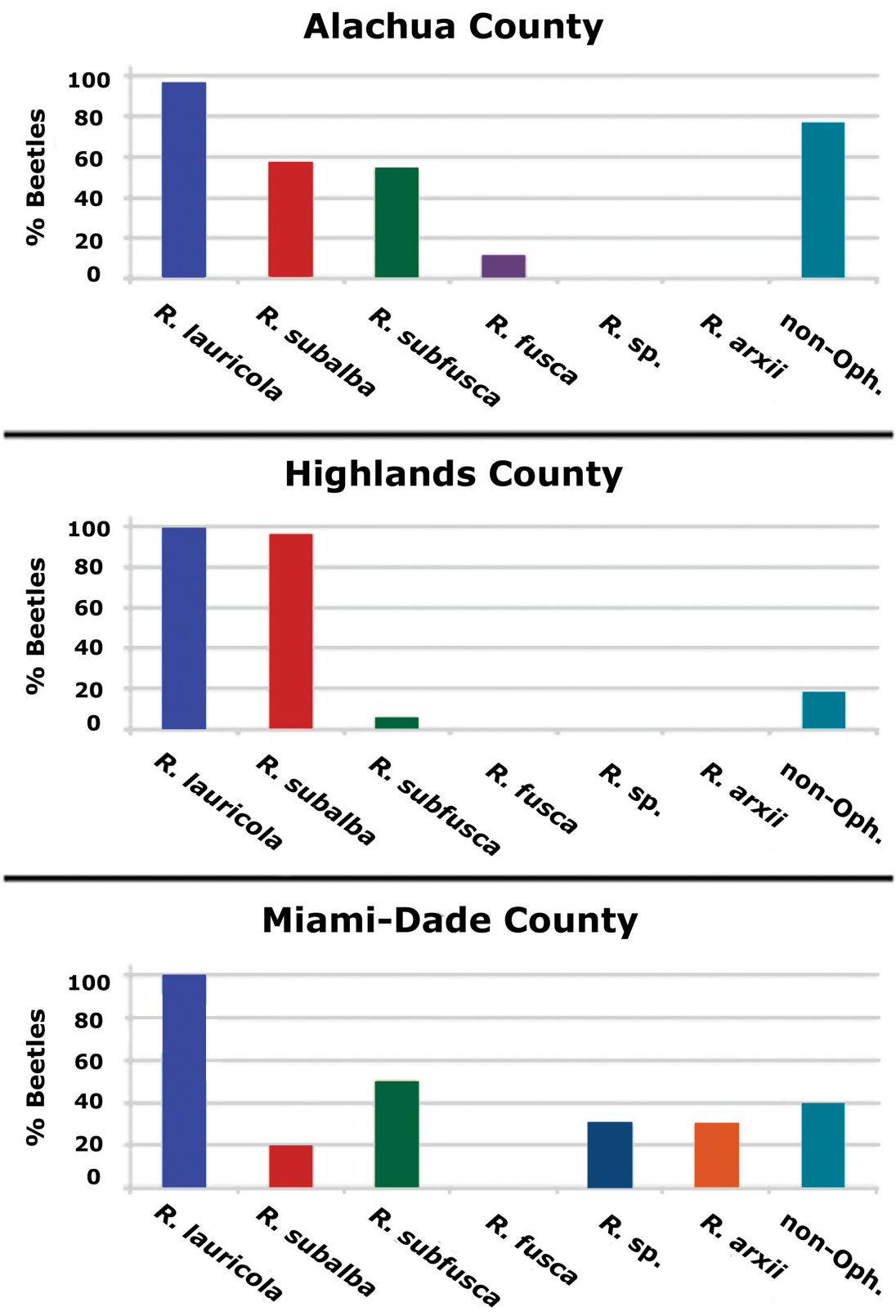

FIG. 1. Percentage of *X. glabratus* beetles in each location that carried a given *Raffaelea* species or non-ophiostomatoid fungi.

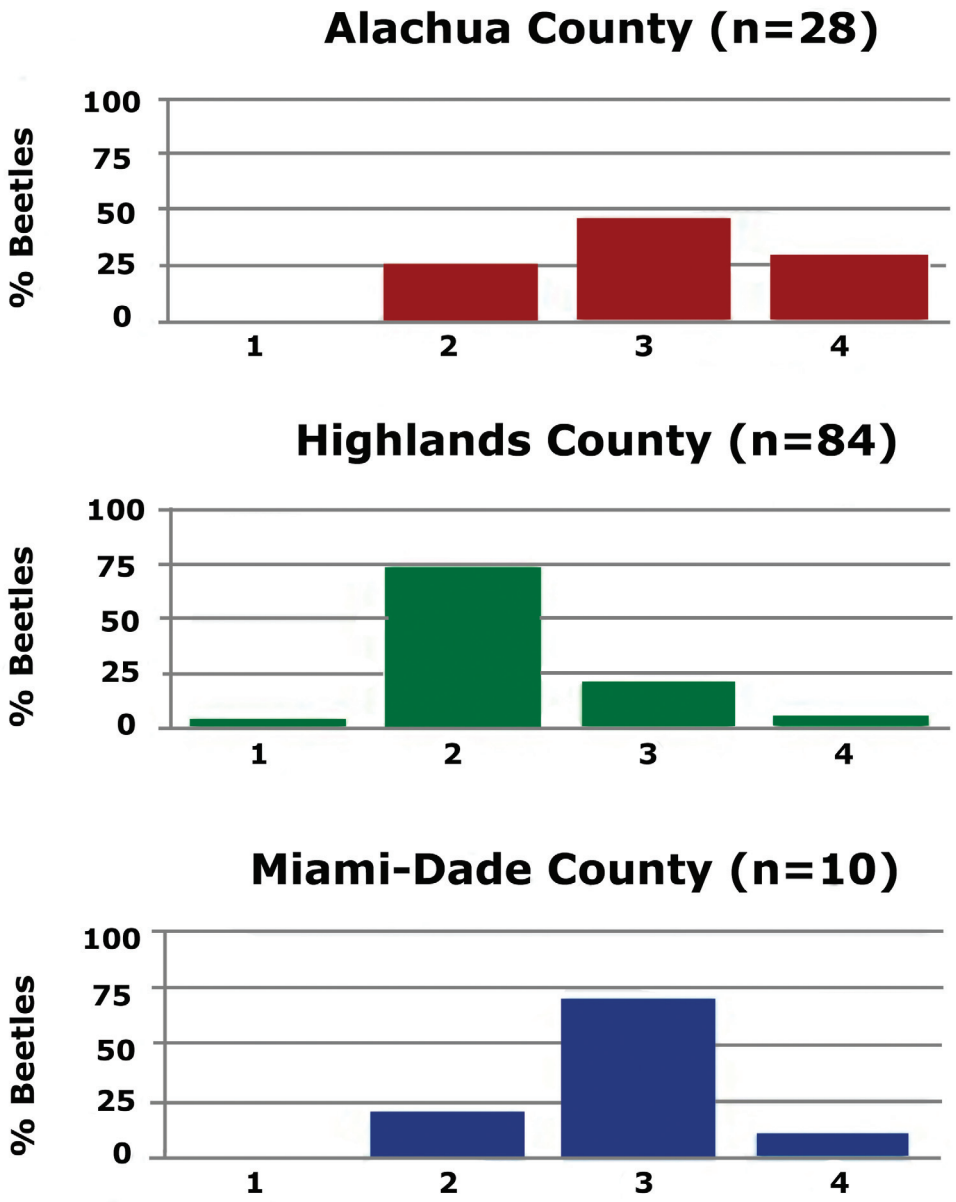

FIG. 2. Percentage of beetles in each location that carried a given number of fungal species (specified at the bottom of each graph). The number of fungal species that co-occurred in an individual's mycangia differed by location (X^2 = 36.1, df = 6, P = 0.0001) and are on the horizontal axes. The percentage of beetles in each location that were carrying a given number of fungal species is specified on the vertical axes. On average individuals from Alachua and Miami-Dade counties carried more species simultaneously in their mycangia, respectively 3.04 (± 0.12) and 2.9 (± 0.20), than those from Highlands County, 2.2 (± 0.07).

R. subalba was found in 98% of those from HC, 57% in AC, and 20% in MDC (FIG. 1).

Phylogenetic analysis of *LSU* sequences of each OTU revealed that phenotype groupings accurately predicted fungal species and these identities were confirmed with a multigene phylogeny (*LSU*, *SSU*, *BT*) (SUPPLEMENTARY FIG. 1). Five previously described species of *Raffaelea* were identified, *R. lauricola*, *R. subalba*, *R. subfusca*, *R. fusca* and *R. arxii*, whereas a sixth represents an undescribed species of *Raffaelea* that is

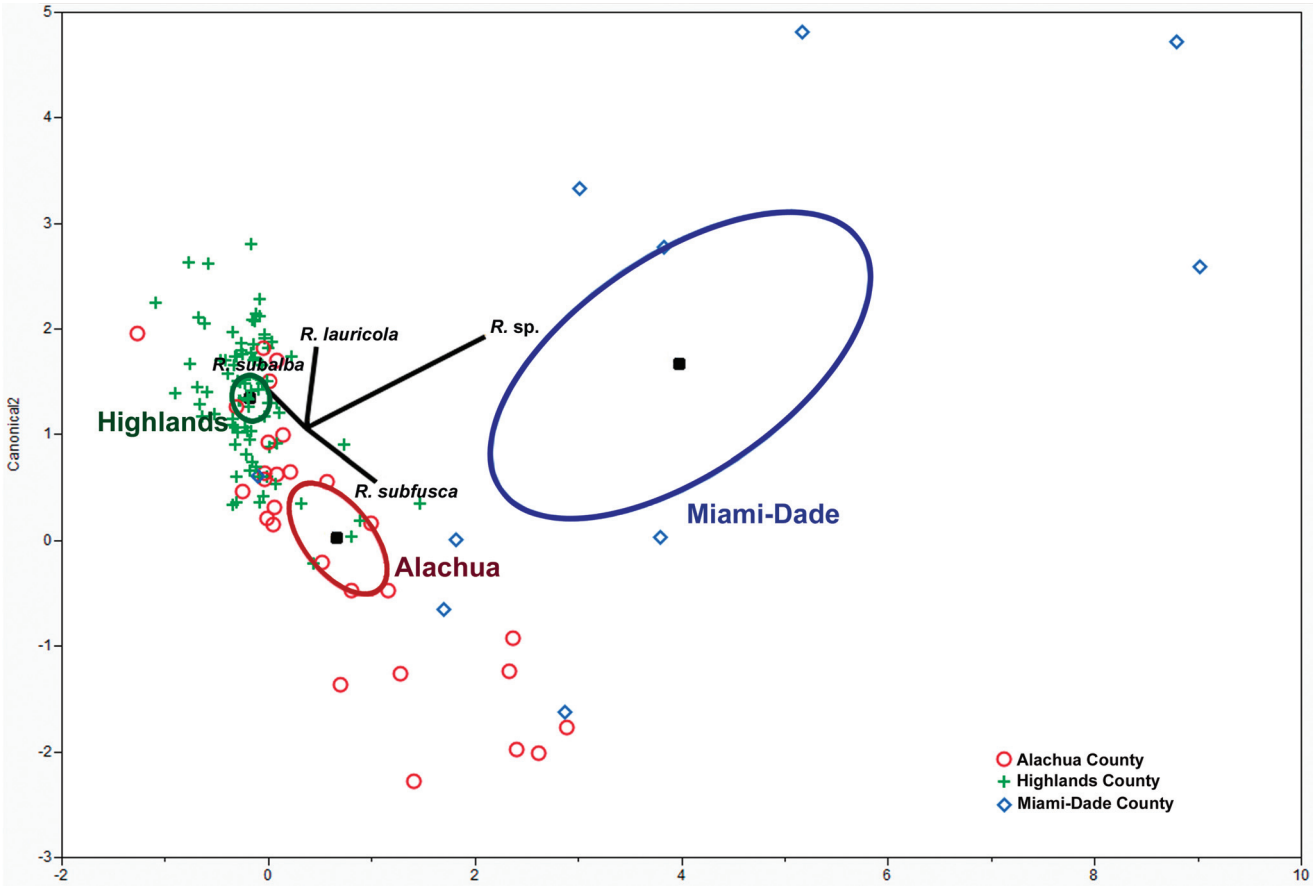

FIG. 3. Canonical plot of differences in mycangial composition of *X. glabratus* among sample locations, which account for species composition (present or absent) and abundance (CFUs). The multivariate mean of each location is a labeled circle, the size of which corresponds to its 95% confidence limit. Ray length indicates the relative importance of each of the six significant variables (fungal taxa) in these communities. The most influential (in decreasing order) were the undescribed *R.* sp., *R. subfusca*, *R. lauricola* and *R. subalba*. The rays of the less important variables (*R. arxii*, and non-ophistomatoid) are not labeled and are too short to be visible.

closely related to *R. subalba*; it was found in three individuals of *X. glabratus* from MDC.

Multivariate analysis of mycangial communities.—Fungal communities in the mycangia of *X. glabratus* from AC, HC and MDC were compared with a discriminant analysis based on CFUs of *R. lauricola*, *R. subalba*, *R. subfusca*, the undescribed *Raffaelea* sp., *R. arxii* and non-ophiostomatoid fungi. Forward stepwise variable selection was used to determine which taxa to add to the model, all except *R. fusca* ($P < 0.16$), were included. Discriminant analysis accounts for species composition and abundance (CFUs) and maximizes differences between locations as illustrated in a 2D-canonical plot (FIG. 3). In the plot multivariate means for each location are significantly different. Ray length indicates the relative importance of each of the six variables (fungal taxa) in these communities (FIG. 3). Although there were not large differences in the importance of each, the most influential (listed in decreasing order) were the undescribed *R.* sp., *R. subfusca*, *R. lauricola* and *R. subalba*, however, the rays of *R. subfusca* and *R. lauricola* were nearly identical in length. The rays of the less important variables (*R. arxii* and non-ophistomatoid) are not labeled in the figure and are too short to be visible.

Raffaelea lauricola *CFUs.*—Mean *R. lauricola* CFUs in 122 *X. glabratus* individuals from AC, HC and MDC varied by location ($F = 8.79$, Num df = 2, Den df = 122, $P = 0.003$), and there was a north to south increase in the amount of the pathogen detected (FIG. 4). Beetles from AC had fewer CFUs (1912 $\pm$ 251) than those from HC (2708 $\pm$ 148) and MDC (3450 $\pm$ 420). Mean CFUs for MDC and HC were not significantly different, perhaps due to the small MDC sample.

Interspecies associations.—Spearman's non-parametric correlations were used to examine relationships among the six *Raffaelea* spp. in *X. glabratus* mycangia.

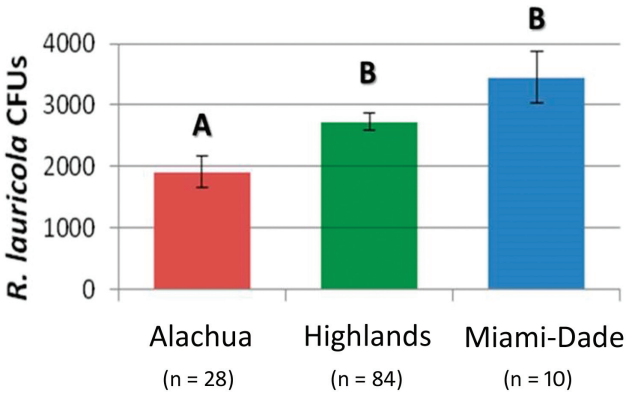

FIG. 4. Colony forming units (CFUs) of *R. lauricola* vary by location where beetle was collected ($P < 0.003$). Untransformed means are listed here. The number of individuals sampled (n) are in parentheses. Columns marked with letters are significantly different at $P < 0.05$. Each column is labeled with error bars that represent the standard error of the mean.

Although *R. lauricola* CFU abundances were not correlated with CFUs of any other fungi, *R. subfusca* was moderately negatively correlated with *R. subalba* ($R_S = -0.45$, $P < 0.0001$); that is beetles with high numbers of *R. subfusca* CFUs tended to have low *R. subalba* CFUs and vice versa.

DISCUSSION

Although the vertical transmission of ambrosia beetle symbionts from natal to newly founded galleries is believed to promote the development of species-specific associations (Six 2003), isolations from *X. glabratus* mycangia indicate the presence of diverse and variable communities. Harrington et al. (2011) and Harrington and Fraedrich (2010) found that these mycangial communities varied by location in terms of both species composition and abundance. The makeup and dynamics of microbial communities of *X. glabratus* and ambrosia beetles in general are poorly understood but might be influenced by host trees, environments and other factors.

Harrington et al. (2010) recovered six different *Raffaelea* spp. from *X. glabratus* mycangia in USA. *R. lauricola* was present in 40 of 41 beetles sampled; it was isolated in the highest numbers, *R. subalba* and *R. ellipticospora* were the next most frequently isolated species and *R. arxii*, *R. fusca* and *R. subfusca* were present only occasionally (Harrington et al. 2011). Several of these fungi also were associated with *X. glabratus* in Taiwan and Japan (Harrington et al. 2011). However, the above results cannot be directly compared with results obtained in this study due to differences in the manner in which the insects were processed. This study used only living beetles, which were assayed soon after being recovered from the field or from rearing facilities. Biochemical studies suggest that glandular cells within beetle mycangia secrete substances favoring the growth of symbiotic fungi at the expense of other microbes (Schneider and Rudinsky 1969). If mycangial composition in *X. glabratus* is affected by secretions or other conditions specific to living beetles, assaying dead beetles would yield different results. In addition, locational comparisons in the present study included only data from extracted mycangia rather than from severed heads, which should have minimized the recovery of secondary contaminants and maximized the recovery of symbionts.

In this study factors that influenced the composition of mycangial communities of *X. glabratus* were explored by assaying individuals from three locations along a 500 km N–S transect in Florida, each of which has a different climate and host tree composition. The Miami-Dade County site (25.8N) was dominated by swamp bay (*P. palustris*), the Highlands County site (27.9N) had both swamp bay and silkbay (*P. humulis*), and the Alachua County site (29.8N) was dominated by redbay (*P. borbonia*). Sites also varied in terms of climate, with the average annual temperatures of 20.4 C in Alachua County, 22.4 C in Highlands County and 25.1 C in Miami-Dade County (www.usclimatedata.com).

This study was designed to maximize the likelihood that recoveries accurately reflected the communities present in living beetles during their dispersal to new natal trees. The general medium that was used, one-half PDA, ensured that a wide range of microbes was detected and only living beetles were used. Nonetheless nonculturable microbes in the mycangia would not have been detected.

Because beetles were collected as they emerged from natal galleries in search of new host trees the fungi that were recovered should reflect the mycoflora to which newly colonized trees would be exposed. Host trees are inoculated with *R. lauricola* as beetles create new galleries in host sapwood (Kendra et al. 2013). Understanding the factors that affect mycangial composition and the amounts of a given symbiont that are carried by an individual are important first steps to possibly manipulating the transmission of a plant pathogen, such as *R. lauricola*, by these insects.

Mean CFUs of *R. lauricola*, in individuals varied by location, as beetles from MDC carried more CFUs (3532), than those from HC (2593) and AC (1829). It was the most abundant *Raffaelea* spp. (in terms of CFUs) in 90% of the individuals from MDC, 83% from HC and 63% from AC. Its predominance may be due to its much higher growth rate and the wider temperature range at which it grows (65mm in 10 d at 25 C and up to 10 mm at 10 C and above 35 C)

TABLE III. Colony-forming units of *Raffaelea* species recovered from mycangia of 122 *Xyleborus glabratus* collected in Florida

Fungi	Mean CFUs (±SE)[a]	Proportion of beetles[b]
R. lauricola	2599 (±125) A	0.98
R. subalba	819 (±67) B	0.80
R. subfusca	211 (±51) C	0.20
R. fusca	36 (±24) D	0.03
R. sp.	20 (±13) D	0.03
R. arxii	13 (±8) D	0.03

[a] Means followed by different letters are significantly different at the $P < 0.05$ family-wise error rate.

[b] Proportion of assayed beetles from which the fungi were recovered.

(Harrington et al. 2008, Harrington et al. 2010). In fact for the species that have been tested there is a positive relationship between recovery frequency and growth rate at 25 C: *R. lauricola* > *R. subalba* > *R. subfusca* > *R. fusca* (TABLE III). The north to south increase in CFUs of *R. lauricola* recovered may be due to this organism having a higher optimum temperature than what occurs in HC and AC.

Alternatively the predominance of *R. lauricola* in mycangia might be a reflection of the high populations of *R. lauricola* that are found in host trees affected by laurel wilt and colonized by *X. glabratus* (Campbell 2014). If mycangial composition is a reflection of garden composition in different host trees, the higher number of CFUs recovered from beetles from MDC may indicate that swamp bay trees support the development of gardens with a higher density of *R. lauricola* compared to host tree species present in the HC and AC sites.

The presence and prevalence of organisms in the fungal gardens within natal galleries should be assayed to determine their relationship to mycangial composition. For example, although *R. lauricola* is assumed to be the primary nutritional symbiont of *X. glabratus*, it was not recovered from some beetles in the present study. Whether other, secondary symbionts can replace *R. lauricola* in the beetle life cycle is not known.

More species co-occurred in individuals of *X. glabratus* from AC and MDC (80% carried ≥ three species) than HC (31%). HC beetles often carried only *R. lauricola* and *R. subalba*, and *R. subalba* also was the next most frequently recovered *Raffaelea* spp. in AC. To date *R. subalba* has been found only in beetles collected in USA. It either occurs in unsampled regions of Asia (Harrington et al. 2011) or was acquired by *X. glabratus* after it was introduced into USA. *Raffaelea arxii* was isolated from *X. glabratus* in Georgia by Harrington et al. (2010) but was found only in MDC, the southernmost location sampled, in the present study. Thus temperatures at which this species grows may not explain its relative abundance. *Raffaelea ellipticospora*, which was reported in Asia and Georgia (Harrington and Fraedrich 2010, Harrington et al. 2011) was not found in the present work.

Based on the present and previous results (Harrington and Fraedrich 2010, Harrington et al. 2011), species of *Raffaelea* appear to be favored colonists of the mycangia of *X. glabratus*. Other than a handful of non-ophiostomatoid fungi only six *Raffaelea* spp. were recovered in this study. Whether the latter species are all nutritional symbionts of the insect or whether its mycangial environment promotes colonization by fungi in this genus is not known. Whether *X. glabratus* can subsist on some of species of *Raffaelea* other than *R. lauricola* should be determined. Carrying multiple fungi may benefit the beetle by increasing its potential to grow in hosts or under environmental conditions in which others are poorly suited. The co-occurrence of closely related species in a confined area such as the mycangium makes interactions among them seem likely. Nonetheless none of the species in this study appeared to influence the presence and prevalence of another in that there were no correlations among the various species.

Fungal communities have been characterized in only a few of the known 3500 ambrosia beetle species (Harrington and Fraedrich 2010, Endoh et al. 2011). Some fungal symbionts differ in terms of their nutritional quality. For example, those of *X. ferrugineous* vary in sterol, lipid and amino acid content (Biedermann et al. 2013).

Xylosandrus mutilatus associates with multiple fungal species. Its gardens are composed of *Ambrosiella* sp., *Candida* sp. and *Paecilomyces* sp., the relative abundance of which vary based on the beetle's developmental stage. *Ambrosiella* sp. dominates during the larval stage and decreases gradually as they grow (Kajimura and Hijii 1992). New *Xylo. mutilatus* females take up at least four fungal species into their mycangia. As they mature processes in the mycangia are thought to select for the *Ambrosiella* sp. (Kajimura and Hijii 1992).

Work is needed to identify and understand the factors that affect community composition. The responses of fungal symbionts to environmental or host factors might determine their relative abundance. Identifying conditions under which *X. glabratus* develops reduced mycangial populations of *R. lauricola* could have important implications for the epidemiology of laurel wilt. Clearly more information is needed to manipulate the environment or mycangial mycota of *X. glabratus* to favor species other than *R. lauricola*, as suggested by Harrington and Fraedrich (2010).

ACKNOWLEDGMENTS

This research was financed partially by USDA NIFA grant 2009-51181-05915. We thank Dr James Colee (UF-IFAS-Statistics Department) for help with the statistical analysis. Thanks also to Josh Konkol, Jill Ploetz and Ronald Wideman for their help with finding and processing the beetles.

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