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HOST MICROBE INTERACTIONS

Detecting Symbioses in Complex Communities: the Fungal Symbionts of Bark and Ambrosia Beetles Within Asian Pines

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Abstract

Separating symbioses from incidental associations is a major obstacle in symbiosis research. In this survey of fungi associated with Asian bark and ambrosia beetles, we used quantitative culture and DNA barcode identification to characterize fungal communities associated with co-infesting beetle species in pines (*Pinus*) of China and Vietnam. To quantitatively discern likely symbioses from coincidental associations, we used multivariate analysis and multilevel pattern analysis (a type of indicator species analysis). Nearly half of the variation in fungal community composition in beetle galleries and on beetle bodies was explained by beetle species. We inferred a spectrum of ecological strategies among beetle-associated fungi: from generalist multispecies associates to highly specialized single-host symbionts that were consistently dominant within the mycangia of their hosts. Statistically significant fungal associates of ambrosia beetles were typically only found with one beetle species. In contrast, bark beetle-associated fungi were often associated with multiple beetle species. Ambrosia beetles and their galleries were frequently colonized by low-prevalence ambrosia fungi, suggesting that facultative ambrosial associations are commonplace, and ecological mechanisms such as specialization and competition may be important in these dynamic associations. The approach used here could effectively delimit symbiotic interactions in any system where symbioses are obscured by frequent incidental associations. It has multiple advantages including (1) powerful statistical tests for non-random associations among potential symbionts, (2) simultaneous evaluation of multiple co-occurring host and symbiont associations, and (3) identifying symbionts that are significantly associated with multiple host species.

Keywords Bark beetles · Microbiome · Mutualism · Ophiostomatales · Platypodinae · Scolytinae

Introduction

Identifying symbioses under conditions of natural complexity is difficult. Simply defining the term "symbiosis" has been a contentious topic since its inception, though the current trend—and our preference—is for a broad definition such as De Bary's original usage, which includes interactions with positive as well negative outcomes [1–3]. Regardless of the precise definition used, the first condition necessary for symbiosis is that the organisms occur in proximity in nature. Surveys of microbial symbionts often verify this first condition alone. Here, we go one step further and employ empirical tests for significant statistical relationships among hosts and potential symbionts. We posit that species that are co-adapted and/or co-dependent upon one another will be found together more often than expected by chance, given their respective frequencies among samples. The null hypothesis of chance associations for observed associations can be tested with

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freely available permutation-based statistical software and quantitative survey data. We demonstrate the value of this approach in a survey of the fungi associated with co-infesting bark and ambrosia beetles of recently dead pines (*Pinus*) in Asian forests, though the same approach could be applied to any system where microbial symbioses are diverse, variable in the breadth of species involved, and hidden among frequent incidental associations.

Bark and ambrosia beetles, which comprise all ~ 7400 species of the Curculionidae subfamilies Scolytinae and Platypodinae [4], have associations with fungi that range from incidental commensalism, to parasitism, to co-evolved obligate nutritional mutualisms [4–7]. Like many symbioses, interactions between beetles and fungi may change with ecological context [8–13]. The term "ambrosia beetle" refers to scolytine and platypodine beetles that farm gardens of "ambrosia" fungi within the galleries they excavate through the nutrient-poor xylem or pith of their plant hosts [14]. Ambrosia beetles do not eat wood, but instead eat fungi either exclusively or nearly exclusively. Reciprocally, ambrosia fungi depend on beetles for dispersal and colonization of new plant tissues [15–17]. The fungal mutualists are transported to new gardens inside pouch- or pit-like organs, termed mycangia, which support growing ambrosia fungi [17]. The ambrosia habit has evolved independently in at least 12 lineages of scolytine and platypodine beetles [18, 19] and in at least 9 lineages of fungi [20–24], and those estimates are likely to increase as more taxa are studied [4]. Many ambrosia beetle species appear to be obligately associated with one or a few fungal taxa [21, 24], though they are often associated with additional commensals [25, 26], parasites [27], and facultative mutualists [8, 15, 28, 29].

In contrast to ambrosia beetles which bore into xylem, bark beetles consume relatively nutrient-rich phloem and typically do not depend exclusively on fungi for nutrition [5]. However, some bark beetle species benefit from mutualistic nutritional fungi as a key component of their diet [30–33], while other associated fungi may be parasites or competitors of bark beetles and their mutualists [34]. Thus, the relationships between bark beetles and fungi are often more variable and dependent on context. Fungal mutualisms as well as parasitic associations may be more prevalent among bark beetles than we currently know, but they are difficult to identify because of inherent variability and the ubiquitous presence of incidental environmental taxa. Consequently, improved observational methods, replicated sampling, and rigorous tests of appropriate null hypotheses are needed to tease apart these complexes and often obfuscated relationships.

In this study, we apply multivariate analyses and multilevel pattern analysis (MPA) to replicated quantitative culture data to test for statistically significant associations between co-occurring beetle and fungal taxa found in Asian pine forests. In typical applications of MPA to community data, sampling units are grouped by habitat characteristics such as successional stage, management regime, land cover, or experimental treatments, but any grouping scheme can be used to identify species with non-random associations to factors of interest [35, 36]. We used MPA to test for non-random associations among host beetle species and potential fungal symbionts, while simultaneously evaluating multiple associations to characterize the breadth of associations among multiple beetle and fungal taxa.

Methods

Study Sites and Collection Methods

We conducted replicated quantitative culture-based sampling of beetle bodies and galleries to test for significant associations between beetle species and fungi in Asian pine forests in Tam Dao National Park, Hồ Sơn, Vinh Phuc Province, Vietnam, and Fuzhou, Fujian Province; Guiyang and Weining, Guizhou Province; and Nanshan, Shaanxi Province, China. During two collecting trips in March 2016 in Vietnam, we collected bark and ambrosia beetles and their galleries from the trunks of eight dead or moribund pines within 1 km^2 in Tam Dao National Park. Plantations of Chinese red pine (*Pinus massoniana*) formed most of the forest canopy at this site. During collections from August 2015 to December 2015 in Central and Southern China, *Xyleborus pinicola* and *Orthotomicus chaokhao* were collected from Chinese red pine in Gushan Mt., Fuzhou, Fujian Province; *X. pinicola* and *Euwallacea fornicatus* were collected from Chinese red pine in Huaxi, Guiyang, Guizhou Province; *Polygraphus* spp., *Tomicus yunnanensis*, *Tomicus minor*, and *Hylurgops longipillus* were collected from Yunnan pine (*Pinus yunnanensis*) in Weining, Bijie, Guizhou Province; and *Dendroctonus armandi* was collected from Chinese white pine (*Pinus armandii*) in Huoditang, Nanshan, Shaanxi Province.

Bark-dwelling beetles were located by removal of sections of bark. Xylem-inhabiting ambrosia beetles and their galleries were excised from the trees using chisels. Individual beetles and sections of their galleries were placed aseptically into sterile 1.5-ml centrifuge tubes modified with small holes through the cap to permit gaseous exchange. To maintain statistical independence among replicated observations, only one representative beetle and gallery sample were taken from extensive galleries with multiple beetles, and galleries were sampled from multiple trees for each beetle species. Beetles remained in their galleries and were stored alive, in the dark, at ambient temperature until processing, which was no longer than 6 days after collection. We also sampled dispersing beetles using bottle traps (design from [37, 38]). Traps were baited with 95% ethanol and a racemic mixture of alpha- and beta-pinene (Synergy Semiochemicals Corporation, Burnaby, BC, Canada; item no. 3076). Our objective was to broadly sample the diversity of fungi and beetles present in these pine forests. Therefore, our sampling effort was

designed to capture as much diversity as possible from many pine trees and traps baited with pine-emulating lures. Sample sizes varied as a natural consequence of varying abundance/detectability among beetle species at the time of sampling. To avoid conflating similarity in host tree preferences with symbiotic affinities between bark beetle and fungal taxa, only pine-specific beetle species were included in the presented analysis. Consequently, fungi that are prevalent in pine, but not biologically dependent on any beetle species, will be found associated with each beetle species at a rate that is not significantly different from random, given the frequencies of each fungus and beetle in the data. Conversely, fungi that are dependent on certain beetle species will be found in association with those beetles at a rate greater than expected by chance. Traps were checked at 2–6-day intervals. Beetles to be sampled for fungi were stored live in sterile microcentrifuge tubes with a small piece of lightly moistened sterile tissue paper prior to processing.

Fungal Isolation

We used standard sampling techniques for the isolation and quantification of fungal propagules from subcortical beetles (e.g., [26, 39–41]). We used non-selective media because our goal was to capture as much of the natural fungal diversity as possible. A sterile scalpel was used to aseptically excise three small (~ 1–3 mm) woodchips from the interior surfaces of each gallery. Excised chips from each gallery were placed together in 1.5-ml tubes with 500 μl of sterile 1X phosphate-buffered saline (PBS) and vortexed at ~ 1500 rpm for 60 s. Gallery samples were then serially diluted to 1:100 and 1:1000 concentrations and plated on sterile potato dextrose agar (PDA) media, using one plate per dilution factor. Chips were also embedded in PDA. For beetle species that were not known to have a mycangium or with a mycangium that is widely opened to the surface (e.g., *Dinoplatypus flectus*), the fungi from the beetle body were isolated as a "body wash" sample in which entire beetles were vortexed individually in 500 μl of sterile 1X PBS and serially diluted to 1:10 and 1:100 concentrations prior to plating on sterile PDA. For beetles with oral mycangia (i.e., *Xyleborus* and *Euwallacea*), the head was aseptically removed, placed in 500 μl of sterile 1X PBS, crushed within the 1.5-ml tube using a sterile plastic pestle, vortexed, and serially diluted to 1:100 and 1:1000 concentrations prior to plating on PDA. Negative controls were prepared simultaneously using the same methods and material, without adding beetle or gallery material. The 1:10 dilution plates were made by plating 50 μl of initial 500 μl PBS wash. The 1:100 and 1:1000 dilution plates were made by transferring 50 μl of original wash to 450 μl of fresh sterile PBS, vortexing, and plating 50 and 5 μl, respectively.

Plates were incubated for 4–7 days in the dark at 25 °C. One representative dilution plate was chosen from each sample for fungal morphotype assignment and counting. The number of colony-forming units was used as a surrogate for the original fungal abundance, which was estimated via multiplication of the number of colonies per morphotype by the inverse of the dilution. Isolates recovered from embedded woodchips were combined with morphotype counts from corresponding dilution plates. From each representative plate, we selected an isolate for each fungal morphotype. Isolates were then subcultured on a fresh plate and photographed after 5–7 days, and another subculture of each was transferred to a sterile 2-ml PDA mini-slant for transportation to a Biosafety Level 2 quarantine facility at the Division of Plant Industries, Florida Department of Agriculture, Gainesville, FL, USA. Isolates were then re-plated on PDA, incubated for 4–7 days at 25 °C, and compared to photographic and descriptive accounts of the original isolates. Isolates were discarded in cases of mismatches or contamination.

DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from approximately 5 μl of mycelia of 1–2-week-old pure cultures in 20 μl of extraction solution (Extract-N-Amp; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 96 °C for 10 min. Then, 20 μl of 3% BSA was added and the mixture was vortexed at ~ 1500 rpm for 5 s and then centrifuged at 2000 rpm for 30 s. The supernatant (20 μl) was used as a genomic DNA template for PCR. Partial sequences for the 28S large subunit (LSU) ribosomal DNA (rDNA) were obtained from all isolates for fungal identification using the primers LR3 (CCGTGTTTCAAGAC GGG) [42] in the Vietnam study, LR5 (ACCCGCTG AACTTAAGC; http://sites.biology.duke.edu/fungi/mycolab/primers.htm) in the China study, and LROR (ACCCGCTG AACTTAAGC) [42] in both studies. PCR reactions contained the following: 1 μl template DNA, 1 μl forward and reverse primers (10 mM), 0.125 μl of Taq polymerase (Takara Ex Taq), 2.5 μl PCR buffer (15 mM $MgCl_2$), 2 μl dNTPs (2.5 mM each), and 20 μl molecular grade DNA-free water. Thermocycler profile was as follows: 3 min at 94 °C, followed by 34 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s. LROR was used in sequencing reactions. Sanger sequencing was performed by the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. We chose LSU over the more commonly used internal transcribed spacer (ITS) barcode region because amplification of ITS is difficult and inconsistent for many of the ophiostomatoid fungi known to associate with bark and ambrosia beetles.

Taxonomic Assignments

Sequence chromatograms were inspected for quality and manually binned into operational taxonomic units (OTUs) at 99%

similarity using Sequencher 5.4.5. Ophiostomatoid fungi were binned at 100% similarity because these taxa often had high sequence similarity but displayed variation at a few loci that was consistent among replicates within collection sources. Coarse identification of consensus sequences was made to the lowest possible taxonomic rank via GenBank National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). We further classified ophiostomatoid fungi using phylogenetic analysis because these fungi are known to be widely important to bark and ambrosia beetle biology and because this group is not well represented or curated in GenBank. Sequences that were identified within the Ophiostomatales using BLAST were aligned and inspected in Geneious 7.1.9. Additional LSU sequences were included from representative taxa within the Ophiostomatales from a recent phylogenetic study [43]. Bayesian inference analyses were performed using MrBayes 3.2.6 [44] on the University of Florida supercomputer (HiPerGator2.0). The nucleotide substitution model was chosen using the Akaike information criterion from jModelTest 2.1.4 [45, 46]. Ten runs were conducted with 5 M generations and subsampling every 1000th generation. The tree was edited in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Isolate names were assigned based on monophyly and closest relatives. For isolates that were lost to contamination, identification was inferred by sequences of representatives from the same morphotype (< 10% of isolates). Representative sequences for each OTU was uploaded to GenBank (accession numbers MF543573–MF543598 and LC314669–LC314681; Table S1).

Data Analysis

Principal coordinate analysis (PCoA) was used to visualize differences in the composition of the fungal communities associated with each beetle species. PCoA was implemented using the cmdscale() function in the base package of R [47]. The Raup-Crick metric was used for all distance-based analyses. This metric is more robust to variation in alpha diversity among samples than other commonly used incidence metrics such as Jaccard and Bray-Curtis [48]. Distance matrices were generated from fungal OTU estimates for each unique combination of beetle species and sample type using the raupcrick() function in the vegan package of R [49]. Permutation-based multivariate analysis of variance (PERMANOVA) [50] was used to test for main and interactive effects of beetle species and sample type (gallery versus beetle body). PERMANOVA was implemented using the adonis() function in the vegan package of R [49]. Because PERMANOVA is sensitive to variation in multivariate dispersion among groups, we also conducted a test of homogeneity of multivariate dispersion [51] using the betadisper() function in the vegan package of R [49].

Heat maps were used to visualize the frequency and abundance of fungi associated with each beetle species. A sample-by-fungus matrix was aggregated by mean CFUs for each fungal taxon per beetle species and sample type (i.e., gallery versus beetle). We performed hierarchical clustering of all combinations of beetle species and sample types according to Raup-Crick distances using the hclust() function in the stats package of R [47]. This hierarchical clustering was then used to sort the columns of the fungal taxon frequency matrix. Rows were sorted by their weighted average scores using the function wascores() in the vegan package of R [49]. The sorted matrices were then visualized as a heat map using custom R scripts.

We used MPA to identify associations between beetle and fungal taxa that occurred significantly more frequently than expected by chance. Multivariate analyses (e.g., PERMANOVA, ANOSIM, BETADISPER) can be used to test for differences in symbiont community composition, but these tests alone do not identify specific significant associations among taxa. For our purposes, MPA offered advantages over other commonly used analytical frameworks such as ANOVA and G-tests (e.g., [9]) or multiple generalized linear models (e.g., "mvabund") [52] because of less stringent assumptions about underlying data type (i.e., occurrence versus abundance) and distributions, and MPA allows for a simultaneous test of associations between all combinations of taxa with appropriate type I error rates [35]. Previous work has also attempted to decompose animal-associated microbial assemblages into "core" and "satellite" community members based on species abundance distributions [9, 53]. However, this approach requires precise estimates of species abundances and requires difficult-to-test assumptions about underlying species abundance distributions [54].

MPA is an expanded form of indicator species analysis (ISA). Although earlier versions of ISA had several limitations such as strict hierarchical groupings of sampling locations, issues caused by variation in niche breadth among species, and significance tests with inflated type I error [35, 55], MPA offers improvements over traditional ISA methods by testing for significant associations between each species and all possible combinations (or a user-defined subset) of sampling groups and thus accommodating variation in niche breadth among species and using improved permutation-based significance tests with appropriate type I error rates [35]. In short, data for fungal communities cultured from beetle bodies and galleries were grouped according to beetle species. Then, matrix permutations were used to identify fungal species that were associated with each beetle species, as well as with all combinations of beetle species, at a frequency greater than predicted by chance, given the frequency of each species in the data [36, 56].

The Vietnam and China datasets were analyzed separately to avoid the confounding influence of sampling region on the degree of association between the respective beetles and fungi

sampled. For instance, if the data were combined prior to analysis, a ubiquitous environmental species in one region might be found to have a statistically significant association with all beetle species sampled in that region. However, such an association would not reflect any degree of ecological relevance for beetles and fungi other than geographic coincidence. We conducted MPA using the multilevel pattern function in the indicspecies package of R (multipatt() function) [57]. Because our sampling effort varied across beetle species, we used group-equalized indicator value indices (IndVal.g) [35, 58] and blocked samples by type (gallery versus beetle body) during permutation tests for significance to avoid conflating the effects of sample type (i.e., beetle bodies versus galleries) with the effects of beetle species [59].

Results

In both the Vietnamese and Chinese datasets, beetle species was a highly significant predictor of fungal species composition on beetle bodies and within beetle galleries. Despite considerable overlap in fungal taxa recovered among beetle species, beetle species explained more than 40% of the variation in fungal community composition among samples in both studies (Table 1). Importantly, quantitative sampling methods and MPA allowed us to test for statistically significant associations among multiple beetle and fungal taxa and identified significant associations even when few beetles could be examined (Table 2). Bark beetles tended to harbor communities composed of several fungi in the genera *Ophiostoma*, *Sporothrix*, and *Leptographium*, whereas ambrosia beetles were frequently associated with species in the genus *Raffaelea* sensu lato. The ambrosia beetle *Xyleborus pinicola* and the bark beetle *Orthotomicus chaokhao* were sampled in both countries. *Xyleborus pinicola* was significantly associated only with *Raffaelea* c.f. *arxii* sp. 1 in both countries and with similar indicator values (IndVal.g = 0.78 in Vietnam and IndVal.g = 0.75 in China). Conversely, the bark beetle *O. chaokhao* was significantly associated with two fungi in Vietnam (*Ophiostoma ips* and *Sporothrix* sp. 1) but showed no significant associations in China. Significant associations between ambrosia beetles and ambrosia fungi tended to be exclusive. However, one species of ambrosia fungi, *Raffaelea* c.f. *fusca* sp. 1, was found associated with both *X. pinicola* and *D. flectus*. This taxon was found in 5 of 17 (29%) *X. pinicola* mycangia in Vietnam, though its association with *X. pinicola* was not statistically significant. *Raffaelea* c.f. *arxii* sp. 1 was numerically dominant in three of the four cases where both were present.

Vietnam

We recovered 116 fungal isolates representing 26 fungal OTUs from 67 beetle and gallery samples. There were significant differences in fungal community composition among beetle taxa (Fig. 1, Table 1). Although there was a significant effect of sample type (gallery versus beetle body) and a significant interaction between sample type and beetle taxa, those effects were small in comparison to the main effect of beetle species, which accounted for 41% of the variation among samples. A test for homogeneity of multivariate dispersion did not detect significant differences in multivariate dispersion among beetle species ($F_{5,65} = 0.89$, $p = 0.487$), indicating that the significant effects found by PERMANOVA reflected differences in fungal community composition among beetle species and not differences in variability among samples (i.e., multivariate dispersion) within beetle species.

Of the 39 observed unique associations between beetles and fungal OTUs, we identified 9 (23%) associations as significantly non-random (Table 2). Each of the beetle species sampled in Vietnam was significantly associated with at least one fungal OTU. The majority of fungi isolated from galleries were also isolated from beetle body samples when both were sampled (see the dendrogram in Fig. 2). A notable exception was *R.* c.f. *arxii* sp. 1. This OTU was abundant in 15 of 17 mycangium samples from *X. pinicola* (prevalence = 0.88, mean abundance = 2559 CFUs), but not detected in their galleries (0 of 8 samples).

China

We recovered 22 fungal OTUs from 37 beetle and gallery samples taken from pines in China. Similar to observations from Vietnam, beetle species explained the largest fraction of variation in community composition among samples (49%), in addition to weaker, but significant, main and interactive effects of sample type (Table 1). There was no significant difference in multivariate dispersion among beetle species ($F_{7,29} = 1.85$, $p = 0.11$), indicating that significant effects found by PERMANOVA reflect differences in species composition among beetle species. As in Vietnam, *R.* c.f. *arxii* sp. 1 was consistently prevalent in mycangium samples from Chinese *X. pinicola* (prevalence = 0.833, mean abundance = 7133 CFUs), but not detected in their galleries (zero of seven samples). Of 28 unique associations observed between beetle and fungal taxa in China, we identified six (21%) as significant (Fig. 3). Of the eight beetle taxa sampled in China, four were found to have significant associations with at least one fungal taxon (Table 2).

Discussion

Our understanding of mutualisms has been evolving from an earlier assumption of rigid pairwise interactions to a paradigm of flexible and more complex interaction networks often composed of generalists and specialist taxa. For instance, *Sirex*

Table 1 Results of permutation-based multivariate analysis of variance tests (PERMANOVA) for beetle-associated fungal communities observed in Vietnam and China, each based on 10,000 permutations of the Raup-Crick distance matrix

Study	Model terms	*df*	Mean squares	Pseudo *F*	R^2	*p* value
Vietnam	Beetle species	5	2.151	9.815	*0.410*	0.001**
	Sample type	1	1.240	5.660	0.047	0.001**
	Species × type	3	0.580	2.647	0.066	0.002**
	Residuals	57	0.219			
	Total	66				
China	Beetle species	7	1.101	4.180	*0.491*	0.001**
	Sample type	1	0.688	2.799	0.044	0.014*
	Species × type	2	0.398	1.634	0.052	0.034*
	Residuals	26	0.269			
	Total	36				

We tested for main and interactive effects of beetle species and sample type (gallery versus beetle body) on fungal community composition. R^2 values in italics highlight the largest fraction of explained variation among samples in both studies

*$\alpha = 0.05$, significant; **$\alpha = 0.01$, significant

wood wasps were thought to have strictly species-specific nutritional symbioses with *Amylosterium* fungi, until recent invasions of European *Sirex* led to rampant symbiont swapping with North American wasps [60, 61]. Likewise, experimental studies of the camouflage symbiosis between sepiolid squid and strains of bioluminescent *Vibrio* bacteria have revealed hierarchies of competitive interactions among symbiotic strains within the light organ [62–64]. A similar trend is unfolding in bark and ambrosia beetle symbiosis research. Improved methods, taxonomy, and mounting observations and experiments have revealed that these symbioses are often diffuse interactions involving multiple fungi and beetle species [4, 9, 15, 24]. Beetles can thrive while consuming alternative fungal food sources [15, 29], and comparative phylogenetic analyses suggest frequent symbiont swapping over evolutionary timescales [65]. The results of our surveys in pines in China and Vietnam mirror this emerging perspective of ambrosia symbioses as often variable at the level of species associations, while generally conserved at broader phylogenetic levels. These findings depict beetle-associated fungi not as simple crops that are strictly managed by the beetle farmers but as dynamic community members with context-dependent competitive abilities and a variety of life history strategies for garnering beetle-facilitated dispersal.

Ambrosia Beetles and Fungi

An inference of co-evolved ambrosial mutualisms is typically considered justified if two conditions are met: high prevalence and abundance of viable spores from a fungal species within the mycangium of a single beetle species, and consistency across that beetle's geographic range [9, 23, 24, 26, 41]. We

Table 2 Results of multilevel pattern analysis (MPA), a type of indicator species analyses, of fungal associations observed in pine forests of Vietnam and China

Study	Beetle taxa	Fungal taxa	Freq. in galleries (%)	Freq. on beetles (%)	IndVal.g	*p* value
Vietnam	*Cyrtogenius luteus*	Ophiostomatales sp.	12.5	30.8	0.58	0.007
	Crypturgus sp.	*Nectriaceae* sp. 1	n.a.	40.0	0.63	0.014
	Dinoplatypus flectus	*Raffaelea* c.f. *fusca* sp. 2	11.1	28.6	0.50	0.024
	Euwallacea c.f. *andamanensis*	*Sporothrix* sp. 2	80	50.0	0.92	0.001
	Xyleborus pinicola	*Raffaelea* c.f. *arxii* sp. 1	0	87.5	0.78	0.014
	Cyrtogenius luteus + *Orthotomicus sp.*	*Ophiostoma ips*	25.0	50.0	0.72	0.004
	Cyrtogenius luteus + *Orthotomicus* sp.	*Sporothrix* sp. 1	37.5	25.0	0.62	0.021
China	*Dendroctonus armandi*	*Ophiostoma* sp. 1	33.3	60.0	1.0	0.001
	Tomicus yunnanensis	*Sporothrix* sp. 3	n.a.	80.0	0.89	0.005
	Xyleborus pinicola	*Raffaelea* c.f. *arxii* sp. 1	0	83.3	0.75	0.015
	Hylurgops longipillus + *Tomicus minor*	*Ophiostoma* sp. 3	100	75	0.82	0.003

Only statistically significant associations are shown. Degree of association was determined by the group-equalized indicator statistic (IndVal.g). Significance of each association was assessed by the permutation test with 10,000 matrix permutations for each putative association ($\alpha = 0.05$)

n.a. not available

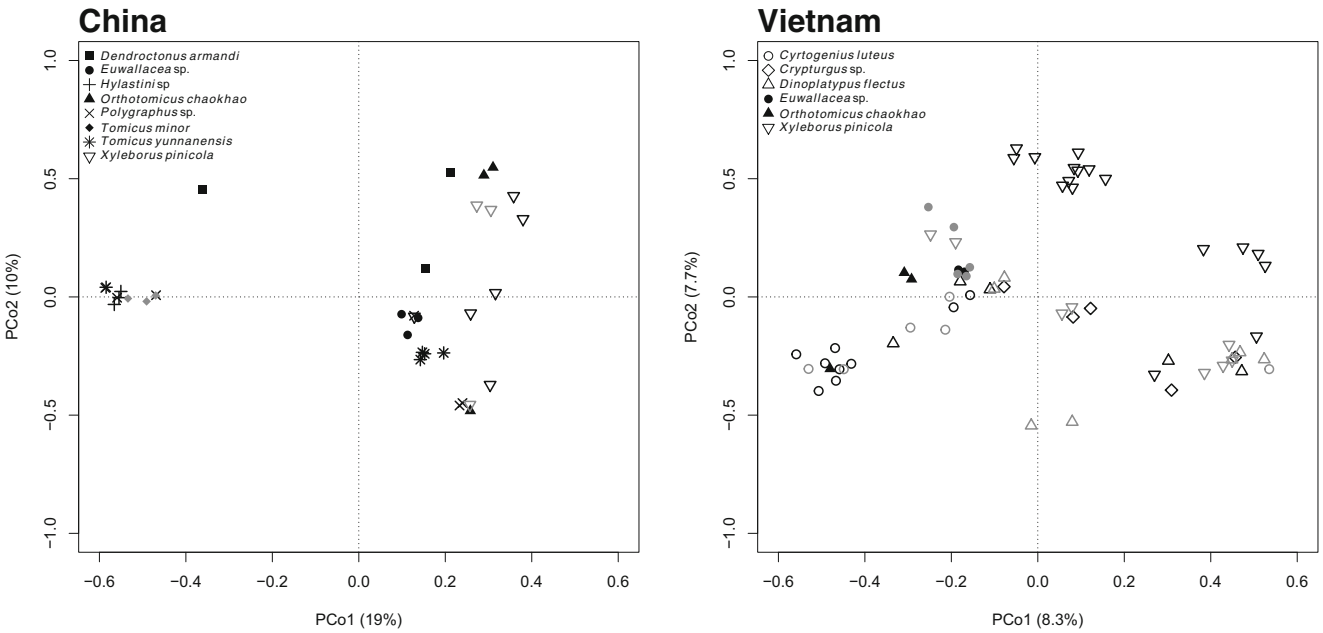

Fig. 1 Principal coordinate analyses (PCoA) showing the effect of beetle species on fungal community composition observed from beetle bodies (black symbols) and beetle galleries (gray symbols) in a pine forest in China and Vietnam. Overlapping points are slightly jittered for clarity. Although beetle species had a highly significant effect on fungal community composition, sample clouds for each species overlapped considerably, emphasizing the need for replicated sampling to accurately characterize fungal taxa specifically associated with each beetle taxon. Ordination was performed on a Raup-Crick distance matrix of CFU estimates

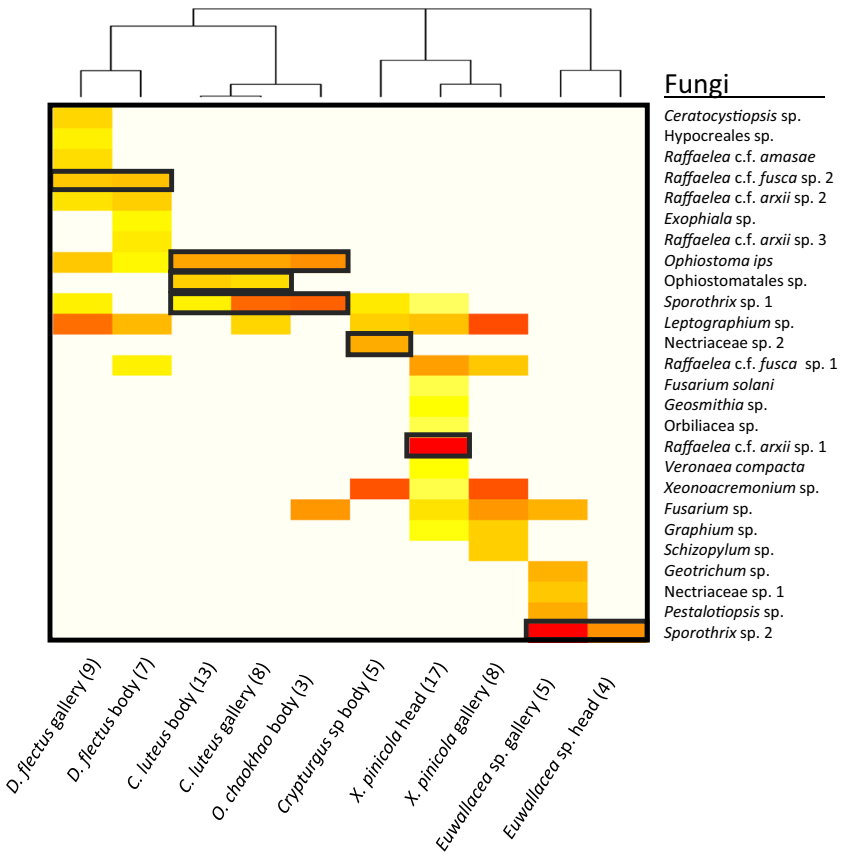

Fig. 2 Heat map illustrating associations between fungi and beetle taxa observed in Vietnam. Columns represent all combinations of beetle species and sample types that were collected. Numbers in parentheses indicate sample sizes. Dendrogram at the top shows results of hierarchical clustering of fungal communities associated with each beetle species/sample type combination. Rows represent fungal taxa and are arranged by weighted average scores obtained from hierarchical clustering. Cell color represents the mean log-transformed colony-forming units; darker colors are more abundant. Black outlines indicate statistically significant associations identified by indicator species analysis

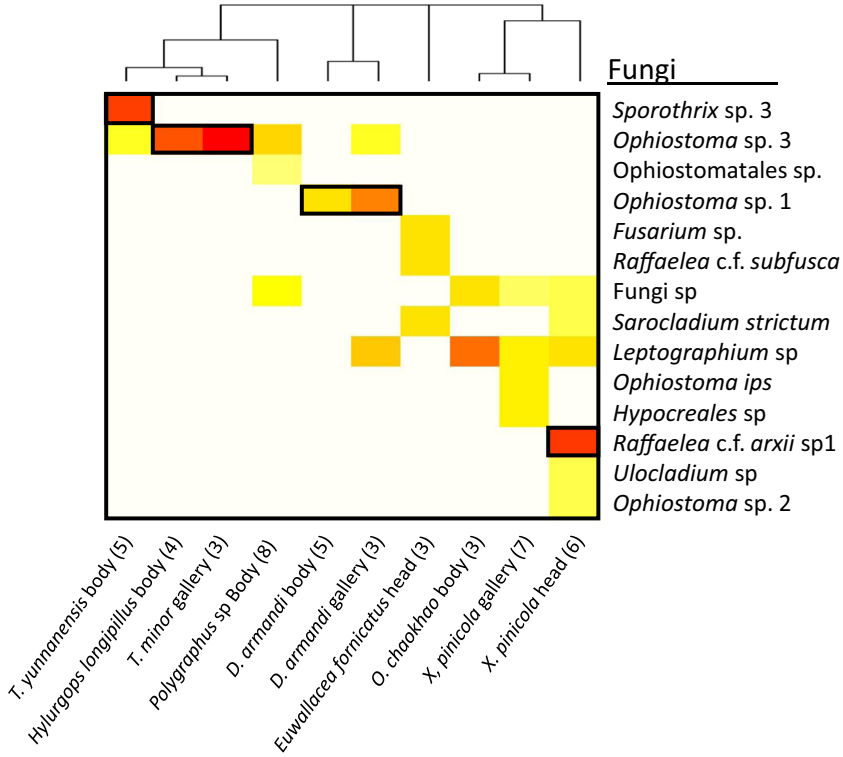

Fig. 3 Heat map illustrating associations between fungi and beetle taxa observed in China. Columns represent all combinations of beetle species and sample types. Numbers in parentheses indicate sample sizes. Dendrogram at the top shows results of hierarchical clustering of fungal communities associated with each beetle species/sample type combination. Rows represent fungal taxa and are arranged by weighted average scores obtained from hierarchical clustering. Cell color represents the frequency of each association; darker colors are more frequent. Black outlines indicate statistically significant associations according to indicator species analysis. "Fungi sp." is a distinct, albeit unidentified strain, not an aggregate group

recovered high CFU estimates of *R.* c.f. *arxii* sp. 1 from the mycangia of *X. pinicola* in both China and Vietnam, and this fungus was not observed in association with any other beetle species. Consequently, *R.* c.f. *arxii* sp. 1 is inferred to be a specialized, prevalent, and dominant mycangial symbiont of *X. pinicola*. Surprisingly, *R.* c.f. *arxii* sp. 1 was not recovered from the galleries of *X. pinicola* in either China or Vietnam. Four related *Raffaelea* species were recovered from galleries of *X. pinicola* and *D. flectus*; thus, it is not likely that the absence of this fungus in gallery samples is due to our methods. Instead, it is possible that *R.* c. f. *arxii* was absent in a particular period of the natural community succession that takes place in ambrosial gardens, during which mycangial symbionts are overrun by secondary colonists [29, 66]. It is also possible that the most abundant and prevalent mycangial symbiont is only one member of a community of fungi introduced and consumed by the beetle, and while it is a competitive dominant in the mycangium, it is less competitive in wood. If this scenario is true, such fungi that are competitive in the mycangium but unimportant to beetle nutrition in the gallery could represent parasitic cheaters.

Maintaining a diversity of fungal associations could buffer ambrosia beetle populations from changes in local conditions or available host plants. While some ambrosial symbionts show high prevalence throughout the ranges of their beetle associates, many more occur at lower frequencies that vary across the geographic range of beetle species. For instance, many species of *Raffaelea* and related fungi are associated with the redbay ambrosia beetle (*Xyleborus glabratus*) in North America, often co-occurring within the mycangium [39, 40] and showing variable prevalence across broad geographic spatial scales [8]. We found *R.* c.f. *fusca* sp. 1 in 29% of *X. pinicola* mycangia in Vietnam, where it was the only ambrosia fungus recovered from two individuals and co-occurred with *R. arxii* sp. 1 in the mycangia of four individuals. *R.* c.f. *fusca* sp. 1 was not recovered from *X. pinicola* in China, suggesting that this fungus is a geographically localized associate of *X. pinicola*. This species may represent a facultative local mutualist, an innocuous commensal, or a fungal antagonist. Similarly, *Dinoplatypus flectus* was found associated with four *Raffaelea* species (three *Raffaelea* spp. s. s. and *Raffaelea amasae* from the "*Estaya*/*Dryadomyces* group") [22], though none of these fungi were highly prevalent across individuals. The only statistically significant associate of this beetle was *R.* c.f. *fusca* sp. 2, which was only recovered from 33% of *D. flectus* body samples and 11% of gallery samples, and not found with any other beetle species. These observations suggest that *D. flectus* and, possibly,

X. pinicola do not depend on a single nutritional symbiont but instead have multiple symbionts. Some evidence suggests that interactions with multiple functionally diverse fungal lineages minimize the risk of *Dendroctonus* beetles losing the benefits of symbiotic fungal partners over a range of variable environmental conditions [67]. However, the ecological and evolutionary consequences of having multiple fungal symbionts remain critically understudied and poorly understood.

One unexpected association was the significant association between *Euwallacea* c.f. *andamanensis* and the ophiostomatoid fungus *Sporothrix* sp. 2, recovered from multiple beetles and galleries, from multiple trees in Vietnam. *Euwallacea* farm many *Fusarium* lineages that together form the monophyletic "ambrosial *Fusarium* clade" (AFC) within the *Fusarium solani* species complex, sometimes co-occurring with *Raffaelea* species [21]. Fungus-feeding bark and ambrosia beetle species that specialize on coniferous hosts are frequently associated with ophiostomatoid fungi [5, 6], suggesting that these fungi are particularly strong competitors within conifers [5]. Perhaps *Euwallacea* form facultative fungal associations that are contingent on the host tree, favoring ophiostomatoid fungi when colonizing coniferous hosts. Recent comparative phylogenetic work suggests that *Euwallacea* species have often switched nutritional symbionts within the AFC [65], and other studies have found the ophiostomatoid genus *Raffaelea* in association with *Euwallacea*. Thus, a switch to symbionts outside of the AFC seems at least plausible.

Partner fidelity, or one-for-one species matching of mutualists (often referred to as "specificity"), is a common theme in studies of ambrosia beetles and their fungi (e.g., [4, 24, 68]). Studies including this one show that fidelity between ambrosia beetles and fungi is asymmetrical, but interestingly, different ambrosial systems appear to display different directions in asymmetry. In this study, ambrosia beetles were often found with multiple species of *Raffaelea*, while each ambrosia fungus was typically only found associated with one beetle species. Four of the five *Raffaelea* OTUs recovered in the Vietnam study and the single *Raffaelea* OTU recovered from the China study were found associated with only one beetle species, despite the mere centimeters that separated the galleries of different co-infesting ambrosia beetles. Similarly, the only significant associate of *Euwallacea* in Vietnam was not recovered from any other beetle. While it is possible that more extensive sampling could uncover shared ambrosia fungi among beetle species, our results indicate that shared ambrosia fungi are not prevalent in this system. In contrast to this study, fungi in other ambrosia systems are known to utilize many beetle species while the beetles are typically restrained to one or a few fungal species. Examples include members of the AFC [21] and the extreme *Flavodon ambrosius* (Basidiomycota) which colonizes many species of beetles, each of which solely relies on this one fungus [69]. Resolving the ecological causes and evolutionary consequences of these differing asymmetries among ambrosia symbioses remains an unexplored and likely fruitful avenue for future research.

Bark Beetles and Fungi

Fidelity among phloem-feeding bark beetles and associated fungi is even less settled [70]. Our results suggest that bark beetle-associated fungi are less restricted in their associations than ambrosia beetle associates occurring in the same trees. While all significant associates of ambrosia fungi were recovered only from one beetle species, several fungal associates of bark beetles were typically found with multiple beetles, and three of seven significant bark beetle associates were significantly associated with multiple beetle species. None of the bark beetles sampled in this study are known to possess a mycangium, suggesting that transmission via external body surfaces or within the gut is less selective. Another possible explanation for greater promiscuity among bark beetle-associated fungi is simply the physical qualities of phloem, which, unlike xylem, is (1) restricted to a thin plane, (2) more accessible to diverse taxa, and (3) relatively high in nutrient content. These qualities encourage higher beetle densities within the phloem and intermingling of galleries belonging to different species which could lead to commonplace fungal exchanges among bark beetles.

Methodological Considerations

Objectively weighing the evidence for putative symbiotic associations is increasingly essential in the inference of symbiotic relationships, especially as powerful and cost-effective new techniques such as high-throughput metabarcoded amplicon sequencing, metagenomics, and metatranscriptomics, provide researchers with richer but potentially noisier datasets [9, 12, 13, 71]. Several factors are likely to introduce noise into microbial symbiont community data including (1) symbiotic taxa that are not always present or detected, (2) ubiquitous environmental taxa that are introduced by stochastic mechanisms (e.g., wind-blown spores, incidental phoresy), and (3) non-indigenous symbionts that may invade from galleries of co-infesting beetle species. Our results show that replicated sampling and permutation-based statistical analyses can rapidly and objectively confirm expected associations and identify unexpected associations between hosts and microbial symbionts amidst a cacophony of incidental interlopers.

Identification of biologically significant associations among many incidental associations can be obscured by natural variation among fungal taxa in propagule production, amenability to culture on media, and competitive ability under

culture conditions [5, 39]. This variability makes assessments of significance based solely on numerical dominance among taxa within mixed samples tenuous. However, carefully selected analytical frameworks can mediate biases associated with variation among taxa. Rather than the relative abundances of multiple taxa within samples, our analysis using MPA emphasized the abundance and frequency of each taxon across replicates within groups of samples, independent of other fungal taxa. Because the statistical significance of each association is being assessed independent of other fungal taxa, the effects of relatively low propagule abundance and/or poor cultural characteristics on the determination of an association's statistical significance are mitigated.

Applying MPA to quantitative culture-based data provided reasonable statistical power even with moderate sample sizes. We were able to identify statistically significant associations with as few as three replicates in some instances, though more replicates would certainly strengthen these inferences. Second, most of the significant associations identified in this study are corroborated by previous records for related beetle species. For instance, we found significant associations of *X. pinicola* and the platypodine *D. flectus* with two *Raffaelea* species. *Raffaelea* species have previously been found associated with other *Xyleborus* species [8, 22, 72], as well as platypodines [73, 74]. Lastly, we consistently found the same species, *Raffaelea* c.f. *arxii* sp. 1, as significantly associated with *X. pinicola* in Vietnam and China, recovering nearly identical indicator values in both studies. Thus, even modestly replicated quantitative sampling, when combined with appropriate statistical tests, can consistently reveal multiple associations of likely biological significance within complex communities of microbes and insects.

There are important limitations to survey-based symbiosis studies such as the one reported here. Surveys alone cannot demonstrate biological outcomes such as the effects (positive or negative) of taxa on one another or if co-evolutionary histories exist between hosts and symbionts. Unknown exogenous factors such as shared microhabitat, host plant preferences, or synchronous successional changes in beetle and fungal communities within dead trees could lead to statistically significant associations in the absence of truly symbiotic interactions. Thus, experimentation and phylogenetic reconstructions are required to demonstrate what type of ecological interactions occurs and what has influenced their evolutionary trajectories. Furthermore, relationships between animals and microbial symbionts may change rapidly with context such as season, tree species, population phase, life history stage, presence/absence of other fungi or animal associates [10, 29, 75–77], and even the sampling method [26]. Consequently, once a putative association is discovered, extensive targeted sampling of specific host and symbiont species, followed by careful analyses, environmental factors may reveal or explain additional and important layers of complexity.

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