Microb Ecol (2012) 64:784–793
DOI 10.1007/s00248-012-0055-5

INVERTEBRATE MICROBIOLOGY

Mycangia of Ambrosia Beetles Host Communities of Bacteria

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Received: 4 February 2012 / Accepted: 5 April 2012 / Published online: 1 May 2012

Abstract The research field of animal and plant symbioses is advancing from studying interactions between two species to whole communities of associates. High-throughput sequencing of microbial communities supports multiplexed sampling for statistically robust tests of hypotheses about symbiotic associations. We focus on ambrosia beetles, the increasingly damaging insects primarily associated with fungal symbionts, which have also been reported to support bacteria. To analyze the diversity, composition, and specificity of the beetles' prokaryotic associates, we combine global sampling, insect anatomy, 454 sequencing of bacterial rDNA, and multivariate statistics to analyze prokaryotic communities in ambrosia beetle mycangia, organs mostly known for transporting symbiotic fungi. We analyze six beetle species that represent three types of mycangia and include several globally distributed species, some with major economic importance (*Dendroctonus frontalis*, *Xyleborus affinis*, *Xyleborus bispinatus–ferrugineus*, *Xyleborus glabratus*, *Xylosandrus crassiusculus*, and *Xylosandrus germanus*). Ninety-six beetle mycangia yielded 1,546 bacterial phylotypes. Several phylotypes appear to form the core microbiome of the mycangium. Three *Mycoplasma* (originally thought restricted to vertebrates), two Burkholderiales, and two Pseudomonadales are repeatedly present worldwide in multiple beetle species. However, no bacterial phylotypes were universally present, suggesting that ambrosia beetles are not obligately dependent on bacterial symbionts. The composition of bacterial communities is structured by the host beetle species more than by the locality of origin, which suggests that more bacteria are vertically transmitted than acquired from the environment. The invasive *X. glabratus* and the globally distributed *X. crassiusculus* have unique sets of bacteria, different from species native to North America. We conclude that the mycangium hosts in multiple vertically transmitted bacteria such as *Mycoplasma*, most of which are likely facultative commensals or parasites.

Electronic supplementary material The online version of this article (doi:10.1007/s00248-012-0055-5) contains supplementary material, which is available to authorized users.

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Introduction

The study of symbioses, whether between humans and their microbiome or insects and their symbionts, has begun to see a shift from research focusing on pairs of species toward the study of symbiotic communities. This is in part because new methods of environmental DNA sampling are revealing many previously unnoticed bacterial and fungal associates. In some symbiotic communities, both coevolved symbionts and incidental associates may play important but different

roles. One such example is the ambrosia symbiosis between wood-boring beetles and diverse fungal and microbial associates. The beetles are often cited as a textbook example of tree disease vectors [1], yet in some cases, the disease agents may be incidental opportunists, rather than the primary symbionts of the beetles [2, 3]. Such complexity of interactions highlights the need to survey the full diversity of microbial associates of ambrosia beetles. Historically, studies of the ambrosia symbiosis have focused on insect interactions with the main fungal symbiont, and few addressed the role of other fungi, and even fewer studies exist on the presence and role of bacteria in this system. Here, we expand the focus of ambrosia symbiosis research to address the prokaryotic component. We combine the approaches of culture-independent microbiology, insect anatomy, and multivariate statistics to analyze bacterial communities inside one of the most frequently evolved organs mediating a symbiosis—the mycangia of ambrosia beetles.

The ambrosia symbiosis of beetles and fungi is one of the most evolutionarily successful and ecologically important insect mutualisms [4], with increasing global economic impact [3]. The symbiosis evolved in at least 11 independent bark beetle ancestors (Curculionidae, Scolytinae, and Platypodinae) and involves over 3,500 beetle species and an unknown but large number of symbiotic wood-decay fungi [4–6, 42, 43]. The fungi are carried in specialized membranous invaginations equipped with secretory glands—mycangia [7, 8]. As most of the research on ambrosia symbiosis focused on mycangial fungi, little is known about bacteria in mycangia. Even the question of whether bacteria are present in beetle mycangia is poorly resolved, and whether the bacterial community is shaped by the type of mycangium, beetle evolutionary origin, or geographic origin has never been tested.

Presence of bacteria in mycangia has been suggested by previous studies [12, 16, 18], but not explicitly tested, as bacterial associates of beetles are typically studied from crushed whole beetles or from external beetle surfaces, and the isolated bacteria might not have been inside mycangia. In this work, we targeted bacteria specifically within beetle mycangia. This often large organ [9] is the functional pivot of the ambrosia symbiosis [5]. Our current understanding is that mycangia are the products of millions of years of mutualistic coevolution between the beetle and fungal symbionts. The mycangium provides an environment in which exposure to UV light, abrasion, and tree defensive chemicals are reduced, and where symbionts are provided nutrients [10]. At the same time, it also excludes most fungi while supporting the few coevolved symbionts [11]. This selectiveness may hypothetically also affect bacteria. Bacteria are abundant and diverse on the body surface and within galleries of ambrosia and bark beetles [12–16], but whether they are also present within the mycangium, or if the mycangium is kept bacteria-free, has been only marginally explored [12]. It has been noted that bacteria are present in the ambrosia beetle niche [17, 18], but their abundance, diversity, composition, and transmission in mycangia have not been analyzed.

We used a high-throughput, culture-independent approach to search for and, if present, identify prokaryotes directly from the mycangia of five species of fungus-farming beetles. In doing so, we addressed four questions:

1. Do ambrosia beetle mycangia, organs originally evolved to host fungi, also contain a significant diversity of bacteria?
2. Is there a "core microbiome" associated with ambrosia beetle mycangia? One hypothesis is that mycangia of all ambrosia beetles, regardless of species, may be dominated by a suite of microbial members shared among samples, the "taxonomic core microbiome" [19]. Alternatively, associations may vary among different beetle species or individuals.
3. If bacteria are present in mycangia and their communities vary among beetles, to what extent is the community composition determined by the geographic origin of the beetle, and to what extent is it the host beetle species? If bacterial communities are more specific to localities than to beetle species, they are likely facultative opportunists from the environment. On the other hand, if bacteria in mycangia display specificity to beetle species regardless of locality, they are potentially symbionts transmitted vertically across beetle generations.
4. If bacterial communities are specific to beetle species, are they also more similar between closely related beetles then between unrelated beetles? Congruence between bacterial community similarity and beetle phylogeny is expected if bacteria are exclusively transmitted vertically within beetle species and families.

By sampling multiple species from nine localities from around the world (not all species were collected everywhere), we are not only assembling the broadest sample of ambrosia beetle symbionts ever collected but also creating a baseline dataset for further studies on global host–symbiont dynamics. This is important for highly invasive groups such as the ambrosia beetles [3]. Representatives of this symbiotic complex are usually not economically important in their native regions but can be devastating in invaded regions [3, 20]. It has not yet been tested whether the increased impact is caused by the change of beetle behavior in the invaded regions, or by acquisition of new symbionts (whether fungal or bacterial). By analyzing several species across their global distribution, we are implicitly testing a key assumption of the latter hypothesis: Is there evidence for acquisitions of new symbionts in newly colonized regions?

Methods

To assure sufficient statistical power of our tests, our sampling included six species of fungus-farming beetles, each represented by eight to 28 different individuals, from nine localities around the world (for details, see Table 1). To include phylogenetically diverse beetle representatives, we included species from three clades of ambrosia beetles, each with its own type of mycangium. The first type is a prothoracic mycangium in *Dendroctonus frontalis*, which has a paired tubular mycangium on the inside of prothoracic pleural plates; the mycangium opens near the procoxa [10]. *Dendroctonus* represents an independently evolved clade of fungus-farming bark beetles. These beetles inhabit tree phloem rather than xylem, and the adults feed primarily on phloem. However, the larvae depend almost entirely on coevolved fungal symbionts for nutrition, thus we include it among ambrosia beetles in this study. The second type is a mandibular mycangium, the ancestral and most widely distributed form of mycangium in the ambrosia beetle tribe Xyleborini, and characteristic of the genus *Xyleborus* [21]. Xyleborini are one of the largest and most economically important tribes of ambrosia beetles [3, 20]. The opening of the mandibular mycangium is in the oral cavity. The third type, representing a different clade within the Xyleborini, is the mesonotal mycangium, a large internal organ with opening between the pronotum and the elytral basis [7]. It appears to have evolved only once within the Xyleborini and coincided with a radiation of a successful clade with many genera, including *Xylosandrus* [21].

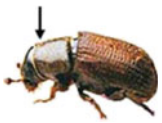
Beetles were obtained either by rearing from colonized logs [22] or from cup traps baited with ethanol. Beetles were preserved in 95 % ethanol and stored at −80°C. To extract bacteria from mycangia, we surface-cleaned each beetle by vortexing in water and ethanol and excised the part of each beetle that contained the mycangium. In *Xyleborus* spp., we sliced off a part of the head between the frons and the joints of mandibles. The taxonomic distinction between *Xyleborus ferrugineus* and *Xyleborus bispinatus* is uncertain [23], thus we treated representatives of the complex as one species. In *Xylosandrus*, we excised the content of the mesonotal mycangium. In *Dendroctonus*, we exposed both mycangia on the inner sides of the thorax and squeezed the content out with a sterile pin. The environmental control composed of two galleries of *Xylosandrus germanus* were collected during the same collecting event as the *X. germanus* beetles. Surface of the gallery containing the fungal garden was scraped into a vial and processed in the same way as the beetle mycangia.

The extracted mycangium was added to 10 μL of Ex-n-Amp™ extraction solution (Sigma-Aldrich), macerated with a pestle, and lysed at 96°C for 10 min. The reaction was deactivated with 10 μL of 3 % BSA; 1 μL of the clear supernatant was used in the polymerase chain reaction (PCR). Amplicons were generated using a universal bacterial/archeal primer pair 515 F and 806R (5′-GTGCCAGCMGCCGCGGTAA-3′ and 5′-GGACTACVSGGGTATCTAAT-3′ [24]). The primer 515 F was appended with a TC linker and a Roche 454 B pyrosequencing adapter; the 806R primer was appended with a 12-bp sample-specific barcode sequence, a CA linker, and a Roche 454 A sequencing adapter. The sample-specific, error-correcting barcode allowed for pooling all amplicons into a single pyrosequencing run. All samples were amplified in triplicated 25-μL reactions using 5 Prime HotMaster polymerase mix (5 Prime, Inc.) under the following cycling conditions—95°C, 1 min, 33×(95°C, 30 s; 50°C, 1 min; 72°C, 1 min). Amplicons were purified using the UltraClean PCR Clean-up kit (MoBio). Concentration of each amplicon was determined using the Quant-iT™ PicoGreen® dsDNA kit (Invitrogen), and equimolar aliquots of all samples were pooled. Pyrosequencing was carried out on a Roche Genome Sequencer FLX system at Engencore, University of South Carolina, USA.

The sequencing output was processed using the package QIIME [25]. All sequences are available upon request. The output was filtered to contain only sequences with lengths >200 and <1,000 bp with an average quality score >25 and no ambiguous characters. Sequences were assigned to samples according to the 12-bp barcode. Sequences that were ≥97 % similar were grouped into phylotypes using the UCLUST method [25], and the taxonomic identity of each phylotype was determined using the RDP Classifier [26]. Phylotypes occurring in >30 % samples that were unclassified by RDP were identified individually in NCBI BLAST. To confirm the genus identity of the three common *Mycoplasma* phylotypes and to rule out possible misclassification in RDP and BLAST, we performed a phylogenetic analysis of the placement of our phylotypes in Mycoplasmataceae. We downloaded 16 S sequences of identified strains of *Mycoplasma* spp. and representatives of other genera within this family from NCBI-GenBank, aligned them with our sequences using MUSCLE [27] (270 bp alignment), and inferred a maximum likelihood phylogeny based on the Tamura-Nei model in MEGA5 [28].

For downstream analyses, a distance matrix was derived using UNIFRAC [30] which takes into account a phylogenetic structure of the community. Representative sequences from all operational taxonomic units were aligned with MUSCLE [27], and the phylogenetic tree and unifrac distance matrix were produced in QIIME. In order to avoid artifacts of PCR, sequencing, and sampling biases [31], we do not use bacterial abundance (read counts) in our analyses; instead, prevalence of a phylotype is calculated as the

Table 1 Ambrosia beetles from which mycangia were dissected and bacterial DNA sequenced

My cangium	Species	Locality	No. beetles	Sum of sequence count
Prothoracic		USA, Arizona, Flagstaff (native)	4	1857
	*Dendroctonus frontalis*	USA, Florida, Lake Alfred (native)	5	2062
		Ghana, Ankasa (native)	5	2231
		Guyana, Iwokrama (native)	5	2021
		Japan, Okinawa (native)	3	1182
Mandibular	*Xyleborus affinis*	Papua New Guinea, Madang (native)	3	1365
		USA, Florida, Lake Alfred (native)	4	766
	Xyleborus bispinatus-ferrugineus	Ghana, Ankasa (native)	5	2139
		Guyana, multiple loc. (native)	2	635
		Panama, Canal zone (native)	3	289
		Papua New Guinea, Madang (native)	1	847
		USA, Florida, Lake Alfred (native)	7	4066
	Xyleborus glabratus	USA, S. Carolina, Myrtle Beach (invasive)	5	4114
		USA, Florida (invasive)	5	3411
		Ghana (unclear)	5	0
		Japan (native)	5	151
	Xylosandrus crassiusculus	Papua New Guinea (unclear)	5	90
Mesonotal		USA, S. Carolina, Myrtle Beach (invasive)	9	1161
		USA, Florida (invasive)	4	264
		Japan, Okinawa (native)	1	0
	Xylosandrus germanus	USA, Carolinas, Myrtle Beach (invasive)	3	0
		USA, Massachusetts, Harvard Forest (invasive)	5	0
Ambient gallery	*Xylosandrus germanus*	Japan, Okinawa (native)	2	1191
Total			96	29842

number of beetle samples yielding one or more reads. Likewise, sample similarities in multivariate analyses were not weighted by abundance. Prior to the analysis, samples were rarefied to 41 reads. Less-strict rarefaction levels were also tested (122 and 330 reads per sample); they did not provide greater explanatory power but resulted in exclusion of most low-yield mesonotal samples. The structure of the bacterial community was visualized using principal coordinates analysis based on the UniFrac distances.

To compare the effect of beetle species and locality on the composition of bacterial communities, we performed a permutational multivariate analysis of variance of pairwise distances between samples (the Adonis function in the Vegan package in R [29]). The factors beetle species, locality, and their interaction were considered fixed factors in the model. Mycangium type was included as a stratifying variable, such that permutations were constrained within mycangium type (the significance of mycangium type was not directly tested, as only one type was sufficiently replicated). To examine pair-wise differences between beetle species in their composition of bacterial communities, we performed Tukey's honestly significant difference (HSD) post hoc tests on a model of bacterial community composition as a function of beetle species identity (using an analysis of multivariate homogeneity of group variances; data were pooled across beetle locality and mycangium type).

To test whether related beetle species have more similar bacterial communities than unrelated beetles, we tested the congruence between a beetle phylogeny and a dendrogram of similarity between bacterial communities summed within species. The beetle phylogeny was derived from concatenated sequences of cytochrome-oxidase I mtDNA and 28 S rDNA of each species from our previous datasets [32] and from NCBI Genbank, using maximum likelihood with the Tamura-Nei model of evolution in MEGA [28]. Records of bacterial phylotypes were summed up within beetle species, distance matrix obtained with unweighted UniFrac, and summarized in a dendrogram using the UPGMA algorithm in QIIME. Jack-knife support for nodes in the dendrogram were derived from repeating this process on successively rarefied matrices from 1,666 reads (the lowest read count, *Xylosandrus crassiusculus*) to 833 reads (50 % of data) per beetle species. The significance of congruence between the two matrices was assessed using Mantel test in PopTools [33] with 1,000 iterations. *X. germanus* was not included in most tests, as it did not yield any bacterial reads.

Results

We successfully amplified bacterial 16 S in 66 out of the initial 96 beetles. In the total of 29,842 reads of sufficient quality, we distinguished 1,546 bacterial phylotypes using a 97 % similarity threshold (see the complete dataset in the Electronic supplementary material).

Several phylotypes occurred in many beetle species and were broadly distributed geographically (Table 2). Several phylotypes of *Mycoplasma*, Burkholderiales, and Pseudomonadales were recovered from more than half of the amplified samples. The genus *Mycoplasma* was represented by three frequently encountered phylotypes, including the most widespread phylotype (present in 75 % of samples). The placement of the three phylotypes in *Mycoplasma*, as opposed to placement in other Mycoplasmataceae more often reported from insect hosts, was confirmed using a phylogenetic analysis of the bacterial family (Fig. 1). The second and third most prevalent phylotypes belonged to unidentified Burkholderiales (probably Comamonadaceae, no closer match was available in RDP and BLAST) found in 69 % and 61 % of beetles, respectively. The last two phylotypes present in more than half of the samples were Pseudomonadales: one *Acinetobacter* and one unidentified Pseudomonadaceae (prevalence 56 % and 48 %, respectively). Several other phylotypes were found in fewer species but still prevalent within certain beetle species (Table 2). Particularly notable is the uniform microbiota of *Xyleborus glabratus*, where four bacterial phylotypes were shared by all beetle individuals and the same phylotypes were infrequent or absent in other beetle species.

The bacterial community as a whole differed strongly among beetle species (Fig. 2; $F_{5,47}=4.298$, $R^2=0.26$; $p<0.001$) and less strongly but still significantly among regions ($F_{7,47}=2.13$, $R^2=0.18$; $p<0.001$). The prokaryotic communities of *X. crassiusculus* and *X. glabratus* are significantly different from several other beetle species (Tukey's HSD post hoc test; Table 3). Similarities between bacterial communities did not correspond to beetle phylogenetic relationships (Mantel test $p=0.149$, Fig. 3). Interestingly, both species with mesonotal mycangia, *X. crassiusculus* and *X. germanus*, repeatedly yielded very poor amplicons of bacterial 16 S (average numbers of reads, 60 and 0, respectively). The two environmental samples from galleries of *X. germanus* yielded a bacterial community as diverse as samples from most mycangia, but the community was significantly different from all mycangial communities.

Discussion

Although mycangia are usually considered to have evolved to house and disperse the fungi on which ambrosia beetles depend, they also host several regularly occurring bacterial phylotypes. Representatives of *Mycoplasma*, Burkholderiales, and Pseudomonadales in particular are widespread in mycangia of multiple beetle species from diverse parts of the world. These appear to be true inhabitants of beetle

Table 2 Dominant phylotypes of bacterial communities in ambrosia beetle mycangia (higher taxon, order, and genus, where available)

Beetle	Bacterial phylotypes present in over half of samples	Prevalence
D. frontalis	Betaproteobacteria; Burkholderiales	0.86
	Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae	0.71
	Mollicutes, *Mycoplasma* 320	0.71
	Mollicutes, *Mycoplasma* 661	0.71
	Gammaproteobacteria	0.57
X. affinis	Mollicutes, *Mycoplasma* 320	0.79
	Betaproteobacteria; Burkholderiales; Comamonadaceae	0.68
	Mollicutes, *Mycoplasma* 1441	0.63
	Betaproteobacteria; Burkholderiales	0.58
	Gammaproteobacteria; Enterobacteriaceae	0.53
X. bispinatus–ferrugineus	Betaproteobacteria; Burkholderiales; Comamonadaceae	0.88
	Mollicutes, *Mycoplasma* 320	0.76
	Betaproteobacteria; Burkholderiales	0.71
	Gammaproteobacteria; Pseudomonadales; *Acinetobacter*	0.53
	Mollicutes, *Mycoplasma* 661	0.53
X. glabratus	Alphaproteobacteria; Rickettsiales; *Rickettsia*	1
	Bacteroidetes; Sphingobacteriales; *Sphingobacterium*	1
	Gammaproteobacteria; Pseudomonadales; *Acinetobacter*	1
	Gammaproteobacteria; Xanthomonadales; *Stenotrophomonas*	1
	Alphaproteobacteria; Caulobacterales; *Caulobacter*	0.9
	Betaproteobacteria; Burkholderiales; Alcaligenaceae	0.9
	Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae	0.8
	Gammaproteobacteria; Xanthomonadales; *Stenotrophomonas*	0.8
	Actinobacteria; Actinomycetales; *Tsukamurella*	0.7
	Betaproteobacteria; Burkholderiales	0.7
	Actinobacteria; Actinobacteria; Actinomycetales	0.6
	BacteroidetesFlavobacteriales; *Chryseobacterium*	0.6
	Bacteroidetes; Sphingobacteriales; *Sphingobacterium*	0.6
	Bacteroidetes; Sphingobacteriales; *Sphingobacterium*	0.6
	Betaproteobacteria; Burkholderiales; *Achromobacter*	0.6
	Betaproteobacteria; Burkholderiales; Comamonadaceae	0.6
	Betaproteobacteria; Burkholderiales; *Delftia*	0.6
	Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	0.6
	Gammaproteobacteria; Pseudomonadales; Moraxellaceae	0.6
	Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae	0.6
	Mollicutes, *Mycoplasma* 320	0.6
X. crassiusculus	Mollicutes, *Mycoplasma* 320	0.78
	Betaproteobacteria; Burkholderiales	0.67
	Gammaproteobacteria; Pseudomonadales; *Acinetobacter*	0.56
	Gammaproteobacteria; Xanthomonadales; *Stenotrophomonas*	0.56
	Mollicutes, *Mycoplasma* 1441	0.56
X. germanus mycangium	No bacterial sequences amplified	
X. germanus, fungal gardens from two galleries	Alphaproteobacteria	1
	Alphaproteobacteria; Rhizobiales	1
	Alphaproteobacteria; Rhizobiales; Brucellaceae	1
	Bacteroidetes; Sphingobacteriales; *Mucilaginibacter*	1
	Bacteroidetes; Sphingobacteriales; *Sphingobacterium*	1
	Bacteroidetes; Sphingobacteriales; *Sphingobacterium*	1
	Betaproteobacteria; Burkholderiales; Alcaligenaceae	1

Table 2 (continued)

Beetle	Bacterial phylotypes present in over half of samples	Prevalence
	Betaproteobacteria; Burkholderiales; *Herbaspirillum*	1
	Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae	1
	Gammaproteobacteria; Xanthomonadales; *Dyella*	1
	Proteobacteria	1

Only phylotypes sequenced from over half of samples of the respective species are listed. Prevalence: Proportion of samples where the phylotype was detected. Note the significantly different composition of the gallery sample

mycangia, rather than environmental or laboratory contaminants: The two control samples of fungus gardens surrounding the beetles were dominated by completely different phylotypes; for example, no *Mycoplasma* was present.

The two most common groups of associates of ambrosia beetles have never (*Mycoplasma*) or only rarely (Comamonadaceae) been recovered from insect hosts. This is the first record of *Mycoplasma* spp. as stable associates of insects. Our discovery is at odds with the definition of the genus as parasites of vertebrates [34] and raises questions about the broader biology of the group. The confamilial genus *Entomoplasma* has been isolated from insects, but the phylotypes from ambrosia beetles do not fall into this clade (Fig. 3). Importantly, the *Mycoplasma* spp. from ambrosia beetles are also not a monophyletic clade, suggesting that several *Mycoplasma* clades colonized ambrosia beetles independently.

Reports of Comamonadaceae from bodies of insects are rare. Several strains have been isolated from insects but not from groups relevant to wood-boring beetles [35–37]. Pseudomonadaceae are reported more commonly. The most relevant is a record of several phylotypes of *Acinetobacter* in the gut of the bark beetle *Dendrocotnus valens* [38]. The 16 S sequence of one phylotype corresponds to the *Acinetobacter* phylotype detected in our samples as the fourth most widespread phylotype (only 1.5 % divergent; other reported strains are not similar). Burkholderiaceae have been previously isolated from crushed whole bodies of another *Dendroctonus*, *D. valens* [15], and Pseudomonadaceae from *Dendroctonus ponderosae* [14], but, in both cases, the isolates represented different 16 S phylotypes than those reported here.

Since beetle species is a stronger predictor for the bacterial community composition than locality, we suggest that more bacterial phylotypes in beetle mycangia are vertically

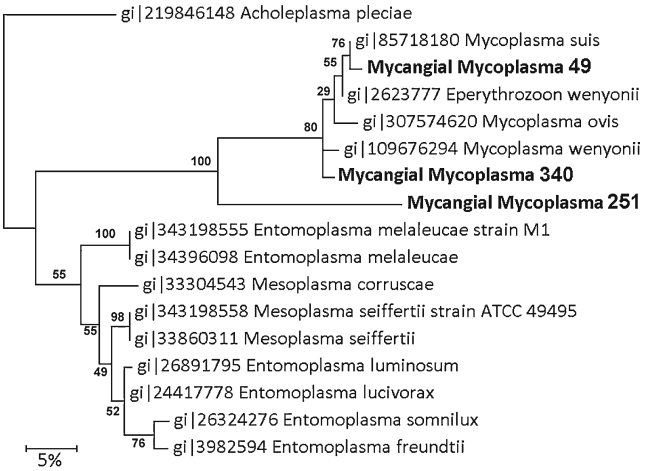


Figure 1 The three most widespread Mycoplasmataceae from ambrosia beetle mycangia are more closely related to *Mycoplasma* spp. from vertebrates than to confamilial genera more often reported from invertebrates. They are also not monophyletic, which suggests repeated colonization of ambrosia beetles by this group of bacteria. Maximum likelihood phylogeny was reconstructed in MEGA [28] based on the Tamura-Nei model; *numbers* at nodes represent bootstrap support. Sequences of identified Mycoplasmataceae were obtained from NCBI-Genbank. The analysis is strictly for taxonomic purposes and did not attempt to reconstruct the actual evolution of the clade

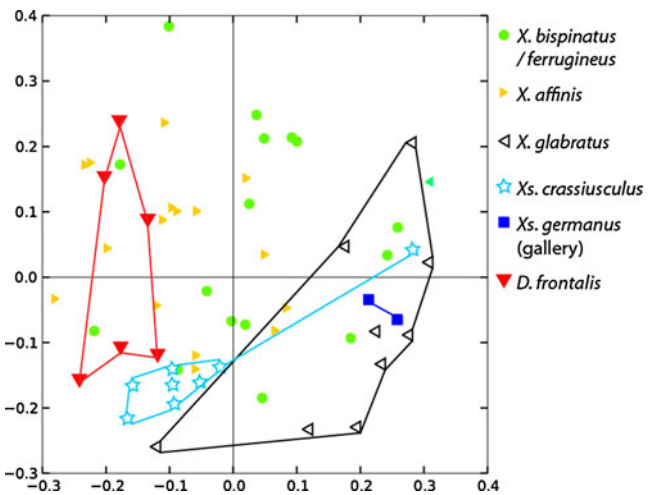


Figure 2 Principal coordinates analysis (*PCoA*) of bacterial assemblages from ambrosia beetle mycangia shows the significant specificity of bacterial community composition to certain beetle species. Clustered are samples from *X. glabratus*, *X. crassiusculus*, *D. frontalis*, and samples from the two galleries of *X. germanus*. Not clustered are the widely dispersed samples from *X. affinis* and *X. bispinatus*/*ferrugineus*. First two axes are shown, explaining 9.04 % and 7.41 % of variation. The PCoA is based on UniFrac distances unweighted by phylotype abundances

Table 3 Pair-wise differences in bacterial community composition between beetle species resulting from post hoc analyses (Tukey's HSD following the analysis of multivariate homogeneity of group variances)

Comparison	Difference	*p* value (adjusted)
X. affinis–D. frontalis	0.009608	0.998436
X. bispinatus/ferrugineus–D. frontalis	0.002645	0.999997
X. bispinatus/ferrugineus–X. affinis	−0.00696	0.998571
X. glabratus–D. frontalis	−0.06972	0.081577
X. glabratus–X. affinis	−0.07933	0.002512
X. glabratus–X. bispinatus/ferrugineus	−0.07236	0.007324
X. crassiusculus–D. frontalis	−0.06102	0.210561
X. crassiusculus–X. affinis	−0.07062	0.019012
X. crassiusculus–X. bispinatus/ferrugineus	−0.06366	0.045119
X. crassiusculus–X. glabratus	0.008703	0.998985
X. germanus* gallery–*D. frontalis	−0.25128	1.02E-06
X. germanus* gallery–*X. affinis	−0.26088	5.11E-08
X. germanus* gallery–*X. bispinatus/ferrugineus	−0.25392	1.02E-07
X. germanus* gallery–*X. glabratus	−0.18156	0.000205
X. germanus* gallery–*X. crassiusculus	−0.19026	0.000132

p values are adjusted for multiple comparisons. In bold are significantly different species pairs

transmitted than sampled from the environment. Given their prevalence (Table 2) and their apparent absence outside of beetle mycangia, several of the widespread phylotypes we found have the potential to be stable associates of ambrosia beetles. Based on our present knowledge, we cannot determine the ecological role of the bacterial associates. The role could be anything from mutualists of the beetles to their parasites, or even endosymbionts of the ambrosia fungi (i.e., Burkholderiales, [40]). In most cases, the beetles seem unlikely to obligately depend on the bacteria, since even the most prevalent bacterial phylotypes in the beetles did not tend to be universally present within a host species. However, an exception in this regard may be the four species-specific associates of *X. glabratus* recovered from all samples of this beetle

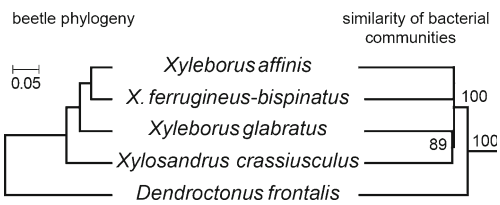


Figure 3 Similarity between bacterial communities in mycangia does not correspond to the beetle phylogeny but distinguishes the conifer-inhabiting *D. frontalis*. *Numbers* on the similarity dendrogram correspond to jack-knife support for nodes; nodes with <50 % support were collapsed

regardless of the locality. These phylotypes in particular deserve additional research to better understand their role in the ambrosia symbiotic complex. It is also worth noting that mycangia are complex organs and may not have been sampled completely. We sampled the lumen of the mycangium, which is where the main mass of symbionts resides, but we did not attempt to explore the ultrastructure of its walls reported, for example, in *D. frontalis* [41].

The conclusion that ambrosia beetles tend to retain species-specific associates in different regions of the world is further corroborated by the unique bacterial communities in the mycangia of *X. crassiusculus* and *X. glabratus* (Table 3). Both species are non-native invaders in North America, and their bacterial communities differ significantly from those of native xyleborine beetles. Particularly intriguing is *X. glabratus*, since its entire non-native population likely originated from a single recent introduction [20, 39]. The bacterial community within its mycangia is unusually uniform (several phylotypes occur in every sample) and dissimilar from bacterial communities in native North American species of *Xyleborus* collected from the same trees. This invasive beetle thus does not appear to have adopted the bacteria of native beetles with which it is now sympatric. An interesting question we cannot resolve here is whether bacterial communities of *X. glabratus* mycangium have changed relative to its ancestors in its native range.

We have repeatedly failed to amplify bacterial 16 S rDNA from mesonotal mycangia of both species of the genus *Xylosandrus*. We are unable to distinguish whether this reflects a true absence of bacteria in the mycangia, or the presence of strong PCR inhibitors. It is however not a result of low quality of the samples. The two control samples from fungal gardens surrounding two different *X. germanus* were collected directly from the gallery around the beetles and processed identically, yet they yielded rich communities of bacteria.

None of the common phylotypes detected here have been previously reported from bark and ambrosia beetles. One reason may be that the majority of previous studies used culturing approaches. Here, we show that the most regular associates belong to groups that are difficult or impossible to culture, such as *Mycoplasma* or *Rickettsia* [34]. Lastly, we also show that focus on specific organs, such as the mycangium, allows refined inference of symbiotic associations, and we recommend that future research on insect symbioses takes insect anatomy into account.

To summarize our findings on the community ecology of the mycangial microbiome, it appears that (1) bacterial communities are more species-specific than locality-specific, however, (2) the core microbiome is nevertheless shared across beetle species, and (3) the similarity of bacterial communities does not reflect phylogenetic relatedness of their host beetles. This suggests that, although beetles tend to retain distinct communities even in newly colonized regions and in

the presence of other ambrosia beetle species, their mycangial associates do experience horizontal cross-infection with a large amount of stochasticity and do not conform to a scenario of long-term co-cladogenesis.

Acknowledgement A number of collaborators contributed beetle samples: Vojtech Novotny, Rich Hofstetter, Sarah M. Smith, Anthony I. Cognato, Shannon Pelini, Hisashi Kajimura, and Kazuki Tsuji. Trudy McKay allowed us to use reagents in her lab. JH was supported by US Forest Service Cooperative Agreement and by NSF-CAREER grant 09533390. RRD was supported by DOE-PER grant DE-FG02-08ER64510, a NASA award NNX09AK22G, and an NSF-CAREER grant 09533390. LLS was supported by a USDA-SCRI grant.

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