Insect–Symbiont Interactions

OXFORD

Fungal Associates of the *Xylosandrus compactus* (Coleoptera: Curculionidae, Scolytinae) Are Spatially Segregated on the Insect Body

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Abstract

Studies of symbioses have traditionally focused on explaining one-to-one interactions between organisms. In reality, symbioses are often much more dynamic. They can involve many interacting members, and change depending on context. In studies of the ambrosia symbiosis—the mutualism between wood borer beetles and fungi—two variables have introduced uncertainty when explaining interactions: imprecise symbiont identification, and disregard for anatomical complexity of the insects. The black twig borer, *Xylosandrus compactus* Eichhoff, is a globally invasive ambrosia beetle that infests >200 plant species. Despite many studies on this beetle, reports of its primary symbionts are conflicting. We sampled adult *X. compactus* and infested plant material in central Florida to characterize the fungal symbiont community using dilution series, beetle partitioning, and DNA-based identification. *X. compactus* was consistently associated with two fungal taxa, *Fusarium* spp. and *Ambrosiella xylebori*. Multivariate analyses revealed that *A. xylebori* was strongly associated with the beetle mycangium while *Fusarium* spp. were associated with the abdomen and external surfaces. The *Fusarium* spp. carried by *X. compactus* are not members of the Ambrosia *Fusarium* Clade, and are probably not mutualists. Fungal community composition of the mycangium was less variable than external body surfaces, thus providing a more consistent fungal inoculum. This is the first report of spatial partitioning as a mechanism for maintenance of a multimember ambrosia fungus community. Our results provide an explanation for discrepancies among previous reports, and suggest that conflicting results are not due to differences in symbiont communities, but due to inconsistent and incomplete sampling.

Key words: *Ambrosiella*, *Fusarium solani*, black twig borer, mycangium, mutualism

One of the most interesting areas of contemporary symbiology is the intersection between the deterministic one-on-one interactions formed by coevolution and physiology, and the more dynamic assembly of communities containing various associates. Both types of relationships are often present, and what often determines the inference is not the biological reality, but rather the assumptions of researchers. For example, our understanding of the ambrosia symbiosis—the association between wood boring beetles and nutritional fungi—has evolved from a traditional focus on a single primary mutualist (Bakshi 1950), through a community ecology view (Haanstadt and Norris 1985, Harrington et al. 2010, Kostovcik et al. 2015), to the contemporary integrative view of a community of members with variable degrees of reciprocity and dependence

(Mayers et al. 2015, Freeman et al. 2015). Changes in how we perceive these associations have run parallel with evolving methodology, and the two are inextricably connected.

Our perception of ambrosia symbioses evolved from pair-wise interactions, to more complex systems of interactions as methodology and research tools developed to gain a broader view of the organisms involved. Methodology has a pervasive influence over the inferences that we draw and the concepts that emerge. For instance, almost any ambrosia beetle can yield either a community of fungi, or a single mutualist ambrosia fungus, depending on the choice of sampling approach. Biases can be introduced by many methodological decisions: whether to use specialized or general media (Whitney et al. 1987, Hsiau and Harrington 1997); whether to place the entire

sample on a culturing plate or use dilution series; whether to focus on particular insect body parts or grind up the entire beetle (McPherson et al. 2013); whether to sample the gallery, the adults, or other developmental stages (Freeman et al. 2015); and which characters or markers to use for the symbiont community identification (Rollins et al. 1987, Alamouti et al. 2009). Consequently, application of new or more rigorous methods can provide novel insights to even the most heavily studied taxa and help advance more robust and comprehensive conceptual frameworks for the study of symbioses in general.

Accurate characterization of the vector–fungus associations is a key component of our ability to monitor invasive species and manage economic and ecological threats. Bark and ambrosia beetles (Coleoptera: Curculionidae, Scolytinae) are normally widespread colonizers of dead or dying wood, but some species attack live trees (Kühnholz et al. 2001, Hulcr and Dunn 2011). Both native and invasive tree-killing beetles pose serious threats to tree crops, lumber industries, landscape ornamentals, and native woody plants, as well as contribute to the spread of plant pathogens. It's not always known what role fungi play in tree death, if any, but in some cases, both the fungus and the beetle contribute (Six and Wingfield 2011). In other cases, it is solely the fungus that acts as a pathogen, enables tree colonization, and is the main reason behind economic damages (Brasier 1991, Fraedrich et al. 2008). Fungus promiscuity in some of the globally homogenized ambrosia communities is already determining the evolution of pathogenicity and invasion ecology, and causing millions of dollars of economic losses (Carrillo et al. 2014).

Xylosandrus compactus Eichhoff, the black twig borer, is one of the most studied ambrosia beetle species worldwide due to its long history as a pest of healthy trees (Brader 1964, Daehler and Dudley 2002). The female bores into the pith of young stems on apparently healthy trees and cultivates symbiotic fungal gardens, causing death from the initial entrance hole to the terminal ends of the branch (Hara and Beardsley Jr 1976, Wood 1982). Well over 200 tree species are recorded as hosts to *X. compactus* (Ngoan et al. 1976). Many commercial crops have experienced substantial losses, such as avocado (Ngoan et al. 1976), mango, tea (Kaneko et al. 1965), coffee (Brader 1964), and cocoa (Hara and Beardsley Jr 1976). Nurseries can be heavily impacted, mostly by aesthetic damages to ornamentals and orchids (Dekle and Kuitert 1968, Ngoan et al. 1976). In island ecosystems such as Hawaii, endangered plants are threatened by the beetle (Nishida and Evenhuis 2000). Despite the diverse and far reaching impacts of *X. compactus*, many basic characteristics of this species remain unknown. This is especially true concerning the identity and stability of its fungal symbiont community.

Many different fungi have been described as associates of *X. compactus*, but three have been reported most consistently: *Ambrosiella xylebori*, *Ambrosiella macrospora*, and *Fusarium solani* (Brader 1964, von Arx and Hennebert 1965, Batra 1967, Bhat and Sreedharan 1988). *A. xylebori* has also been described in association with other beetle species, including taxa that are congeneric (e.g., *Xylosandrus crassiusculus* (Motschulsky) (Gebhardt et al. 2005)) and those that are more distantly related (e.g., *Corthylus columbianus* (Hopkins) (Batra 1967)). Morphological species recognition frequently fails within *Ambrosiella* and *Fusarium* due to their phenotypic uniformity. Thus, the reports of *A. macrospora* from a broad range of beetles, including *Ips acuminatus* (Gyllenhal) infesting *Pinus* spp. in Europe (Francke-Grosmann 1952, Batra 1967) and *X. compactus* (Muthappa and Venkatasubbaiah 1981), need to be confirmed using DNA sequence data.

Fusarium solani is a name given to a complex of over 45 morphologically cryptic species (O'Donnell et al. 2008). Members of the *F. solani* complex (hereafter referred to as the FSSC) have been reported in association with many distantly related scolytine beetles, including *Xyleborus ferrugineus* (Fabricius, 1801) (Baker and Norris 1968), *Hypothenemus hampei* (Ferrari) (Rojas et al 1999), *Scolytodes unipunctatus* (Blandford) (Kolarik and Hulcr 2008), *Xylosandrus compactus* (Ngoan et al. 1976), and several other beetle genera (Hulcr and Cognato 2010). A clade of true ambrosia fungi was recently discovered within the FSSC that has coevolved with ambrosia beetles (the Ambrosia *Fusarium* Clade (AFC); Kasson et al. 2013). Therefore, it is possible that the *Fusarium* species reported from *X. compactus* belong to the mutualistic AFC clade, or it may represent a new ambrosial lineage, persistent commensals or parasites, or transient environmental contaminants.

In order to characterize the symbiosis between *X. compactus* and its fungal associates for use in future research on the symbiosis, this study was initiated to:

1. Quantitatively characterize the fungal community in terms of species identity, diversity, frequency, and abundance distribution.
2. Determine whether different fungal symbionts co-occur in beetle mycangia during transport or are spatially separated to different beetle body parts.

Materials and Methods

Sampling and Isolation

Fungi were isolated from live adult beetles and infested plant material during the spring of 2013 and 2014. All beetles included in fungal isolations were collected in-flight from ethanol-baited traps. Five adult *X. compactus* females from each of the six sites in Gainesville, FL, and one specimen from Venus, FL, were used for fungal isolation ($n = 31$ total). Five beetle galleries were also selected from Gainesville, FL, for fungal isolation.

Beetles captured in flight were stored on lightly moistened paper tissue at 15°C for a maximum of three days prior to fungus extraction. Only females were examined because Xyleborini males are not primary gallery founders: they lack mycangia, are flightless, and may not leave natal galleries for most species (Kirkendall 1983).

Fungi were isolated from four locations from adult female beetles: the surface, mycangium, and from within the anterior (head and pronotum) and posterior (mesonotum to abdomen) halves of the beetle. The surface of each beetle was washed by vortexing in a 1 ml sterile solution of 1% Tween 80 (Sigma Chemical Co, St. Louis, MO) and phosphate buffer saline (PBS) and then was serially diluted prior to plating. Following the wash, beetles were vortexed at 2,100 rpm for 15 s in 1 ml sterile PBS and allowed to dry on tissue paper. Live beetles were then secured onto paraffin wax using minuten pins (Bioquip, Rancho Dominguez, CA). Minuten pins were used to pry the pronotum away from the mesonotum, revealing the mycangium (Fig. 1). Under 60× magnification (Fisher Scientific Stereomaster, Fisher Scientific, Suwanee, GA), the fungal clump was transferred into a 2-ml microcentrifuge tube containing 0.5 ml PBS using a flame sterilized 000 insect pin (Bioquip). Beetles were then aseptically severed between pronotum and mesonotum. Each half of the beetle was crushed in separate microcentrifuge tubes containing .5 or 1 ml sterile PBS using plastic pestles (Fisher Scientific, Suwanee, GA).

Direct plating of beetle galleries occurred first by surface-sterilizing the wood samples with 95% ethanol, and then by placing
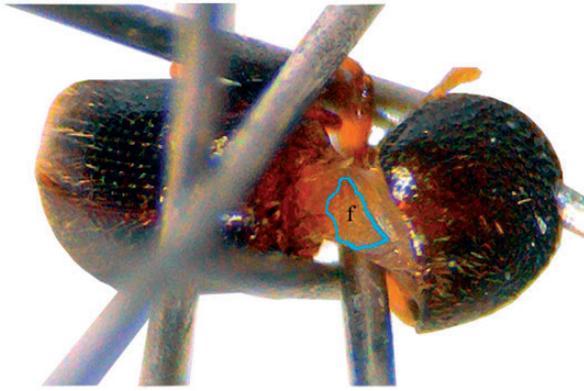
Fig. 1. Procrustean method: *Xylosandrus compactus* undergoing dissection of fungi (f) from its mesothoracic mycangium.

~3 mm gallery samples removed with a sterile scalpel onto the media.

Serial dilutions (1/10, 1/100, and 1/1,000) were made from each sample (i.e., surface wash, head, mycangium, and abdomen). Dilutions were plated on Potato Dextrose Agar (Difco Labs, Detroit, MI) amended with 1.4% additional agar (Fisher Scientific, Fairlawn, NJ). Cultures were incubated in an incubator in the dark at 25°C for up to 2 wk. Fungal colony-forming units (CFUs) were assigned to morphotypes and relative abundances calculated as in Bateman et al. (2015). Morphotype designations were confirmed by comparing pure cultures and by DNA sequencing as described below.

DNA Sequence Acquisition and Phylogenetic Analysis

Pure cultures between 1–2 wk old were used for DNA extraction. Mycelium (10–20 μl) was added to 20 ul of Sigma-Aldrich Extraction Solution (Extract-N-Amp, St. Louis, MO) in 0.25-ml PCR tubes, which were then incubated in a thermocycler (Eppendorf Mastercycler) at 96°C for 10 min. After incubation, 20 μl of 3% BSA (Fermentas) was added to each tube, vortexed for 5 s and then centrifuged at 2,000 rpms for 30 s. The upper half of this solution was used as genomic DNA template for PCR.

Partial sequences from two nuclear ribosomal DNA (rDNA) encoding regions, the internal transcribed spacer (ITS) and partial 28S (LSU) rDNA regions, were obtained for all morphotypes found on greater than 15% of the beetles sampled. For the *Ambrosiella* morphotype, seven isolates were included in sequencing. For the *Fusarium* morphotype, 13 isolates were selected for sequencing, including an additional three protein-encoding genes for some isolates: RNA polymerase 1 & 2 (RPB1, RPB2) and trans-elongation factor 1-α (EF1-α).

PCR reactions contained a final volume of 25 μl: 1 μl of template DNA, 1 μl of forward and reverse primer (10 μM), 0.125 μl of Taq (Takara ExTaq), 2.5 μl PCR buffer (15 mM MgCl$_2$), 2 μl dNTP mix (2.5 mM each dNTP), and 20 ul sterile water. PCR and sequencing primers are listed in Table 1. PCR products were purified using Exosap-IT, following the manufacturer's protocols (USB Corp., Cleveland, OH). PCR reactions were performed in an Eppendorf Mastercycler Pro following parameters specific for each marker, as listed in Kim et al. (2004).

BLAST queries of the NCBI GenBank database were performed with each sequence to gain a rough identification of the fungi that were isolated. Final taxonomic identity for the *Ambrosiella* morphotype was determined by maximum likelihood (ML) phylogenetic

analysis in PhyML within the Microascales using the concatenated dataset and parameters described for *Ambrosiella* spp. in Bateman et al. (2015). Identity of isolates under the *Fusarium* morphotype was determined using (ML) phylogenetic analysis using RaxML and parameters described in O'Donnell et al. (2013). *Fusarium* sequences included in the phylogenetic analysis were downloaded from the Fusarium multilocus sequence typing website (MLST: http://www.cbs.knaw.nl/fusarium, last accessed July 1, 2016 O'Donnell 2013), including partial ITS+LSU rDNA, RPB1, RPB2, and EF1-α sequences, but only ITS+partial LSU sequences were included for *Fusarium* isolates recovered in this study. Sequences generated from representative isolates in this study have been deposited in the NCBI GenBank database (Supp. Table 1 [online only]).

Statistical Analysis

We used a combination of multivariate ordination and inferential statistics to evaluate differences in fungal species composition and variability in species composition among the body parts of the beetles sampled. Nonmetric multidimensional scaling (NMDS) was used to visualize fungal community composition from each of the beetle parts. NMDS was conducted on a Bray-Curtis distance matrix using the metaMDS function of the vegan package for R (Oksanen et al. 2013). Because they varied over orders of magnitude, estimates of fungal CFUs were standardized using a Wisconsin double standardization (Bray and Curtis 1957). Dimensionality was chosen by visual examination of a scree plot of stress values versus several candidate numbers of dimensions. We used a permutations-based multivariate analysis of variance to statistically test for significant difference in fungal community composition among the sampled beetle parts (PERMANOVA; Anderson 2001). PERMANOVA was conducted on a Bray-Curtis dissimilarity matrix using the adonis function of the vegan package for R (Oksanen et al. 2013). In addition to beetle body part, collection site was used as a cofactor to account for variation in fungal communities among localities. To identify significant associations between specific fungal taxa and beetle parts, indicator species analysis was used (De Cáceres and Legendre 2009), individually and in combination (De Cáceres, Legendre and Moretti 2010). Indicator species analysis was conducted using the multipatt function in the indicspecies package for R and 10,000 random permutations to test for significance (De Cáceres and Legendre 2009).

In addition to assessing differences in fungal community composition among beetle body parts, we also tested if the mycangium contains a less variable fungal community than other body parts. Therefore, we tested for a significant difference in the within-group variation among the sampled beetle parts. We conducted a test of homogeneity of multivariate dispersion (Anderson 2006) using the betadisper function of the vegan package for R (Oksanen et al. 2013) and a one-way ANOVA of within-group distances to their respective centroids. A post hoc Tukey's honestly significant difference (HSD) test was used to determine significant pairwise differences in multivariate dispersion among beetle body parts (alpha = 0.05).

Results

Fungi Associated with *Xylosandrus compactus*

Seven fungal morphotypes were isolated from adult *X. compactus* females captured in flight in north central Florida from at least 15% of beetle specimens (Tables 2 and 3). The two most common morphotypes, each isolated from over 90% of beetles and also from galleries, were identified as *Ambrosia xylebori* and *Fusarium* spp.

Table 1. Primers used for PCR and DNA sequencing

Locus	Gene product	Primer		Use		Reference
		Name	Sequence (5′-3′)[a]	PCR	Sequencing	
SSU rDNA	Nuclear ribosomal small subunit (18S)	NS1	GTAGTCATATGCTTGTCTC	•	•	(White et al. 1990)
		NS4	CTTCCGTCAATTCCTTTAAG	•	•	(White et al. 1990)
ITS rDNA	Internal transcribed spacer	ITS1	TCCGTAGGTGAACCTGCGG	•	•	(White et al. 1990)
		ITS4	TCCTCCGCTTATTGATATGC	•	•	(White et al. 1990)
LSU rDNA	Nuclear ribosomal large subunit (28S)	NL1	CTTGGTCATTTAGAGGAAGTAA	•	•	(Vilgalys and Hester 1990)
		LR3	CCGTGTTTCAAGACGGG	•	•	(Vilgalys and Hester 1990)
		LROR	ACCCGCTGAACTTAAGC	•	•	(Vilgalys and Hester 1990)
EF-1α	Translation elongation factor 1α	EF1	ATGGGTAAGGARGACAAGAC	•	•	(O'Donnell et al. 1998)
		EF2	GGARGTACCAGTSATCATG	•	•	(O'Donnell et al. 1998)
		EF3	GTAAGGAGGASAAGACTCACC		•	(O'Donnell et al. 2008)
		EF22T	AGGAACCCTTACCGAGCTC	•	•	(O'Donnell et al. 1998)
RPB1	RNA polymerase largest subunit	Fa	CAYAARGARTCYATGATGGGWC	•	•	(Hofstetter et al. 2007)
		G2R	GTCATYTGDGTDGCDGGYTCDCC	•	•	(O'Donnell et al. 2010)
		R8	CAATGAGACCTTCTCGACCAGC	•	•	(O'Donnell et al. 2010)
		F5	ATGGGTATYGTCCAGGAYTC		•	(O'Donnell et al. 2010)
		F6	CTGCTGGTGGTATCATTCACG	•	•	(O'Donnell et al. 2010)
		F7	CRACACAGAAGAGTTTGAAGG		•	(O'Donnell et al. 2010)
		F8	TTCTTCCACGCCATGGCTGGTCG		•	(O'Donnell et al. 2010)
		R9	TCARGCCCATGCGAGAGTTGTC	•	•	(O'Donnell et al. 2010)
RPB2	RNA polymerase second largest subunit	5f2	GGGGWGAYCAGAAGAAGGC	•	•	(Reeb et al. 2004)
		7cr	CCCATRGCTTGYTTRCCCAT	•	•	(Liu and Hall 2004)
		7cf	ATGGGYAARCAAGCYATGG	•	•	(Liu and Hall 2004)
		11ar	GCRTGGATCTTRTCRTCSACC	•	•	(Liu and Hall 2004)

[a] D = A, G, or T; R = A or G; S = C or G; W = A or T; Y = C or T.

Table 2. Average fungal colony counts from different parts of *X. compactus* indicate the abundance of *A. xylebori* but not *Fusarium* spp. in mycangia

Species	Surface	s.e.	Head	s.e.	Mycangium	s.e.	Abdomen	s.e.
Acremonium sp.	47.1	33.0	100.0	96.7	387.1	326.9	137.7	99.0
Ambrosiella xylebori	0.0	0.0	108.7	45.7	3,946.1	732.4	91.3	45.1
Cladosporium sp.	67.7	64.4	37.7	29.5	14.8	9.1	180.0	151
Cryptococcus sp.	2,556.5	2,514.9	134.2	99.3	12.6	7.2	3.5	2.2
Fusarium spp.	262.9	116.2	54.2	33.2	13.2	12.9	681.9	235.7
Pestalotiopsis sp.	0.0	0.0	52.6	48.4	33.5	32.2	41.9	33.7
Phialemonium sp.	58.4	40.6	67.7	64.5	0.0	0.0	754.8	648.4

Table 3. Prevalence (% of samples) of fungi isolated from different parts of *X. compactus* shows *A. xylebori* and *Fusarium* spp. to be the more prevalent isolates

Species	Surface	Head	Mycangium	Abdomen	Total prevalence
Acremonium sp.	22.6	6.5	6.5	16.1	25.8
Ambrosiella xylebori	0.0	35.5	90.3	25.8	90.3
Cladosporium sp.	9.6	16.1	9.7	12.9	19.4
Cryptococcus sp.	18.4	16.1	16.1	9.7	35.5
Fusarium spp.	58.1	29.0	6.5	61.3	90.3
Pestalotiopsis sp.	0	12.9	6.5	6.5	16.1
Phialemonium sp.	9.7	6.5	0.0	9.7	16.1

Ambrosiella xylebori was identified based on a 100% match to partial SSU, ITS, and LSU rDNA sequence data from the holotype strain of *A. xylebori* (CBS 110.61). The *A. xylebori* isolates also formed a monophyletic group with the type strain in the phylogenetic analysis. The *Fusarium* spp. morphotype was not assigned to a single species within the genus. Maximum likelihood analysis showed *Fusarium* isolates generated in this study formed a monophyletic group, but did not group with members of the AFC (Supp. Fig. 1 [online only]). Some *Fusarium* isolates in this study did show differences in ITS sequences, including two isolates which had a maximum of 72 base pair differences from other isolates, but these differences were not phylogenetically informative, possibly due to instances of highly divergent paralogs or xenologs at this locus in some groups within the *Fusarium solani* species complex (O'Donnell and Cigelnik 1997, O'Donnell et al. 1998). Abundance and prevalence of *Fusarium* isolates where such sequence divergence was observed was not analyzed further here because morphotypes could not be standardized between genotypes.

A three-locus typing scheme, employing portions of the ITS+LSU rDNA, EF-1α, and RPB2, was used to identify one *Fusarium* isolate as a novel species within the *F. solani* species complex, which was designated FSSC 45-a. Arabic numbers and lowercase Latin letters are used to identify, respectively, phylospecies and haplotypes within the FSSC (O'Donnell et al. 2008). Isolate *Fusarium* sp. FSSC 45-a was accessioned in the ARS Culture Collection as NRRL 62797 where it is available upon request (http://nrrl.ncaur.usda.gov/, last accessed July 1, 2016).
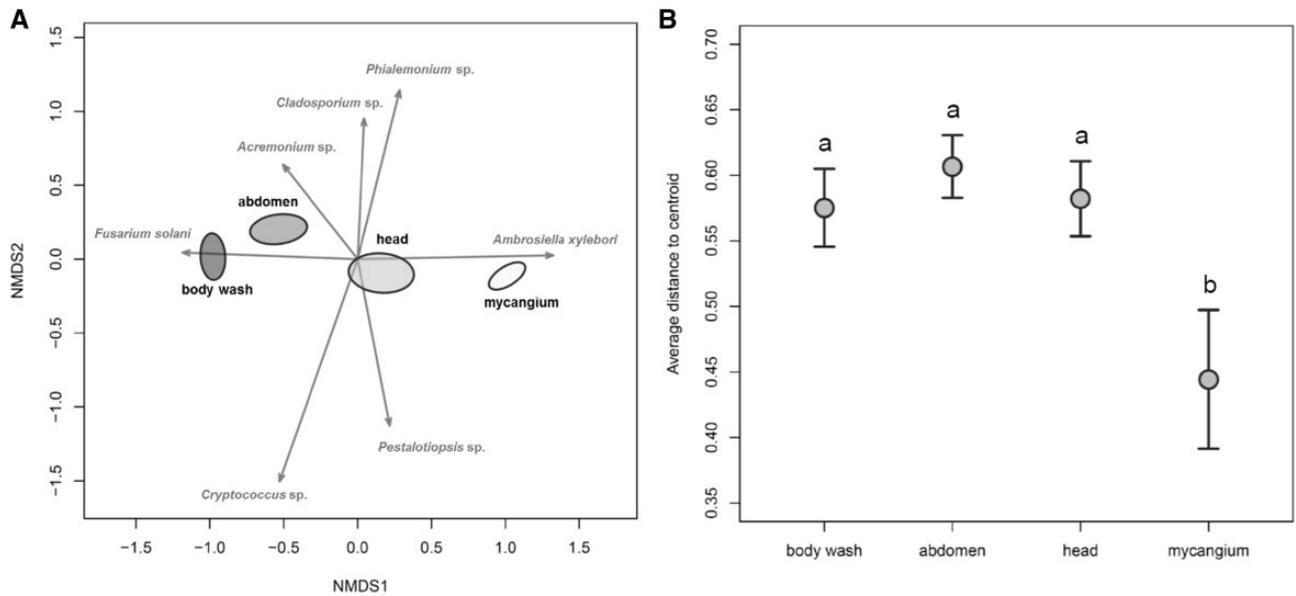
Fig. 2. (**A**) NMDS ordination of fungal community samples collected from multiple beetle body parts (Bray-Curtis dissimilarity, stress = 0.083). Shaded ellipsis represent 1 standard error surrounding the centroid of all samples collected from each beetle part. Gray arrows represent the species scores for fungal taxa observed during the study. *Ambrosiella xylebori* was strongly associated with the mycangium, whereas *Fusarium* spp. were associated with body washes and the abdomen. (**B**) Variation in multivariate dispersion of fungal composition among beetle parts showing that the mycangium was significantly less variable than all other parts examined. Symbols indicate the mean distance from all points within a group (body part) to the centroid of the group. Error bars represent 1 standard error around the mean. Letters represent significant among-group differences determined by post hoc analysis.

Additional fungi were also recovered, but with overall lower prevalence. A yeast species in the genus *Cryptococcus* was recovered from 35.5% of the beetles and an *Acremonium* sp. from 25.8% of the beetles. CFU counts of these two fungi were high in a few samples, but their association with specific body parts was inconsistent (Tables 2 and 3). The remaining fungal species were isolated from <15% of the beetles.

The Two Dominant Fungal Associates Are Spatially Segregated During Transport

Different beetle parts carried distinct combinations of fungal taxa (Fig. 2A). The factors "beetle part" and "sampling site" had significant effects on fungal inoculum composition and together these factors explained ~28% of the variation among samples (PERMANOVA, Table 4). Indicator species analysis identified two significant associations between specific fungal taxa and beetle parts; *Ambrosiella xylebori* was significantly associated with the mycangium (stat = 0.916, $P < 0.001$) and *Fusarium* spp. were significantly associated with a combination of the abdomen and body wash (stat = 0.872, $P < 0.001$). There were no fungal taxa significantly associated with the head. In addition to differences in fungal composition, we also observed significant differences in multivariate dispersion among groups (ANOVA; $F = 3.712$, $P = 0.014$, df = 3,90). Post hoc tests revealed that the fungal composition of the mycangium was significantly less variable among samples than all other beetle parts examined (Fig. 2B).

Discussion

A review of previous studies would suggest that *X. compactus* is associated with either *Ambrosiella* or *Fusarium*. In contrast to the historical "either-or" perspective, rigorous quantitative sampling of individual body parts and multivariate data analyses presented here confirm that both *A. xylebori* and *Fusarium* spp. are associates of

Table 4. Results of PERMANOVA showing significant effects of beetle body part and sampling site on fungal species composition of collected inoculum

Parameter	df	Sums of squares	F	R^2	P-value
Body part	3	7.577	7.719	0.20	0.001*
Sampling site	6	3.121	1.590	0.08	0.006*
Residuals	84	27.486			
Total	93	38.185			

* Indicates significant effect (alpha = 0.05).

X. compactus. Moreover, each symbiont taxon had a unique, spatially segregated association with its host. *A. xylebori* was isolated almost exclusively from the mycangia, while *Fusarium* spp. were recovered primarily from the body surface, particularly the beetle abdomens. Furthermore, the *Fusarium* association is less consistent, partly because of its presence only on the beetle surface, but also because there appear to be several species, one dominant and several incidental ones. This is the first record of an ambrosia beetle that is associated with multiple spatially segregated fungi. Although our samples were limited to Florida, reports of *Ambrosiella* and *Fusarium* associated with *X. compactus* from Hawaii (Daehler and Dudley 2002, Kuo 2010) and Japan (Hayato 2007) suggest these associations are distributed worldwide.

Our discovery that *Ambrosiella* is the most prevalent fungus in the mycangium of *X. compactus* suggests they are engaged in a close-knit nutritional symbiosis. This finding appears to corroborate reports of this association from around the world (Muthappa and Venkatasubbaiah 1981, Daehler and Dudley 2002, Hayato 2007), including the original description of *Ambrosiella* from *X. compactus* infesting coffee in Ivory Coast (Brader 1964). Portions of the partial SSU, ITS, and LSU rDNA sequences of *A. xylebori* in Florida were identical to those from the holotype strain (CBS 110.61). Moreover,

our finding that the mycangium is significantly less variable in fungal composition provides evidence that the mycangium is adaptive because it provides a more consistent and predictable fungal inoculum than external body surfaces. At present, broader geographic taxon sampling and more informative marker loci are needed to understand exactly where the symbiosis originated and whether symbiont fidelity is maintained worldwide.

The role that *Fusarium* plays in the ecology and life history of *X. compactus* is currently unknown but deserves further study. Since it is carried on the surface and probably in the gut, but much less frequently in the mycangium, we speculate that the association is either not a result of coevolution, or is evolutionarily recent and less specific than that between beetles and coevolved nutritional symbionts. The *Fusarium* species associated with *X. compactus* are not a part of the AFC, which contains highly coevolved nutritional mutualists (Kasson et al. 2013). Conversely, *X. compactus* is the first xyleborine ambrosia beetle consistently associated with *Fusarium* species that are not members of the AFC. Members of the genus *Fusarium* were also abundant in the gallery, and are routinely isolated from heavily stained areas around galleries in twigs attacked by *X. compactus* (Bateman and Hulcr personal observation). This nonmycangial but consistent association may be explained by three hypotheses. 1) *Fusarium* spp. are opportunists that sporulate prolifically and are competitive in the gallery as a nutritional mutualist. 2) *Fusarium* spp. are so prevalent because they are phytopathogens and suppress defense mechanisms in the twig, thus increasing the fitness of the entire gallery. The difficulty of distinguishing phoretic opportunists from beneficial mutualists has been discussed extensively within the scolytine–fungus literature (Six and Wingfield 2011). 3) *Fusarium* spp. have minor biological significance in the *X. compactus* system, but they are prolific spore-formers, which makes them appear as abundant on culture media as the mycangial coevolved fungus.

It is unclear why *A. xylebori* was not recovered from 9.7% of the *X. compactus* sampled. The absence of *Ambrosiella* from some beetle samples may be a methodological artifact or due to interactions between the mycangium and fungus. Mycangia are thought to provide important nutrients for growth and storage of fungi during transport to new substrates (Francke-Grosmann 1952). We observed that mycangia of beetles taken from galleries always appeared empty, while those caught in flight were typically filled with fungus. Fungi inhabiting the mycangium may need time to grow sufficiently, or rely on cues from the beetle before inoculation and growth in a new gallery (Kinuura 1995). The beetles that did not yield *Ambrosiella* may have been captured before the symbiotic fungus had sufficiently colonized the mycangium. The high variability in past studies in reporting *Ambrosiella* from *X. compactus* may be due to inconsistent methodology, or due to development-induced variation in symbiont abundance.

A yeast in the genus *Cryptococcus* was also recovered from more than half of the beetles as well as from a gallery. An *Acremonium* species was the next most prevalent fungus isolated from beetles, but its significance is unknown. A closely related species, *Paracremonium pembeum*, was recently found to be a frequent opportunistic associate of some *Euwallacea* ambrosia beetles (Lynch et al. 2016). Laboratory rearing experiments are needed to assess what role, if any, the less frequent fungi play in the life history of *X. compactus*.

Our study revealed that isolation from different *X. compactus* body parts yielded symbiont communities with different dominant members. However, the dominant fungi from all body parts were also found in beetle galleries. This finding suggests that mechanisms may exist that restrict these symbionts to different parts of the beetle body, or that they are adapted for transport on beetles differently. For example, the abundant *Fusarium* spores may easily adhere to the beetle surface and/or survive passage through the gut, while *Ambrosiella* appears to be selected for or is competitive within the mycangium. Secretions in the mycangium may provide an advantage to specialized coevolved symbionts (i.e., *Ambrosiella*).

A more detailed explanation of this multimember fungus community may be revealed after studies are performed considering anatomical and ontological complexity of other ambrosia symbioses. Several other *Xylosandrus* spp. reportedly yield diverse fungi, including *Ceratocystis*, several *Fusarium* spp. (Kessler 1974, Anderson and Hoffard 1978, Weber and McPherson 1984, Ploetz et al. 2013), *Pestalotiopsis* (Pennacchio et al. 2012), and various yeasts (Saccharomycetales) (Kinuura 1995, Dute et al. 2002) just to name a few. The relevance of these reports needs to be tested more rigorously.

Similar symbiont partitioning is likely to occur not just in ambrosia beetles, but many other insects, animals, and plants. A noteworthy example is the mountain pine beetle, *Dendroctonus ponderosae*, which carries multiple associates with varying affinities to the beetle surface and mycangium (Six 2003). These results are important to consider, as bark and ambrosia beetles are increasingly transported across the globe, and may carry pathogens that could be overlooked depending on sampling methodology.

Based on these results, we suggest that the traditional approach of processing whole beetles is not appropriate for capturing the fine-scale community structure of the symbionts. This and many such beetle–fungal symbioses are ecologically and economically destructive, and need to be studied carefully to elucidate theoretical and practical aspects of their biology. Future studies that focus on the rapid emergence of fungal pathogenicity and virulence in formerly harmless ambrosia beetles are needed to devise effective methods for the detection, control, and eradication of these exotic pests.

Acknowledgments

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