

Bacterial Communities Associated With the Pine Wilt Disease Vector *Monochamus alternatus* (Coleoptera: Cerambycidae) During Different Larval Instars

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Abstract

We investigated the influence of larval instar on the structure of the gut bacterial community in the Japanese pine sawyer, *Monochamus alternatus* (Hope; Coleoptera: Cerambycidae). The diversity of the gut bacterial community in early, phloem-feeding larvae is significantly higher than in later, wood-feeding larvae. Many of these associates were assigned into a few taxonomic groups, of which Enterobacteriaceae was the most abundant order. The predominant bacterial genus varied during the five instars of larval development. *Erwinia* was the most abundant genus in the first and fifth instars, *Enterobacter* was predominant in the third and fourth instars, and the predominant genus in the second instars was in the Enterobacteriaceae (genus unclassified). Actinobacteria were reported in association with *M. alternatus* for the first time in this study. Cellulomonadaceae (Actinobacteria) was the second most abundant family in the first instar larvae (10.6%). These data contribute to our understanding of the relationships among gut bacteria and *M. alternatus*, and could aid the development of new pest control strategies.

Key words: gut bacteria, pyrosequencing, long-horned beetle, Enterobacteriaceae

Larvae of long-horned beetles (Cerambycidae) are xylophagous, which feed in subcortical tissues of healthy, dead, or decaying woody plants (Haack and Slansky 1987, Grünwald et al. 2010). Larval development occurs entirely within the host, requires at least several months, and can kill trees (Allison et al. 2004). Bacterial communities associated with subcortically feeding beetles are known to play important roles in facilitating larvae in surviving and developing within their host plants (Douglas 2009; Scully et al. 2013, 2014; Alves et al. 2016). Bacterial communities are reported to contribute to their host beetles' reproductive success, community interactions and niche diversification (Cardoza et al. 2006, Scott et al. 2008, Douglas 2009, Morales-Jiménez et al. 2013). Bacteria can contribute to the nutrition of phloeophagous and xylophagous larvae, which rely on a nutrient-poor food source, by exploiting nitrogen and carbon compounds in woody substrates and providing nutritional supplements that are absent from the substrate, such as amino acids and essential vitamins (Dillon and Dillon 2004, Geib et al. 2008, Morales-Jiménez et al. 2009, Berasategui et al. 2017).

The Japanese pine sawyer, *Monochamus alternatus* (Hope; Coleoptera: Cerambycidae), is the most important vector in Asia for long-distance transport of the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Steiner and Buhner) Nickle 1970, the invasive pathogen that causes pine wilt disease (PWD), (Morimoto

and Iwasaki 1972, Teale et al. 2011). This insect-transmitted pathogen has caused significant losses of pines in Japan, Korea, China, and Portugal (Rodrigues 2009, Chen et al. 2013, Alves et al. 2016, Van Nguyen et al. 2017). One of the major strategies to manage the nematode is to reduce between-tree transport by controlling *M. alternatus*. Owing to the importance of *M. alternatus*, various aspects of its physiology and genetics have been studied, such as its pheromones (Teale et al. 2011), transcriptome (Wu et al. 2016), pathogens (Ma et al. 2009), symbiotic fungi (Maehara et al. 2005) and tracheal bacteria (Alves et al. 2016). However, symbiotic intestinal bacterial communities are not well known for *M. alternatus*.

Like other *Monochamus* spp., *M. alternatus* feed on different sections of the wood during different larval stars. After hatching, early larvae feed first on phloem under bark. Later larvae feed in the xylem, including sapwood and heartwood, and form long, irregular mines (Yanega 1996). Thus, their food source changes substantially during development, and previous studies have not yet categorized how corresponding gut communities respond (Park et al. 2007, Ma et al. 2009, Scully et al. 2014, Alves et al. 2016).

A deeper understanding of the structure of the microbiome of the insect vector is required, and may contribute to the development of new approaches to managing PWD. To better understand how do symbiotic intestinal bacterial communities relate to larval feeding

stage, we used a metagenomics approach to investigate the gut-associated bacteria diversity and community structures.

Materials and Methods

Insect Collection and Dissection

Larvae of *M. alternatus* in different instars were removed from recently attacked *Pinus massoniana* (Lamb; Pinales: Pinaceae). Larvae in the first and second instar were collected from phloem and third, fourth, and fifth instar larvae were collected from sapwood and heartwood. Larvae were placed on ice, and then transported to the laboratory in sterile vials containing sterile moist paper. Sampling was performed in the town of Guan Tou, Lianjiang county in Fujian Province (N 26.15046°; E 119.59261°) in August 2015. All larvae were manually removed directly from galleries using fine forceps. Instars first through fifth were separated according to the width their head capsules (Liu et al. 2008) (Table 1). Three larvae in each instar were prepared for 16S rRNA analysis, for a total of 15 samples.

The larvae were surface sterilized with 70% ethanol for 1 min, and then rinsed twice with sterile water. After placing in 10 mM sterilized phosphate-buffered saline (138 mM NaCl and 2.7 mM KCl, pH 7.4), the larvae were dissected under a stereomicroscope using insect pins to obtain mid-guts and hindguts. One gut from each larva was transferred to a 1.5-ml microcentrifuge tube with 500 µl of tris-EDTA (10 mM tris-HCl [pH 8.0], 1 mM EDTA) separately and then homogenized several times with a plastic pestle, followed by vortexing for 3 min at the speed of 2500 r/min. The homogenate was centrifuged at 4000 r/min for 15 s to separate the microbial cells from the gut wall tissues and undigested food (Hu et al. 2013). The supernatant (containing bacteria) was transferred to new tubes for DNA extraction. All procedures were completed in a sterile environment.

DNA Extraction and PCR Amplification

DNA was extracted from the samples using QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany). Successful DNA isolation was confirmed by agarose gel electrophoresis. DNA concentration was assessed by a Nanodrop (Thermo Scientific) and quality was determined by agarose gel electrophoresis. The V3-V4 regions of the bacteria 16S ribosomal RNA gene were amplified by PCR using the following primers: 338F 5'-barcode-ACTCCTACGGGAGGCAGCA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3' (barcode is an 8-base sequence unique to each sample). The PCR products were then extracted from 2% agarose gels, and further purified by using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences) and quantified by QuantiFluor-ST (Promega).

High-Throughput Pyrosequencing and Bacterial Community Analysis

The purified DNA amplicons were then added with Illumina adapters by ligation (TruSeq DNA LT Sample Prep Kit), and the adapter-ligated DNA fragments were further amplified on an Illumina MiSeq

platform (San Diego, CA) for sequencing according to the standard protocols at Majorbio Bio-Pharm Technology, Shanghai, China. Raw fastq files were then demultiplexed and quality-filtered by using QIIME (version 1.17).

After pyrosequencing, the quality of the raw Miseq sequencing reads was checked with FastQC (Margulies et al. 2005, Andrews 2014). Raw reads were quality screened by using an average minimum quality score of 20. Barcodes and primers sequence were trimmed by using the Trimmomatic. After quality control and barcode assignment, operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using Usearch (version 7.1) and chimeric sequences were identified and removed using UCHIME. Mothur (<http://www.mothur.org/>) was used to sort sequences exactly matching the specific barcodes into different samples. Then, Sickle tool (<https://github.com/najoshi/sickle>) was used to perform the quality filtering to remove the reads with average quality score <50 or with any unknown bases. Then, the reads were assembled by Mothur command 'make.contigs' with the criteria of 'maxambig = 0', 'maxhomop = 8', and 'minoverlap = 10'. The quality-filtered reads were then processed by Mothur with commands 'trim.seq', 'pre.cluster', and 'chimera.uchime' to remove chimera and sequencing noise (Guo et al. 2015). Taxonomic classification of each sample was individually conducted using Ribosomal Database Project (RDP) (<http://rdp.cme.msu.edu/>) Classifier (version 2.6) with a confidence threshold of 50% (DeSantis et al. 2006, Wang et al. 2007, Cole et al. 2009, Quast et al. 2013). 'Aligner' and 'Complete Linkage Clustering' were applied to calculate richness and diversity indices including OTUs, Shannon Index, and Chaos index (Schloss et al. 2011). Sequences were rarefied to the lowest number of reads in the samples using QIIME script single_rarefaction.py. before statistical analysis. Rarefaction curve methodology was used to estimate the relationship between the expected OTU richness and sampling depth (Colwell et al. 2004).

Statistical Analysis

Principal component analysis (PCA) was performed using the vegan package for R (version 2.1) (Wang et al. 2012). Potential significant differences were analyzed by *t*-test, analysis of variance (ANOVA) and chi-square in SPSS version 18.0. (SPSS Inc.: Chicago, IL). Normality of the data was evaluated with the Kolmogorov Smirnov test.

Results

Pyrosequencing

A total of 13 samples were successfully sequenced, which excluded the data from one first instar and one third instar. The raw data set of all 13 samples contained 408,318 reads. After stringent quality assessment and data filtering, 79.2% high-quality reads were available for analysis. The average length of valid sequences was 448 bp. A total of 159,106 valid sequences (123 Mb in total) obtained from genomic DNA harvested from these gut-associated bacteria have been deposited into GenBank database (accession number: SRX2251637-SRX2251649).

Table 1. Mean width of larval heads and pronotum (\pm SE) of *Monochamus alternatus*, $n = 3$ samples per instar

Width of samples (mm)	Instar				
	1 st	2 nd	3 rd	4 th	5 th
Larval head	1.08 \pm 0.04	1.66 \pm 0.05	2.68 \pm 0.03	3.14 \pm 0.04	3.49 \pm 0.01
Larval pronotum	1.57 \pm 0.12	2.03 \pm 0.09	3.84 \pm 0.04	4.64 \pm 0.18	5.32 \pm 0.06

Bacterial Diversity Analysis and OTUs

To determine community richness and diversity, the Shannon index of diversity (H') and Simpson index (S) were determined for all samples (Table 2, Supplementary Table S1). The value of H' ranged from 0.09 to 1.48, and the value of S ranged from 0.29 to 0.97. The H' index showed that there were no significant differences among different stages (Kruskal-Wallis tests, $\chi^2 = 5.5238$, $df = 4$, $P = 0.238$). The S index, which gives more weight to dominant species, showed that significant differences among different stages (Kruskal-Wallis tests, $\chi^2 = 10.341$, $df = 4$, $P = 0.035$). The diversity of bacteria in early larvae (first, second instars) was significantly higher than that of the later larvae (third, fourth, fifth instars) ($P < 0.05$), with Student's t -tests for S index ($F = 27.3$, $df = 11$, $P < 0.001$) and H' index ($F = 5.5$, $df = 11$, $P = 0.038$).

OTUs were identified at genetic distances of 0.03 (species level), 0.05 (genus level) and 0.2 (phylum level) by using quality sequences with a read length of ≥ 50 bp per sample. A total of 75 OTUs_{0.03} were obtained from the 13 samples (Supplementary Table S1, S2, Fig. S1). The number of OTUs ranged from 9 to 44 clusters per sample. There were no significant differences among different instars (Kruskal-Wallis tests, $\chi^2 = 5.329$, $df = 4$, $P = 0.255$), and no significant differences between early versus later larvae (t -tests $F = 0.6$, $P = 0.454$) in the number of OTUs. There were 145,085 reads belonging to six clusters (OTU3, OTU9, OTU28, OTU40, OTU55, and OTU62), which accounted for 91.2% of the total 159,106 reads (Supplementary Table S2). According to the analysis of the shared and unique OTUs and reads between the different instars (Fig. 1), the gut associated bacterial communities hosted by *M. alternatus* early larvae displayed some commonalities with later larvae. A total of 54 OTUs and 20,758 reads were shared between early larvae (including first and second instars) and later larvae (including third, fourth, fifth instars) (Fig. 1c and 1f).

Taxonomic Assignment of Bacterial Symbionts and Dominant Taxa

After taxonomic-based analysis, all OTUs were assigned to four different phyla: Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. At the phylum level, the composition of the bacterial community structure was similar across all five instars (Fig. 2, Supplementary Fig. S2). Proteobacteria was the dominant phylum, representing on average 93.1% of the reads in each sample, followed by Firmicutes (3.2%), Bacteroidetes (2.6%), and Actinobacteria (1.0%) (Fig. 2, Supplementary Table S3). In first instar larvae, Proteobacteria comprised 86.9% and Actinobacteria comprised 12.4%. Proteobacteria accounted for 97.7% in second instar and 98.6% in third instar larvae. In the fourth instar larvae, Proteobacteria comprised 85.0%, Firmicutes comprised 13.0% and

Bacteroidetes comprised 2.0%. In fifth instar larvae, Proteobacteria accounted for 97.3% (Fig. 2).

Enterobacteriaceae (Proteobacteria) was the most abundant family in all samples, representing 86.1%, 94.5%, 97.7%, 86.6%, and 91.9% from first to fifth instar larvae, respectively. The Enterobacteriaceae present included *Enterobacter*, *Erwinia* and Enterobacteriaceae-like genera shown in the Fig. 3. Cellulomonadaceae (Actinobacteria) was the second most abundant family in the first instar larvae (10.6%). At the genus level, all samples share some taxa. *Enterobacter* (Enterobacteriaceae, γ -Proteobacteria), *Erwinia* (Enterobacteriaceae, γ -Proteobacteria), *Gordonia* (Moraxellaceae, Actinobacteria), and *Chryseobacterium* (Flavobacteriaceae, Bacteroidetes) were each present in at least one larval gut of all *M. alternatus* instars (Fig. 3).

Combining the relative abundance of reads and the results of the taxonomic-based analysis, *Erwinia* is the most abundant bacterial genus in first ($58.3 \pm 3.7\%$) and fifth ($82.0 \pm 16.9\%$) instar larval guts. *Enterobacter* is the predominant bacterial genus in third ($97.1 \pm 2.3\%$) and second ($49.2 \pm 14.2\%$) instar guts, and of variable relative prevalence in first ($9.8 \pm 2.5\%$), second ($31.7 \pm 20.1\%$), and fifth ($4.4 \pm 7.4\%$) instar guts. Enterobacteriaceae are predominant in the second instar guts ($60.7 \pm 9.5\%$). Its frequencies in other stages were: first: $18.0 \pm 2.7\%$, fourth: $30.5 \pm 9.6\%$, fifth: $5.0 \pm 8.3\%$. Genera belonging to Cellulomonadaceae comprised the third most abundant bacteria in the first ($10.6 \pm 4.5\%$) instar guts. The genus *Lactococcus* (Streptococcaceae, Firmicutes) was the third abundant bacteria in fourth ($13.0 \pm 19.8\%$) instar guts.

PCA indicated potentially correlated variables among bacterial distributions. The first two principal components (PCs) explained 67.2% of the variance of the bacterial communities (Fig. 4). PCA showed that samples obtained in different instars clustered into five separate groups, while, B3 (second instar) and E1 (fifth instar) were clustered with D (fourth instar). This analysis indicated that the gut bacterial communities from larvae within the same instar tended to be close.

Discussion

This study provides new insight into the gut bacterial diversity of *M. alternatus* among different larval instars by high-throughput pyrosequencing. Overall, the diversity of gut-associated bacteria in early larvae was higher than in later larvae, which might be related to dietary differences between them. Proteobacteria was the most dominant phylum, representing on average 93.1% of the reads in each *M. alternatus* sample. In particular, genera in the family Enterobacteriaceae (γ -Proteobacteria) represent a major fraction, and occurred in the guts of all larvae sampled. γ -Proteobacteria

Table 2. Total number of reads obtained by pyrosequencing for samples of each instar, and the respective indexes of diversity including the number of OTUs, Shannon-Wiener index (H') and Simpson index (S)

Larval stage	Reads	Similarity threshold (0.97)					
		OTU	Ace	Chao1	Coverage	H'	S
1 st	10858	24	36	29	0.999355	1.24 ± 0.01	0.40 ± 0.02
2 nd	12525	22	37	27	0.999491	0.92 ± 0.10	0.50 ± 0.01
3 rd	11406	18	23	21	0.999606	0.19 ± 0.07	0.94 ± 0.02
4 th	11924	19	30	24	0.999406	1.26 ± 0.07	0.33 ± 0.01
5 th	13744	36	38	37	0.999661	0.74 ± 0.19	0.71 ± 0.08

Data of Reads, OTU, ace, chao and coverage are means; data of H' and S are means \pm SE, $n = 2$ (1st and 3rd), and $n = 3$ (2nd, 4th, 5th).

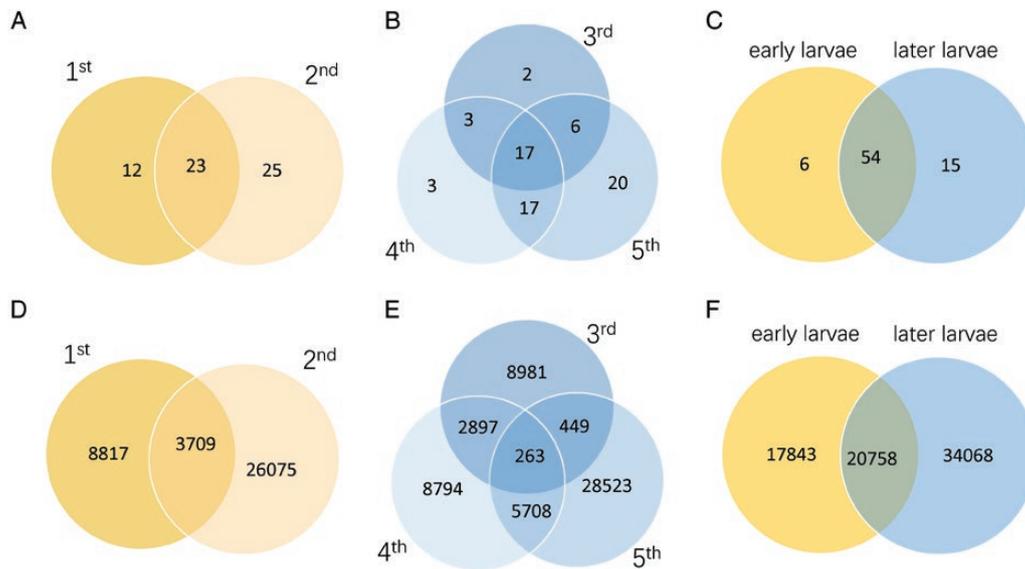


Fig. 1. Venn diagrams representing the number of shared and unique OTUs and reads. (A) OTUs between first and second instar larvae; (B) OTUs among third, fourth, fifth instar larvae; (C) OTUs between early larvae and later larvae; (D) reads between first and second instar larvae; (E) reads among third, fourth, fifth instar larvae; (F) reads between early larvae and later larvae.

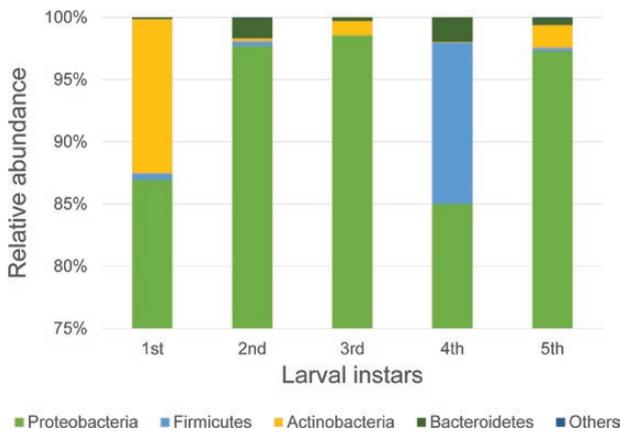


Fig. 2. Relative abundance of the predominant bacterial phylum from each insect sample (more than 0.1% of the total number of reads). Data shown are means.

(87.9%) was also reported to associate with *Monochamus galloprovincialis* (Olivier; Coleoptera: Cerambycidae), an important vector of PWN in Europe (Sousa et al. 2001, Naves et al. 2007, Vicente et al. 2013). The predominance of Enterobacteriaceae in the gut bacterial community of *M. alternatus* is in agreement with reports from other phytophagous insects from several feeding guilds (Broderick et al. 2004, Delalibera et al. 2005, Schloss et al. 2006, Hu et al. 2016). Enterobacteriaceae were also found to be the predominant family (52.2%) in the bacterial communities colonizing the trachea of *M. galloprovincialis* and *M. alternatus* (Alves et al. 2016). In the abdomen of *M. galloprovincialis*, the most abundant genus was *Serratia* (95%) (Enterobacteriaceae) (Vicente et al. 2013), whereas no *Serratia* was detected in *M. alternatus* gut in this work. The genera composed bacterial community might varied among different *Monochamus* spp. In the only culture-dependent study of gut-associated bacteria of *Monochamus* spp., 14 of 16 strains were identified as γ -Proteobacteria in the gut of *M. alternatus*, with the other two strains belonging to Firmicutes (Park et al. 2007). Xylanase activity

could be detected in one of these isolates (Park et al. 2007). Likewise, in our other culture-dependent research, several cellulolytic bacteria belonging to Proteobacteria, Firmicutes, and Bacteroidetes were also found in the guts of *M. alternatus* larvae. It was confirmed that gut associated bacteria could help host insects degrade wood fabric.

Like other wood-boring beetles, *M. alternatus* was associated with a core group of microbiota, which seem likely to influence host success (Hu et al. 2013, Mason et al. 2016). *Erwinia* and *Enterobacter* are consistently found in a variety of insect guts, including Diptera, Lepidoptera, Homoptera, Coleoptera (Harada et al. 1997, Watanabe and Sato 1998, Basset et al. 2003, Hu et al. 2013, Aylward et al. 2014, Mason et al. 2016). Interestingly, *Enterobacter* is also associated with PWN carried by *M. alternatus* in China (Han et al. 2003). This supports the hypothesis that PWN can harbor bacteria from the insect. These representatives of the *Enterobacteriaceae* might benefit xylophagous hosts because of their ability to hydrolyze many polysaccharides (Scully et al. 2013). A few species can persist in the gut's harsh environment, which consists of digestive enzymes, high redox potential and high ionic strength (Vallet-Gely et al. 2008). *Erwinia* are likely to have been acquired during larval feeding, based on documentation of this pathway in *Drosophila melanogaster* Meigen and *Frankliniella occidentalis* Pergande (De Vries et al. 2001, 2004; Basset et al. 2003). *Erwinia* produce a set of depolymerizing enzymes, such as pectinases, cellulases, proteases, phospholipases and xylanases that can degrade plant cell wall components, and include some phytopathogenic species (Barras et al. 1994). Interestingly, *Erwinia* was also the dominant genus, and its relative abundance changed during different development stages, in the guts of thrips (De Vries et al. 2001). Thrips benefitted from gut *Erwinia* when they fed on a diet of only leaves, but experienced negative effects of *Erwinia* when they fed on a mixed diet of leaves with pollen (De Vries et al. 2004). This suggests that *Erwinia* might represent a diet-dependent switch from mutualism to parasitism in some host insects. The prevalence of *Erwinia* in all five instars might also be related to digestion of lignocellulose. The relative abundances of *Erwinia* among different instar larvae might be influenced by differences in feeding substrates.

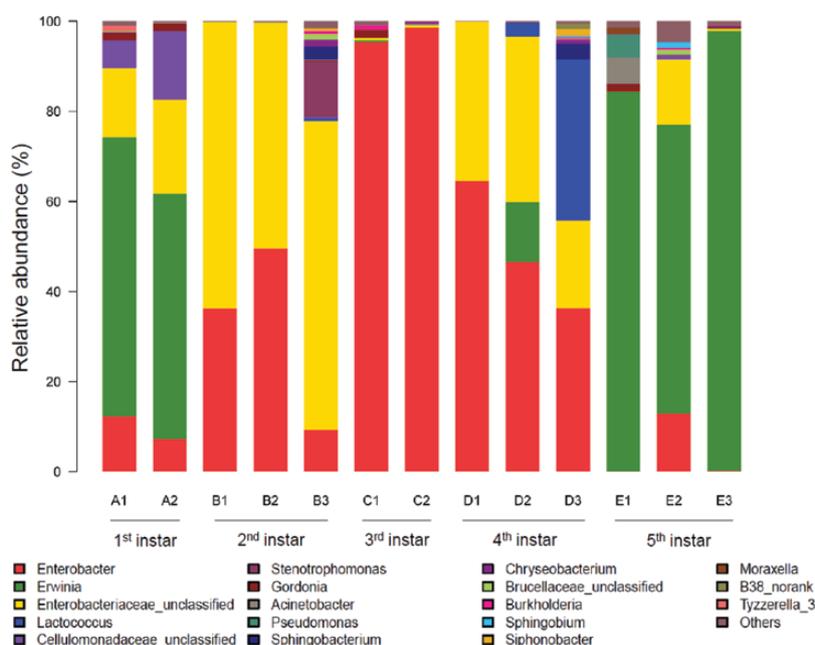


Fig. 3. Relative abundance of the predominant bacterial genus for each insect sample (only genera with more than 0.1% relative abundance were shown).

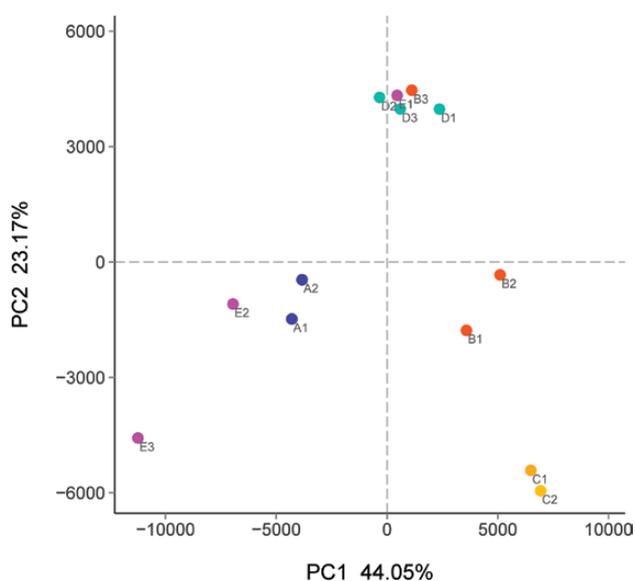


Fig. 4. Principal component analysis (PCA) showing the potentially correlated variables of the bacterial distribution pattern in each *Monochamus alternatus* sample: A1, A2 represent first instar samples; B1, B2, B3 represent second instar samples; C1, C2 represent third instar samples; D1, D2, D3 represent fourth instar samples; E1, E2, E3 represent fifth instar samples.

Cellulomonadaceae (Actinobacteria), another major component in first instar larval gut, are known to produce a large variety of hydrolytic starch, xylan and cellulose-degrading enzymes (Stackebrandt et al. 2006). Actinobacteria were reported for the first time in association with *M. alternatus* in this study. *Lactococcus* (Firmicutes) is the third abundant genus of fourth instar larval gut bacteria. In nature, *L. lactis* occupies a niche consisting of plant or animal surfaces and the animal gastrointestinal tract. It is believed to be dormant on the plant surfaces and to multiply in the intestinal tract after feeding (Bolotin et al. 2001). All these dominant gut

bacteria may be sources of enzyme that contribute to nutrition of the host insect.

The highest levels of *Enterobacter* were found in the third and fourth instar communities, with a high abundance also showing in other instars. *Enterobacter* was also predominant in the larval gut of long-horned beetle *Rhagium inquisitor* L. (Coleoptera: Cerambycidae) (Grünwald et al. 2010).

These results could potentially lead to improved protection of pine from the *M. alternatus*-*B. xylophilus* complex. *Enterobacter gergoviae*, a gut bacterium of the pink bollworm (*Pectinophora gossypiella* Saunders), was exploited as a biopesticide vector by transforming it to express Cyt1A, an insecticidal protein lethal to mosquitoes and black fly larvae (Kuzina et al. 2002). Also, the gut bacterium *Enterobacter cloacae* was used to control the mulberry pyralid (*Glyphodes pyloalis* Walker; Lepidoptera: Crambidae) by transforming the ice nucleation gene to increase the supercooling point of *G. pyloalis*, thus causing increased mortality (Watanabe et al. 2000). Detailed knowledge about the functions of these gut bacteria and their modes of transmission are necessary before similar strategies could be successfully devised and implemented against invasive longhorned beetles or their phytopathogenic symbionts. The results enlarged our understanding of the relationships among gut bacteria and *M. alternatus*, and could encourage the development of new pest control strategies in the future.

Supplementary Material

Supplementary material can be found at *Journal of Insect Science* online.

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