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HOST MICROBE INTERACTIONS

Microbial Community Profiling to Investigate Transmission of Bacteria Between Life Stages of the Wood-Boring Beetle, *Anoplophora glabripennis*

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Abstract Many insects harbor specific bacteria in their digestive tract, and these gut microbiota often play important roles in digestion and nutrient provisioning. While it is common for a given insect species to harbor a representative gut microbial community as a population, how this community is acquired and maintained from generation to generation is not known for most xylophagous insects, except termites. In this study, we examined acquisition of gut microbiota by the wood-feeding beetle, *Anoplophora glabripennis*, by identifying and comparing microbial community members among different life stages of the insect and with microbes it encounters in the environment. Automated ribosomal intergenic spacer analysis was employed to compare bacterial communities present in the egg and larval stages of A. *glabripennis* as well as with microbes found in the oviposition site and the surrounding woody tissue. Multivariate analyses were used to identify relationships between sample type and specific bacterial types (operational taxonomic units). From this analysis, bacteria that were derived from the environment, the oviposition site, and/or the egg were identified and compared with taxa found in larvae. Results showed that while some larval microbes were derived from environmental sources, other members of the larval microbial community appear to be vertically transmitted. These findings could lead to a better understanding of which microbial species are critical for the survival of this insect and to development of techniques that could be used to alter this community to disrupt the digestive physiology of the host insect as a biological control measure.

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Introduction

Insects often exploit beneficial symbiotic relationships to augment their physiological capabilities and facilitate their expansion into challenging niches. Obligate mutualists are usually intracellular, transmitted vertically, and enable survival of many insects on nutritionally deficient diets

such as blood, plant sap, or wood [2, 9, 36]. In addition to intracellular obligates, many insects harbor secondary facultative symbionts that display a wider range of tissue tropism and can be intracellular [40, 48], located in the gut of the insect [4, 6, 12, 17, 18, 28, 41, 49], or associated externally [1, 11, 13, 33]. Secondary symbionts primarily serve a nutrient provisioning role in their hosts, which may include cellulose digestion, nitrogen fixation, and synthesis of vitamins, amino acids, lipids, and sterols [5, 8, 50]. The source of symbionts may be through vertical transmission, horizontal transmission, or acquisition from diet or environmental sources [15, 20, 24, 26].

The Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) is a wood-feeding insect in the family Cerambycidae. Native to China and Korea [30, 51], *A. glabripennis* grows and develops in a broad range of host trees [21, 37–39, 52], attacking apparently healthy, vigorous trees [22, 30] with the larval stages of this insect burrowing into the inner wood of the host tree, growing, and developing by feeding on lignocellulose. Preferred host tree species include maple (*Acer* spp.), poplar (*Populus* spp.), and willow (*Salix* spp.). Reproduction and oviposition occur when free living adults emerge from host trees, maturation feed (i.e., feeding that is required to reach reproductive maturity) on twigs and leaves, mate, and then oviposit (egg lay) under the bark of a chosen host. During oviposition, the adult female chews a small hole into the bark of the tree to reach the phloem and then carefully places an egg into that niche with her ovipositor. Additional fluid is secreted by the female with the egg, perhaps to secure the egg in place under the bark or to protect it from desiccation. Although gut microbes in xylophagous insects play important roles in host nutrition [18], particularly in insects that feed on wood, the acquisition and maintenance of microbes has received very little attention. Although cellulose is a rich source of nutritional carbohydrate, lignin interferes with the accessibility and digestibility of cellulose [27] and protein [53], which is reflected in the inverse relationship between plant lignin content and its digestibility by animals. In addition, nitrogen is limiting for phytophagous insects [16] and this is particularly true for insects that live on wood. While it is likely that gut bacteria play an important role in the digestive physiology of ALB, transmission of gut microbiota between generations is unknown.

Few studies have examined microbial symbionts in cerambycids, but the gut microbial community of *A. glabripennis* has received some attention. A wide diversity of bacteria was found in the gut of larval *A. glabripennis* collected from willow trees in China [44], from insects reared in a research colony and field-collected insects in an established population in the USA (Geib et al., unpublished data). Interestingly, regardless of the beetle's geographic origin or host, the gut microbial community profiles exhibited considerable similarity, suggesting that some members of the gut community are conserved in space and time. Therefore, of particular interest is discerning which bacterial species are environmentally derived, and which are vertically transmitted. Our hypothesis is that vertically transmitted bacterial species may be the key in maintaining the ability of this insect to feed on lignocellulose, utilizing specific bacteria for wood degradation and to obtain nutrition.

The purpose of this study was to determine the source of the gut microbial community harbored by larval *A. glabripennis*. Microbial community profiling was performed using automated ribosomal intergenic spacer analysis (ARISA) on a variety of insect- and environmentally derived samples to identify and distinguish microbial taxa that are transmitted from the adult to larval stages, versus taxa that are acquired from the environment. By creating replicate profiles from each treatment group, multivariate analysis techniques were used to explore relationships between gut bacterial communities and sources of these bacteria. This information is crucial to focus future research on particular bacterial species that are more likely to be of critical importance to the insect and may help identify potential obligate versus facultative symbionts.

Materials and Methods

Insect Rearing and Experimental Design

Insects were derived from a quarantine research colony maintained on artificial diet at Penn State University [19, 39]. This colony is of mixed ancestry from invasive populations of *A. glabripennis* obtained within the USA and has been in culture for over 5 years. While maintenance of this colony on artificial diet could have some impact on the diversity and complexity of the microbial community, these insects are still able to grow and develop on trees, suggesting that critical microbes are still present in the insects. Mating pairs were collected from pupae generated from this colony, and maturation fed on Norway maple (*Acer platanoides*) twigs until used in this experiment (5–7 days of maturation feeding). Norway maple trees of approximately 5 to 12 cm in diameter were cut down from a woodlot located in University Park, Centre County, PA, USA and sectioned into 30 cm sections. These sections were coated with paraffin wax at both cut ends to maintain wood moisture and placed into sterile 1 gallon glass wide mouth jars to be used as oviposition material for adult oviposition. Three to five pencil-sized Norway maple twigs were also added to the jars to provide food for the single mating pair of *A. glabripennis* in each jar. Overall, ten mating jars were used in this experiment. During the entire setup process, care

was taken to reduce microbial contamination by performing all steps on sterilized surfaces with sterile gloves and tools. Adults were allowed to mate and oviposit into the wood sections for 2 weeks, after which the wood sections were removed from the jars.

Collection of Insect and Environmental Samples for Community Analysis

Seven different sample types were collected for community analysis as follows: (1) oviposition site bark/phloem, (2) oviposition site wood, (3) non-oviposition site bark/phloem, (4) non-oviposition site wood, (5) non-surface sterilized egg, (6) surface sterilized egg, and (7) 2-week-old larvae. The first six sample types were collected as follows: To obtain five samples, each of the non-oviposition site bark/phloem (#3 above) and wood (#4 above), five of the ten oviposition wood sections containing eggs were chosen at random (the remaining five logs were used to collect larvae, described below). From each of these logs, a section of the wood distant from the oviposition sites was selected, and a flame-sterilized 10-mm cork borer was used to cut a circular hole through the bark, phloem, and inner wood approximately 3 mm deep. This wood section was removed from the log using sterile forceps, and the bark/phloem layer was separated from the inner wood by pulling these sections apart. Each of these sections was carefully diced with a sterile scalpel and placed into a sterile micro-centrifuge tube.

Next, the cork borer was dipped in 70% ethanol and flame-sterilized, and this process was repeated in a location on the log where an oviposition site was located, identified by a small slit through the outer bark chewed by the female before laying an egg. The cork borer was used to cut a plug and the bark/phloem and inner wood were pulled from the log, separated and placed into individual sterile micro-centrifuge tubes. During separation of the bark and wood layers, the egg was located, removed from the wood, and placed into a micro-centrifuge tube. For each log, a total of six eggs were collected by peeling the bark layer from additional oviposition sites and excising the eggs with sterile forceps. One-half of the eggs were surface-sterilized before placing into micro-centrifuge tubes by dipping them in ethanol for 1 min, followed by three rinses in sterile water. All of these samples were collected from the remaining four logs and all samples were stored at −20°C until DNA extraction. In total, five non-oviposition bark, five non-oviposition wood, five oviposition site bark, five oviposition site wood, 14 surface-sterilized eggs, and 14 non-sterilized eggs were collected from these five logs.

The remaining five logs from the mating jars were placed into new sterile 1 gallon wide mouth glass jars and incubated at room temperature for 2 weeks to allow eggs to hatch and larvae to feed and develop under the surface of the bark. After 2 weeks, these logs were removed from the jars, and in a sterile laminar flow hood, the bark was peeled from the logs to reveal developing larvae. These larvae were typically first or second instar and had fed within the phloem layer, but had not yet burrowed into the inner wood. These larvae were collected, surface-sterilized as described above for the eggs, and placed into a sterile micro-centrifuge tube. In total, two to three larvae were collected from each log for a total of 14 larvae from the five logs.

DNA Extraction

DNA extraction was performed in a laminar flow hood to maintain sterility using sterile dissection tools. Total DNA was extracted using the FastDNA® SPIN for Soil Kit (MP Biomedicals, Solon, OH, USA) using the FastPrep® Instrument (BIO101 Inc, La Jolla, CA, USA) for tissue homogenization following the manufacturer's protocol for all 62 samples collected (five non-oviposition bark, five non-oviposition wood, five oviposition site bark, five oviposition site wood, 14 surface-sterilized eggs, 14 non-sterilized eggs, 14 larvae). This kit was used due to the complexity of the samples to ensure complete and consistent DNA extraction from all organisms. A control DNA extraction was also performed on the sterile water used in the collection and preparation of all experimental samples, thus serving as a negative control, to confirm that no contaminating DNA was extracted. DNA concentration was determined for each sample by measuring absorbance at 260 nm and samples were stored at −20°C until use.

PCR Amplification and ARISA Community Analysis

PCR was performed using a bacterial automated ribosomal intergenic spacer analysis primer set, ITSF and ITSReub [10]. These primers amplify the intergenic space between the 16S and 23S ribosomal subunits by amplifying from position 1423 on the 16S ribosomal RNA (rRNA) subunit to position 38 on the 23S rRNA subunit. Because the intergenic space is a non-coding region, the length of this region is not conserved between different species of bacteria, so polymerase chain reaction (PCR) amplification of a mixed bacterial sample produces PCR products of multiple lengths, with each unique fragment length representing a bacterial type. PCR reactions were performed for each sample in 25 μl volumes with the following components: 5 μl of 5× GoTaq green reaction buffer, 0.5 μl GoTaq DNA polymerase (1.25 U, Promega, Madison, WI, USA), 1 μl 10 μM dNTP mix, 2 μl of 10 μM forward primer (ITSF, 5′-GTCGTAACAAGG TAGCCGTA-3′), 2 μl of 10 μM labeled reverse primer (ITSReub-HEX, 5′-GCCAAGGCATCCACC-3′), and 20 ng

of template DNA. PCR conditions were 95°C denaturation for 3 min, 25 cycles of 95°C for 30 s, 55°C for 1 min, 72°C for 1.5 min, with a final extension at 72°C for 5 min. Control DNA, extracted from sterile water using the FastDNA® SPIN for Soil Kit also underwent PCR to ensure that there was no contaminating DNA during extraction, and positive and negative PCR control reactions were also performed. A second PCR reaction was performed using the same procedures, except that the fluorescently labeled reverse primer was replaced with an unlabeled primer so that the fragments could be cloned and sequenced. All PCR products were verified by gel electrophoresis, and stored at −20°C. One microliter of each fluorescently labeled PCR product was analyzed with an internal standard on an ABI PRISM 3100 Genetic Analyzer at the Penn State University Nucleic Acid Facility, University Park, PA. Resulting fluorograms were analyzed using GeneScan Software, where fragment length and relative abundance of each peak were recorded. Relative abundance of individual phylotypes, described as operational taxonomic units (OTUs) and defined as fragments of unique length, was inferred using the fluorescence of each individual peak normalized to total fluorescence within a profile to account for run-to-run variation during fragment analysis.

Cloning, DNA Sequencing and Sequence Analysis

For each sample type, a representative sample was chosen, based on complexity and fragment diversity. This sampling was then used for cloning and sequencing of fragments to provide taxonomic information to ARISA fragments. Unlabeled PCR products were ligated into the pCR® 2.1 TOPO vector (Invitrogen Corp., Carlsbad, CA, USA) following the manufacturer's protocol. The vector was then transformed into chemically competent *Escherichia coli* cells (TOP10, Invitrogen Corp.) by heat-shock and clone libraries were created.

Insert DNA from each of these clones was amplified from the M13 priming sites of the vector using direct PCR. Twenty-five microliter PCR reactions were set up in 96-well format with the following components: 5 μl of 5× GoTaq green reaction buffer, 0.5 μl GoTaq DNA polymerase (1.25 U, Promega), 1 μl 10 μM dNTP mix, 2 μl of 10 μM forward primer (M13Universal), and 2 μl of 10 μM reverse primer (M13Rev). Individual colonies were picked from the clone library using a sterile pipette tip and immersed into the PCR mix to allow the bacteria cells to enter the PCR reaction. The PCR program had an initial 95°C denaturation step for 10 min to rupture bacterial cells, followed by 30 cycles of 95°C for 30 s, 55°C for 1:00 min, 72°C for 1:30 min, with a final extension at 72°C for 5 min. PCR products were visualized by gel electrophoresis and samples of unique fragment length were chosen for sequencing. For each fragment length, several clones were chosen to account for similar sized clones. For the clones chosen for sequencing, 4 μl of the PCR product was cleaned up for sequencing by adding 0.8 μl of ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and incubating the sample at 37°C for 15 min, followed by 80°C for 30 min. Two microliters of this reaction was then used to sequence from the forward direction, from the M13Universal priming site and 2 μl for the reverse from the M13Rev site. Sequencing using the BigDye Terminator method was performed at the Penn State Nucleic Acid Facility. ARISA 16s–23s intergenic spacer sequences were constructed based on alignments of the forward and reverse sequences for each clone analyzed. Editing and alignment of forward and reverse sequences was performed using MEGA 4 [45]. Identity of these sequences was determined by non-redundant BLASTn search of the NCBI database. The amplified product was then analyzed using the methods described previously for community ARISA [25, Newton, 2006 #677]. This procedure matches the AFL (measured as the length of the amplification from ITSF to ITSReub) from an individual clone to the fragment lengths obtained from the ARISA community fingerprints. It was then possible to apply taxonomic definitions to ARISA peaks in the profile.

Multivariate Analyses

Multivariate analysis was performed to determine associations between sample types and microbial OTUs present in the samples. We used cluster analysis and unconstrained ordination to describe and visualize the structure of the data across sample types. We tested whether the sample type explained OTU composition with constrained ordination and permutational multivariate analysis of variance (PERMANOVA).

Hierarchical Cluster Analysis of ARISA Profiles

To determine if there were characteristic microbial community profiles for each sample type, ARISA profiles from all samples (14 profiles each from non-sterilized egg, surface-sterilized egg, and larvae, five profiles each from oviposition wood, oviposition bark, non-oviposition wood, and non-oviposition bark) were compared by performing a cluster analysis based on the normalized abundance of each fragment within the samples. Hierarchical cluster analysis was performed using R version 2.6.1 [46], with the "cluster" package [31]. A Bray–Curtis dissimilarity matrix was created from the proportioned data, and this matrix was used to perform the cluster analysis using Ward's method [34].

Ordination Analysis of ARISA Profiles

Unconstrained ordination analysis was performed on all of the profiles by correspondence analysis (CA) in CANOCO 4.5 [29, 47]. CA was used because the profile data were determined to be unimodal by detrended correspondence analysis (beta diversity greater than 4) [29]. For CA, the relative abundance of each fragment in ARISA profiles was used as the input data and analyzed using Hill's scaling. We created a bi-plot to visualize the data using CanoDraw [47], plotting the unconstrained axes that explained the most variance. Each sample was plotted and coded according to sample type, and we plotted the 30 microbial OTUs that best fit the model (Fig. 2).

We used constrained ordination analysis to determine whether sample type significantly explained microbial OTU composition. Due to different sample sizes among treatments, we analyzed surface-sterilized eggs, non-sterilized eggs, and larvae (n=14) separately from oviposition and non-oviposition wood and bark (n=5). Again, detrended correspondence analysis was performed on these subsets of the data, and in both subsets the beta diversity was greater than 4, so unimodal methods were performed. Ordination of the microbial data was constrained by axes created from the treatment variables. Canonical correspondence analysis (CCA) was performed with Hill's scaling. Treatment variables were assessed using Monte Carlo simulations with 999 iterations. We created bi-plots using CanoDraw [47] with the 29 OTUs (egg and larvae plot) and 18 OTUs (wood and bark plot) that best fit the model.

PERMANOVA was used to examine the significance of the sample types on the microbial community composition [3]. The data were standardized to create proportions, and single occurrence OTUs were removed from the dataset to reduce the impact of outliers. Again, surface-sterilized eggs, non-sterilized eggs, and larvae were analyzed separately from oviposition and non-oviposition wood and bark. This analysis tests the response of multiple dependent variables (microbial species) to multiple treatment (sample) types based on distance measures using permutational methods. Analysis was performed using pair-wise a posteriori methods based on Bray–Curtis distance measures with 4,999 permutations [3].

Results

ARISA Community Profiling

Overall, 62 profiles were collected from all sample types with 60 unique fragment lengths (bacterial OTUs) present in these profiles, varying in 16s–23s rRNA intergenic spacer length from 173 to 656 bp. Fragment lengths greater than 700 bp could not be accurately resolved from the profiles on the genetic analyzer due to the size standard used and have not been included in the ARISA analysis. The percentage of replicate samples for each sample type containing fragments within fragment length groupings was calculated to demonstrate differences in fragment composition between sample types (Table 1). Fragment length

Table 1 Proportion of replicates from each sample type containing ARISA fragments of a given length

ARISA fragment length	Bacteria classes contained in region	Larvae (%)	Non-sterile eggs (%)	Surface-sterilized eggs (%)	Oviposition wood (%)	Oviposition bark (%)	Non-oviposition wood (%)	Non-oviposition bark (%)
173–199	Unknown	36	21	100	100	100	100	100
200–223	Unknown	64	100	0	20	0	0	20
224–360	Bacilli	93	64	100	100	100	100	80
360–389	Alphaproteobacteria	7	7	0	60	80	100	100
390–407	Actinobacteria	14	7	0	100	100	100	80
408–423	Proteobacteria, Bacilli, Actinobacteria	0	0	0	60	60	100	60
424–430	Actinobacteria, Alphaproteobacteria	0	0	0	20	0	100	40
431–459	Actinobacteria	64	64	21	100	80	100	40
460–468	Betaproteobacteria	0	7	0	40	60	40	0
469–470	Actinobacteria	0	7	0	60	60	40	0
470–642	Gammaproteobacteria	93	93	100	80	100	80	60
643–656	Actinobacteria	7	50	0	0	0	0	0

The percentage of replicates for each sample type that contained fragment lengths within the specified regions is listed, demonstrating regions that were present in certain sample types and not in others. ARISA OTU groupings are arbitrary, but based on the taxonomic identification of the fragment lengths (Table 2)

groupings were based on microbial classes described by the intergenic spacer fragment lengths, and their correlation with identified sequences in the clone libraries. Relative abundance of each bacterial OTU was calculated for cluster analysis and multivariate analysis described below.

Designation of Taxonomic Identification to ARISA Fragments

Cloning and sequencing of unlabeled amplifications of ITSF-ITSReub permitted taxonomic identification of the ARISA fragments [7]. Overall, seven clone libraries were created from separate amplifications of representative samples, with at least one sample from each sample type. Approximately 40 clones from each library were sequenced and analyzed, revealing 55 unique sequence lengths giving OTUs over a broad diversity of bacteria, including Actinobateria, Firmicutes, and Proteobacteria (Table 2). While there were some sequence discrepancies among sequences with shared nucleotide lengths, these discrepancies represented only a few nucleotides over the entire fragment. BLASTn matching of these fragments to the NCBI database displayed identical results, despite these sequence variations. Only one fragment length, 579 bp, was associated with highly divergent sequences with different BLAST results; one sequence matched to γ-Proteobacteria, while the other matched to δ-Proteobacteria. All fragments lengths from the clone sequences were matched to the ARISA profile fragment when the fragment length was within 1 bp, giving taxonomic identification to 26 of the 55 fragment lengths, except for those longer than 700 bp, which could not be matched to ARISA profiles as mentioned previously (Table 2).

Cluster Analysis

Hierarchical clustering of the ARISA profiles based on relative abundance and diversity of fragments in each profile gave a dendrogram showing high clustering of samples within sample type (Fig. 1). Groups within this tree that are derived from common branches have greater similarity in their bacterial community profiles. Consequently, the top group in Fig. 1 represents larval *A. glabripennis* communities. This group branches with the surface-sterilized egg group, indicating that bacterial profiles of larvae and surface-sterilized eggs share more taxa than with any other sample type. These two sample types cluster with groups representing bacterial communities of the non-sterilized eggs and the outer bark of oviposition sites. This cluster is distinct from the lower cluster of the dendrogram, which represents non-oviposition site-derived wood and bark samples, as well as oviposition site wood samples, indicating that these profiles and microbial communities are the most distinct from the larval *A. glabripennis* gut bacterial communities. The number of microbial OTUs present in each sample type is listed on the dendrogram; diversity decreases from samples associated with the environment to samples associated with the insect (Fig. 1).

Ordination Analysis

Similar to cluster analysis, the unconstrained correspondence analysis shows clustering of the sample types. The first axis of the bi-plot explains 11.3% of the variation in community composition (Fig. 2). This axis separates the environmental samples (non-oviposition bark and wood, oviposition wood), which have positive values, from the insect related samples (surface-sterilized and non-sterilized eggs, larvae), which have negative values (Fig. 2). Oviposition bark samples have an intermediate position on this axis, indicating input from both insect and environmental microbes. The second axis, which explains 8.2% of the variation, does not further explain variation in the environmental samples since they all have values close to zero. However, among the insect samples, microbial communities of larval and surface-sterilized eggs separate from the non-sterilized egg microbial communities. Larval and surface-sterilized egg communities share similar OTUs, represented by the overlapping samples with positive values on the second axis. Most non-sterilized egg samples have negative values on the second axis (Fig. 2). Also, communities in non-sterile eggs and larval samples were more variable than in sterile eggs. The 30 microbial OTUs that explained the greatest difference between the samples were also plotted; their positions relative to the samples on the bi-plot represent the sample communities to which they are most related.

The centroid principle can be applied to these data, in which the distance between samples and bacterial OTUs represents the abundance of the OTUs in each sample. Samples that are closest to a given OTU have the highest abundance of that OTU, while samples more distant from a given OTU have less abundance of that OTU. A large group of OTUs was associated with the environmental samples, and numerous OTUs were associated with the insect samples as well (Fig. 2). OTUs 220 and 238 were associated with non-sterilized egg microbial communities, while OTU 198 was associated with larval microbial communities. OTUs 224 and 581 were associated with both larval and egg microbial communities, falling within the groupings of these samples (Fig. 2).

CCA analysis of eggs and larvae (Fig. 3) determined that the variation in OTU composition was significantly explained by sample type (first axis: $F=5.816$, $P=0.0020$; all axes: $F=5.146$, $P=0.0020$). The first axis explains 13.0%

Table 2 ARISA OTUs identified by cloning and sequencing

Length	Phylum	Class	Order	Family	Genus
224	Fimicutes	Bacilli	*Bacillales*	*Bacillaceae*	*Geobacillus*
314	Fimicutes	Bacilli	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*
334	Fimicutes	Bacilli	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*
360	Fimicutes	Bacilli	*Lactobacillales*	*Streptococcaceae*	*Streptococcus*
365	Proteobacteria	Alphaproteobacteria	*Rhizobiales*	*Methylobactriaceae*	*Methylobacterium*
389	Actinobacteria	Actinobacteria	*Actinomycetales*	*Streptomycetaceae*	*Streptomyces*
409	Proteobacteria	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Bordetella*
409	Fimicutes	Bacilli	*Lactobacillales*	*Enteroccaceae*	*Enterococcus*
424	Actinobacteria	Actinobacteria	*Actinomycetales*	*Nocardiaceae*	*Nocardia*
428	Proteobacteria	Betaproteobacteria	*Burkholderiales*	*Alcagenaceae*	*Bodetella*
431	Actinobacteria	Actinobacteria	*Actinoycetales*	*Nocardiaceae*	*Nocardia*
435	Actinobacteria	Actinobacteria	*Actinoycetales*	*Microbacteriaceae*	
459	Actinobacteria	Actinobacteria	*Actinoycetales*	*Corynebacteriaceae*	*Corynebacterium*
460	Proteobacteria	Betaproteobacteria	*Burholderiales*		
461–466	Proteobacteria	Gammaproteobactria	*Enterobacteriales*	*Enterobacteriaceae*	*Pantoea*
469	Actinobacteria	Actinobacteria	*Actinomycetales*	*Corynebacteriaceae*	*Corynebacterium*
472	Proteobacteria	Gammaproteobactria	*Enterobacteriales*	*Enterobacteriaceae*	*Pantoea*
480	Proteobacteria	Gammaproteobactria	*Enterobacteriales*	*Enterobacteriaceae*	*Pantoea*
543	Proteobacteria	Gammaproteobactria	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*
546	Proteobacteria	Gammaproteobactria	*Enterobacteriales*	*Enterobacteriales*	*Yersinia*
579	Proteobacteria	Deltaproteobacteria	*Myxococcales*	*Polyangiacease*	*Sorangium*
	Proteobacteria	Gammaproteobactria	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*
581–583	Proteobacteria	Gammaproteobactria	*Xanthomonadales*	*Xanthomonadaceae*	*Xanthomonas*
598	Proteobacteria	Gammaproteobactria	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella*
611	Proteobacteria	Gammaproteobactria	*Xanthomonadales*	*Xanthomonadaceae*	*Xanthomonas*
630	Proteobacteria	Gammaproteobactria	*Enterobacteriales*	*Enterobacteriaceae*	*Cronobacter*
642	Proteobacteria	Gammaproteobactria	*Xanthomonadales*	*Xanthomonadaceae*	*Xanthomonas*
654–655	Antinobacteria	Antinobacteria	*Actinomycetales*	*Microbacteriaceae*	*Curtobacterium*
659	Antinobacteria	Antinobacteria	*Actinomycetales*	*Microbacteriaceae*	*Curtobacterium*
661	Proteobacteria	Gammaproteobactria	*Enterobacteriales*	*Enterobacteriaceae*	
665	Proteobacteria	Gammaproteobactria	*Enterobacteriales*	*Enterobacteriaceae*	*Pantoea*
678	Proteobacteria	Alphaproteobacteria	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*
691	Proteobacteria	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Bordetella*
692	Proteobacteria	Betaproteobacteria	*Burkholderiales*	*Alcaligenaceae*	*Bordetella*
693	Proteobacteria	Betaproteobacteria	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*
711	Proteobacteria	Gammaproteobactria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
732	Proteobacteria	Betaproteobacteria	*Burkholderiales*	*Comamonadaceae*	*Acidovorax*
739	Proteobacteria	Gammaproteobactria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
769	Proteobacteria	Betaproteobacteria	*Burkholderiales*	*Comamonadaceae*	*Pelomonas*
782	Proteobacteria	Gammaproteobactria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
787	Proteobacteria	Gammaproteobactria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
798	Proteobacteria	Gammaproteobactria	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*
801	Proteobacteria	Alphaproteobacteria	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*
837	Proteobacteria	Alphaproteobacteria	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*
893	Proteobacteria	Alphaproteobacteria	*Rhizobiales*	*Brucellaceae*	*Ochrobactrum*

Clones from libraries from a representative sample from each sample type were sequenced and matched using the NCBI database. Length of the sequence, representing the ARISA OTU, is listed followed by consensus taxonomic identification of the BLASTn hits. Only one fragment length had multiple sequences revealing different taxonomic identification (OTU 579).

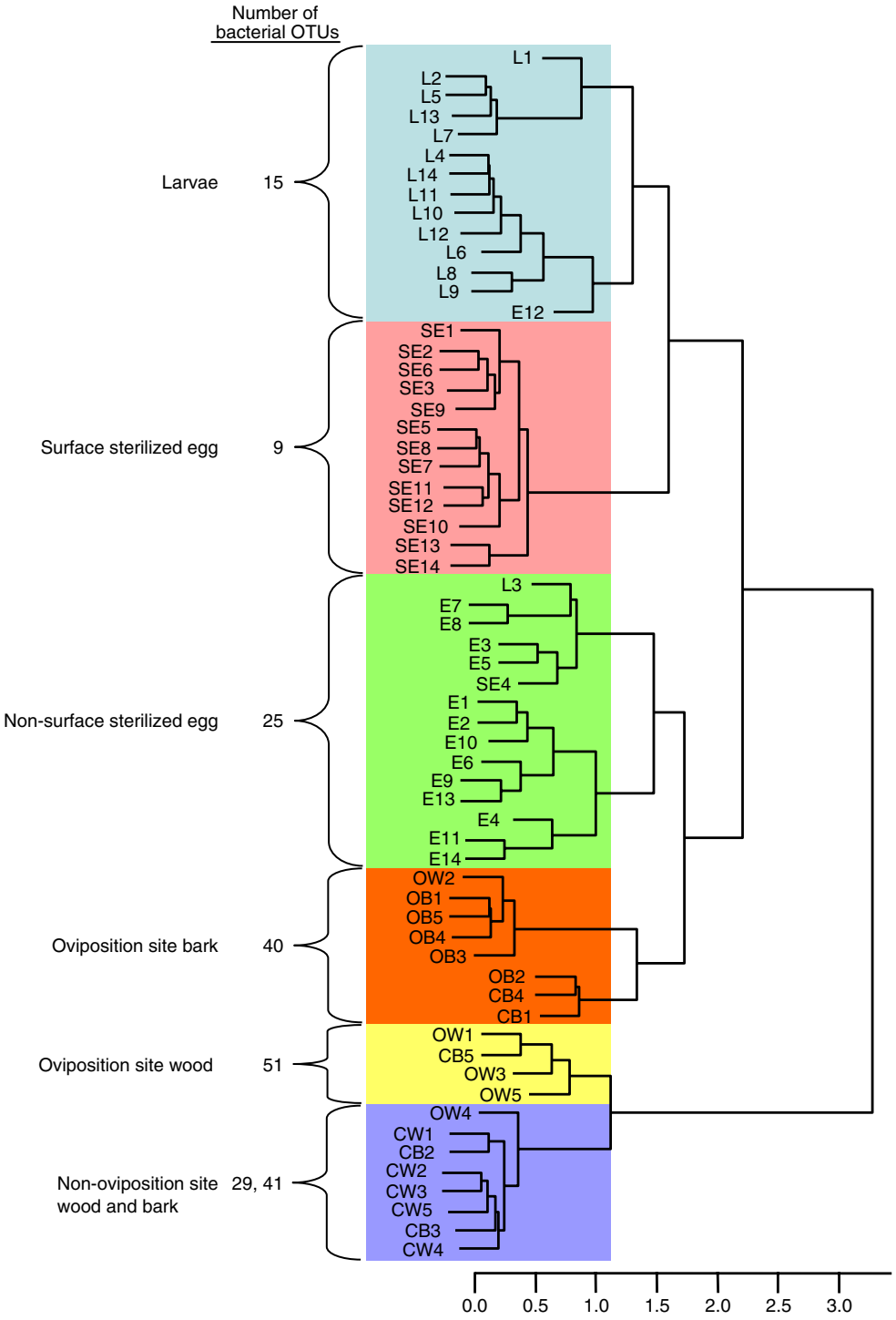

Figure 1 Cluster analysis of ARISA profile data. Branching represents relative relationships between individual samples, based on their ARISA profiles. Sample types are larvae (*L*), non-sterilized eggs (*E*), surface-sterilized eggs (*SE*), oviposition bark (*OB*), oviposition wood (*OW*), non-oviposition bark (*CB*), and non-oviposition wood (*CW*). The general grouping of the clusters are defined with *colored boxes and brackets on the left*, with the sample type and number of ARISA OTUs present in each sample type listed

of the variance in community composition (F=5.816, P=0.0020), defined generally by the surface-sterilized and non-sterilized egg treatments (Fig. 3). The second axis, describing differences between the egg treatments and larvae, explained 7.9% of the variability in the species data (Fig. 3). Using the centroid principle, based on distance between OTUs and treatments, OTUs 224 and 581 showed similar relative abundance in both surface-sterilized egg and larval samples, while OTUs 458, 295, and 440 showed similar relative abundance in non-sterilized egg and larval samples (Fig. 3). Also, many OTUs were most abundant in each of the treatments, since they were most closely positioned to the treatment.

PERMANOVA examined the effects of sample type on microbial community composition. There were significant differences between the communities associated with larvae

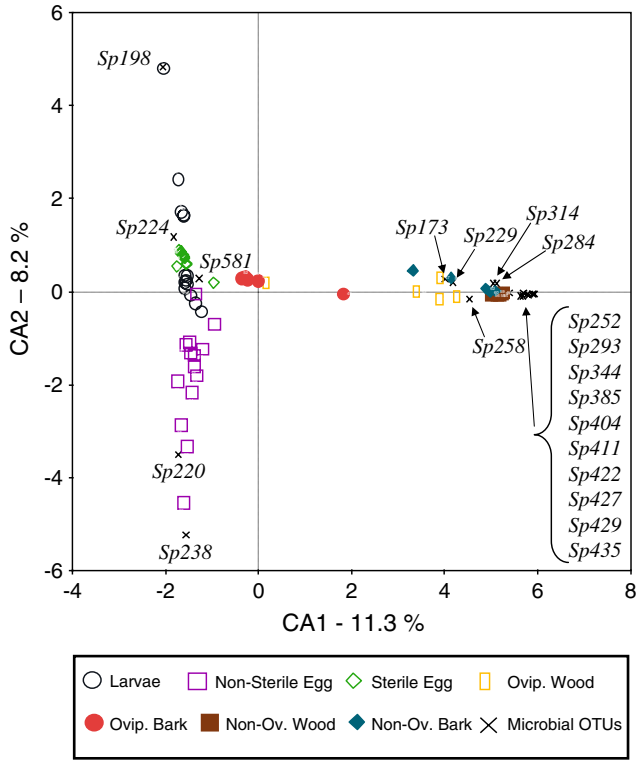

Figure 2 Correspondence analysis bi-plot showing association between samples and microbial OTUs. Sample types include larvae, non-sterilized eggs, surface-sterilized eggs, oviposition wood, oviposition bark, non-oviposition wood, and non-oviposition bark. *Solid filled shapes* represent insect-derived samples, *hatched shapes* represent insect–plant samples, and *unfilled shapes* represent plant samples. Microbial OTUs that are denoted with an "×" and the naming (Sp###) represent "species type" and the length of the ARISA fragment that is denoted by the OTU. See Table 2 for taxonomic identification of ARISA fragment lengths. Species shown are the 30 OTUs that are best explained by the model

and non-sterilized egg sample types (P=0.0002), larvae and surface-sterilized egg sample types (P=0.0002), and non-sterilized and surface-sterilized egg sample types (P=0.0002).

According to the oviposition and non-oviposition wood and bark CCA, sample type significantly explained community composition (first axis: F=3.157, P=0.0040; all axes: F=2.208, P=0.0020). The first canonical axis defines differences between non-oviposition samples and oviposition bark samples, describing 16.5% of the variance in the microbial community composition (Fig. 4). The second axis, which defines differences between wood and bark samples, described 7.1% of the variance in the microbial samples. In this analysis, all microbial OTUs were most closely related to a single sample type, with little evidence of shared OTUs between sample types (Fig. 4). PERMANOVA analysis examined the effects of sample type on microbial community composition. In this case, the Monte Carlo P values were used to determine significance, due to the low number of replicates for each sample type, resulting in a relatively low number of possible permutations [3]. There were significant differences in the communities associated with oviposition wood and bark (P=0.0170), oviposition and non-oviposition wood (P=0.0156), oviposition bark and non-oviposition wood (P=0.0002), and oviposition and non-oviposition bark (P=0.0032). There was no significant difference between non-oviposition bark and oviposition wood or between non-oviposition bark and non-oviposition wood.

Discussion

ARISA community profiling permits individual fingerprints of replicate samples to be created with relatively little effort, allowing for semi-quantitative comparison of microbial community structure within and between sample types in an environment. Among community profiling techniques, ARISA has been shown to be very sensitive, repeatable, and unbiased compared to other fragment length polymorphism techniques in certain environments [10, 14, 32, 42, 43]. In this study, a wide diversity of bacterial OTUs lengths were recovered representing a broad diversity of bacterial types over many classes (Table 2), although sequence match to the NCBI database was not always high. This is likely because

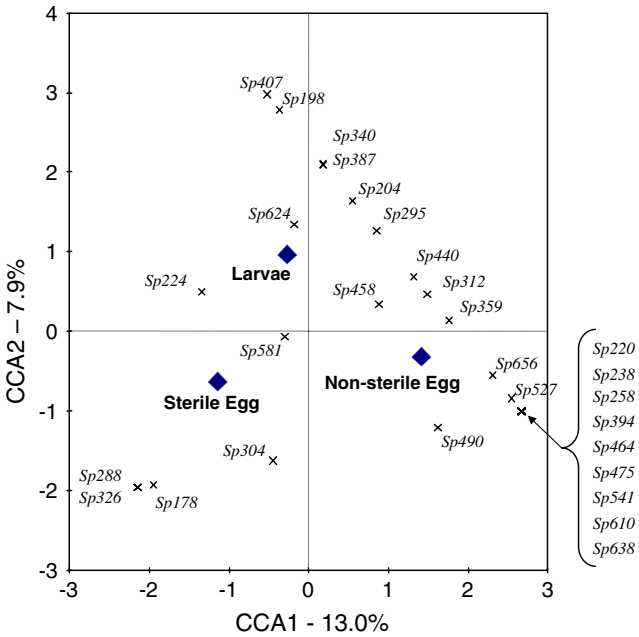

Figure 3 Canonical correspondence analysis bi-plot showing association between larvae, surface-sterilized eggs, non-sterilized eggs, and microbial OTUs. Microbial OTUs are denoted with an "×" and the naming (Sp###) represents "species type" and the length of the ARISA fragment that is denoted by the OTU. See Table 2 for taxonomic identification of ARISA fragment lengths. Species shown are the 28 OTUs most influenced by the sample types. The relative position of each bacterial OTU in relation to each sample type represents the relative influence of that OTU in defining the sample type based on microbial community

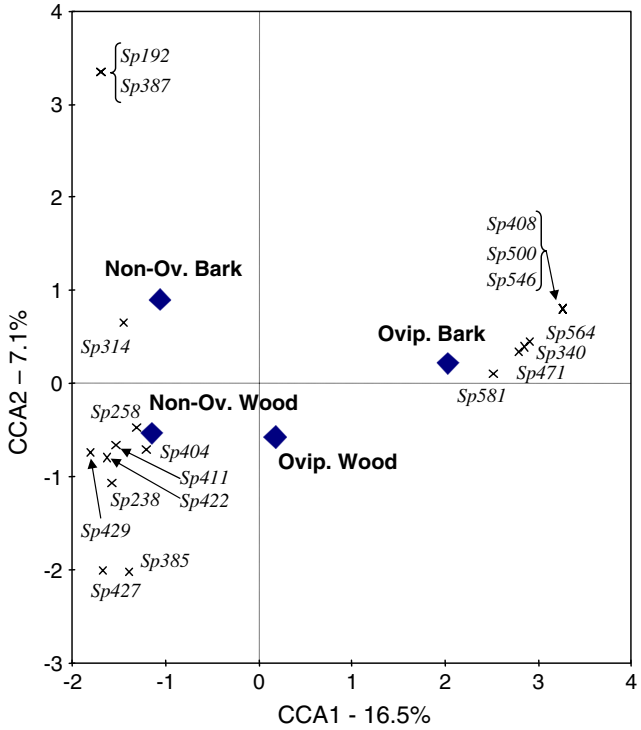

Figure 4 Canonical correspondence analysis bi-plot showing association between oviposition and non-oviposition wood and bark samples, and microbial OTUs. Microbial OTUs are denoted with an "×" and the naming (Sp###) represents "species type" and the length of the ARISA fragment that is denoted by the OTU. See Table 2 for taxonomic identification of ARISA fragment lengths. Species shown are the 18 OTUs most influenced by sample type. The relative position of each bacterial OTU in relation to each sample type represents the relative influence of that OTU in defining the sample type based on microbial community

the ITS region is not commonly sequenced in uncultured microbes. Unique OTU lengths also typically produced a single OTU unit, except for one case where clone sequences of the same length produced two different fragment sequences from bacteria in different phyla (fragment length 579, Table 2). Despite this, it is likely that each OTU represents several species of closely related bacteria because sequencing of fragments of equal length produced only minor sequence discrepancies (single nucleotide differences within the entire fragment length). This could be investigated further by sequencing the adjoining 16S rRNA region, where database information is much greater. Interestingly, grouping the fragments into general classes revealed that there were fragments that were always found in environmental samples, but were present in very low numbers in insect samples (fragments 360–470, Table 1), including many of the α- and β-Proteobacteria, and bacilli clones. At the same time, other fragment groups were more common to these insect samples (fragments 200–223 and 643–656, Table 1), which include Actinobacteria species, while other groups were common in all samples (Table 1), suggesting they may be environmentally derived.

Evaluation of the cluster analysis dendrogram, based on microbial community similarities between samples, revealed that the microbial communities of the samples accurately described the sample types, since these types largely clustered together (Fig. 1). Also, the relative position of the clusters within the tree followed the relative "relatedness" of the sample types to each other based on their bacteria community composition. The insect samples, particularly the larvae and surface-sterilized eggs, clustered strongly together, as did the non-sterilized eggs and oviposition bark samples (Fig. 1). This was expected, since the larvae were derived from the eggs, and the non-sterilized eggs were derived from the bark layer of the oviposition site. The microbial communities from environmental samples, which included non-oviposition wood and bark, as well as oviposition site wood, were the least similar to the eggs and larvae (Fig. 1). This suggests that larvae do not randomly acquire their gut microbial community from the environment, and that there is some contribution of microbes by the female during egg laying to the oviposition site and the surface of the egg. Manipulation of the oviposition site appeared to be limited to the bark layer, since there was little similarity in microbial communities among the oviposition wood, the larval gut, or the oviposition bark samples (Fig. 1).

To examine the relationship between specific bacterial OTUs and each sample type in more detail, constrained and unconstrained unimodal ordination analyses were performed. Initial unconstrained ordination of all samples and microbial OTUs demonstrated that there was a strong separation of insect and oviposition bark samples from environmental and oviposition wood samples (Fig 2, axis 1), and that these two groups were associated with strikingly different microbial communities. This ordination also demonstrated that the majority of the OTUs plotted were largely present in the environmental samples, but there were several OTUs highly correlated with eggs and larvae. For example, OTUs 224 and 581 were placed within the cluster of larvae and surface-sterilized egg samples (Fig. 2). Thus, these were likely not environmentally derived OTUs because they were not present in any other samples. Clone library analysis assigned these OTUs to microbial families Bacillaceae and Xanthomonadaceae, respectively (Table 2). Interestingly, both of these bacterial families were found in previous studies of the microbial community in *A. glabripennis* larvae collected in China [44], but their role in the microbial community is not known.

While unconstrained ordination explores total variability in the dataset, constrained ordination is necessary to determine how much variance can be explained by sample type. Using CCA, we showed that sample type significantly explained variation in community composition in

both egg and larvae and wood and bark samples. PERMANOVA allowed us to complete pair-wise comparisons to specifically determine which sample types differed from one another. All sample types were significantly different from one another, except for the relationship between non-oviposition bark and both non-oviposition wood and oviposition wood. The microbial profile of the non-oviposition bark samples was highly variable, which can be expected since these samples represent the passive microbial community present on the surface of a tree. These microbes could be impacted dramatically by environmental conditions.

According to CCA, OTUs 224 and 581 were found to be abundant in both surface-sterilized egg and larval samples (Fig. 3). Interestingly, OTU 581 was also abundant in the oviposition bark samples (Fig. 4), suggesting it may be associated with oviposition and may not be an internal egg symbiont. While the egg was surface-sterilized, this may not have completely removed all bacteria, particularly if the bacteria were associated with oviposition fluid used to attach the egg to the surface of the wood. Also, OTU 581 is not particularly abundant in the non-sterilized egg sample. This is likely because the data were analyzed according to relative abundance. In a diverse sample with many species, such as the non-sterilized eggs, a less abundant species will be less represented. Examination of the ARISA profiles revealed that OTU 581 was present in the non-sterilized egg samples, but represented on average only 22% of the microbial community in these samples, compared to 28–57% of the communities in the surface-sterilized egg, larvae, and oviposition bark samples. OTU 224 was only present in surface-sterilized egg and larval samples, suggesting that it was vertically transmitted, i.e., it came from inside the egg (Fig. 3). In contrast to what we would expect, OTU 224 was not present in the non-sterilized egg samples, but again this may be due to the relative abundance of this OTU within the microbial community population. A relatively low number of bacteria may be present inside the egg compared to the plethora of bacteria on the surface of the egg, and thus would not be represented strongly in the community profiles. In other insects, the number of bacteria vertically transmitted can vary from as few as tens of thousands in aphids [35] to 3×10^7 in stinkbugs [23].

In summary, our results demonstrate that specific microbial OTUs are associated with *A. glabripennis*, which are transmitted between generations. Also, microbial community profiles (ARISA) can be used to accurately predict the sample types investigated in this study. Overall microbial diversity decreased from samples that were environmental in nature (non-oviposition wood and bark), to insect and environmental samples (oviposition wood and bark), to microbes associated most strongly with the insect (surface-sterilized and non-sterilized eggs, and larvae; Fig. 1). This reduction in diversity and consistency of microbial profiles within the insect samples suggests that the insect is able to manipulate environmental microbial communities, probably by deposition of microbial species onto the oviposition site, by transmission of bacteria in association with the egg, and by selective larval acquisition of microbes, in order to ensure transmission of a refined community of bacteria that is likely important for the survival and growth of its offspring. These findings could lead to better understanding of which microbial species are critical for the survival of this insect and the development of techniques to alter this gut community to disrupt the digestive physiology of the host insect. This could have applications for control of wood-boring insects through gut microbial community manipulation.

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