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The Bacterial Community of Entomophilic Nematodes and Host Beetles

Sneha L. Koneru^{1,2}, Heilly Salinas¹, Gilberto E. Flores¹, and Ray L. Hong^{1,*}

¹Department of Biology, California State University, Northridge, Northridge, California, United States of America

Abstract

Insects form the most species-rich lineage of Eukaryotes and each is a potential host for organisms from multiple phyla, including fungi, protozoa, mites, bacteria, and nematodes. In particular, beetles are known to be associated with distinct bacterial communities and entomophilic nematodes. While entomopathogenic nematodes require symbiotic bacteria to kill and reproduce inside their insect hosts, the microbial ecology that facilitates other types of nematode-insect associations is largely unknown. To illuminate detailed patterns of the tritrophic beetle-nematode-bacteria relationship, we surveyed the nematode infestation profiles of scarab beetles in the greater Los Angeles area over a five-year period and found distinct nematode infestation patterns for certain beetle hosts. Over a single season, we characterized the bacterial communities of beetles and their associated nematodes using high-throughput sequencing of the 16S rRNA gene. We found significant differences in bacterial community composition among the five prevalent beetle host species, independent of geographic origin. Anaerobes *Synergistaceae* and sulfate-reducing *Desulfovibrionaceae* were most abundant in *Amblonoxia* beetles, while *Enterobacteriaceae* and *Lachnospiraceae* were common in *Cyclocephala* beetles. Unlike entomopathogenic nematodes that carry bacterial symbionts, insect-associated nematodes do not alter the beetles' native bacterial communities, nor do their microbiomes differ according to nematode or beetle host species. The conservation of Diplogastrid nematodes associations with *Melolonthinae* beetles and sulfate-reducing bacteria suggests a possible link between beetle bacterial communities and their associated nematodes. Our results establish a starting point towards understanding the dynamic interactions between soil macroinvertebrates and their microbiota in a highly accessible urban environment.

Keywords

microbiome; entomophilic nematodes; beetles; bacteria; next-generation sequencing

*corresponding author: ; Email: ray.hong@csun.edu

²Current address: Imperial College London, Department of Life Sciences, London, United Kingdom

Data Accessibility

- DNA sequences: Genbank accessions JX649208-JX649221; NCBI SRA: PRJNA294696.

Introduction

A species' microbiome is increasingly recognized as an ineluctable part of its characteristic physiology. In response to changing environments, microorganisms can alter host phenotypes because their shorter generation time allows faster adaptations than variations in the host genome (Dheilly et al. 2015). Bacterial symbionts can also enable novel changes in the host life cycle and trophic level, as in nematodes that have evolved strong associations with larger organisms, including plants and animals, through specific bacterial associations (Goodrich-Blair and Clarke 2007; Hallem et al. 2007; Johnson and Rasmann 2015). In one extreme of the insect-nematode-bacteria association, entomopathogenic nematodes (EPN) are obligate parasites that infect and kill insects by releasing their gut-associated bacterial symbionts into the hosts (Kaya and Gaugler 1993; Adams et al. 2006). The most well-studied EPNs belong to the genera *Steinernema* and *Heterorhabditis* that carry species-specific strains of enteric *Xenorhabdus* and *Photorhabdus* bacteria, respectively (Poinar 1972; Grewal et al. 1993a). Since recruitment of bacterial symbionts for entomopathogenicity evolved more than once in nematodes (Poinar 2009; Sudhaus 2009), identifying the bacterial taxa associated with entomophilic, but non-pathogenic nematodes will enable us to better understand how existing nascent partnerships contribute to the continual evolution of nematode parasitism.

Within this framework, the recruitment of previously casual bacterial associations may be considered part of the preadaptations necessary for entomophilic nematodes to evolve into parasites. Non-parasitic insect-nematode associations include phoresy and necromeny, which do not require having their insect hosts to complete their life cycles (Kiontke and Sudhaus 2006; Mayer et al. 2009). For example, the phoretic *Caenorhabditis drosophilae* nematode larvae associate with a cactus fly *Drosophila nigrospiracula* only for dispersal (Kiontke 1997; Kiontke and Sudhaus 2006). Necromenic nematodes, however, wait for their invertebrate hosts to die before resuming development on the microorganisms of the decaying carcasses (Poinar 1986; Sudhaus and Schulte 1989). *Pristionchus* and *Diplogasteroides* are necromenic nematodes that associate with living scarab beetles as dauer juveniles and resume reproductive development after their hosts die (Manegold and Kiontke 2001; Herrmann et al. 2006b; Weller et al. 2010). Because necromenic nematodes consume the bacteria from their deceased insect hosts, and are interacting directly with the host microbiome, necromeny is considered an intermediate step towards entomopathogeny under the preadaptation concept (Sudhaus 2009).

Recent studies suggest the existence of such transitional necromeny that may lead to obligate entomopathogeny, if the nematodes are able to gain a more specific mutualistic relationship with the bacteria (Ye et al. 2010; Torres-Barragan et al. 2011a). For example, several well-studied species of *Oscheius* nematodes exhibit the necromenic lifestyle, including *O. myriophila* and *O. colombiana* (Poinar 1986; Sudhaus and Schulte 1989; Stock et al. 2005). A related species, *O. carolinensis*, can penetrate, reproduce, and kill insect hosts as a facultative entomopathogenic nematode when associated with the bacteria *Serratia marcescens*, although *O. carolinensis* does not require *Serratia* for survival as a free-living nematode (Ye et al. 2010; Torres-Barragan et al. 2011b). Unlike the internal bacterial symbionts of the Steinernematid and Heterorhabditid EPNs, *S. marcescens* bacterium is only

associated externally with *O. carolinensis*. This ability of entomophilic nematodes to associate with pathogenic bacteria suggests that entomopathogenicity may evolve from non-parasitic entomophilic nematodes into parasitic ones through initially low specificity and non-obligate affiliations with bacteria (Dillman et al. 2012).

Could entomophilic nematodes transition towards parasitism by first associating with certain bacteria that can alter the microbiome of their host insects without symptoms of pathogenicity? Such ‘transitional’ entomophilic nematodes may be identified by measuring the potential impact of their associated bacteria on the bacterial communities of their insect hosts. To address this question, we surveyed scarab beetles with varying degrees of nematode infestations and used high-throughput direct sequencing of the 16S rRNA gene to characterize the entire bacterial communities of beetles and their associated nematodes. Specifically, we asked if beetle species differ significantly their bacterial communities, and if nematode infestation can affect the bacterial diversity of their host beetles.

Materials and Methods

Beetle collection

We captured active beetles near lights after dusk in the two months from late May to late July from 2010 to 2014 around Los Angeles County (California, USA) and Wooster, Ohio. Although the specific life history traits of these beetles are not well studied, previous investigations of related species suggest the beetle larval “white grubs” live underground for at least a year by feeding on plant roots, and are recognized as economically important garden pests (Koppenhofer et al. 2000). Beetles were captured alive with minimal gloved-hand contact near light sources during the first three hours after sunset and kept at 4°C until dissection for nematode isolation and DNA extraction within two weeks. Each beetle was dissected sagittally from mouth to anus on a petri-dish lid using forceps and scissors, which were surface sterilized in between dissections with 5% hypochlorite solution (bleach), followed by 70% ethanol. Beetle species were identified by the carapaces color and morphology. In 2014, one half of each beetle was transferred to a fresh 3.5 cm diameter NGM plate (Nematode Growth Medium, 1 L: 34 mM NaCl, 22 mM KH₂PO₄, 2.9 mM K₂HPO₄, 0.4% Bacto-tryptone, 2% Bacto-agar, 5 ug/ml cholestrol) and allowed to decompose on the plate. The other half of each beetle was frozen immediately at –20°C until extraction for microbial DNA. The plates with decomposing beetles were monitored using a Leica S6E microscope for nematodes every two days for up to 14 days. However, most nematodes appeared on the plate within 10 days.

Most *Cyclocephala* beetles can be categorized based on morphological descriptions— *C. pasadenae* has a slenderer body than *C. hirta*, with no dent at the edge of the pronotum and with a dark brown carapace, while *C. hirta* is bulkier with a slight dent at the edge of pronotum and a lighter tan carapace. However, many beetles captured possessed intermediate traits and thus it is likely that we found hybrids between *C. pasadenae* and *C. hirta* (Evans and Hogue 2006)(J. Hogue, Pers. Comm.). *C. longula* has a distinct ebony colored carapace and red pronotum that are easily distinguishable from *C. pasadenae* and *C. hirta*. The infestation rate of *Amblonoxia* included three beetles from 2009 in addition to the 2010–2014 study.

Nematode culture

The nematodes that appeared in the plates with decomposing beetles were transferred into sterile NGM plates to maintain both native bacteria and diversity of the wild nematodes. Nematodes grown on the native bacteria were used in the extraction of microbial DNA for nematode microbiome characterization. A single hermaphrodite or gravid female was transferred into a NGM plate seeded with OP50 bacteria to establish isogenic lines and to identify nematode species. Because more than one nematode emerge from each beetle carcass, consistent effort was made to isolate at least two isogenic females per beetle for subsequent 18S rRNA identification. The mating system of the nematodes was noted as hermaphroditic or gonochoristic (male/female). The low frequency of males after three generations indicated that the nematode species is hermaphroditic. In the cultures that had males, single virgin females were isolated and checked for progeny later. The absence of progeny confirmed that the strain is gonochoristic. Using the available genetic mutants for *P. pacificus*, we tested for conspecifics based on the ability of males from the wild strains to cross with the *pdl-5* dumpy-allele to produce non-dumpy F1 progeny before utilizing these strains in the chemoattraction assay.

Molecular identification of nematode species

To extract genomic DNA for species identification, five adult nematodes from the isogenic lines were taken into 10 µl of single worm lysis buffer (30 mM Tris pH 8, 8 mM EDTA, 100 mM NaCl, 7% NP400, 7% Tween, 100 µg/ml Proteinase K) and incubated at 65 °C for 1 h, then heated to 95°C for 15 min. This worm lysate was diluted by adding 70 µl of dH₂O and stored at -20°C. The small subunit ribosomal RNA gene (SSU) was amplified by using the primers RH5401 (SSU18A)(5'-AAAGATTAAGCCATGCATG-3') and RH5402 (SSU26R) (5'-CATTCTTGGCAAATGCTTTTCG-3') (Blaxter et al. 1998; Floyd et al. 2002; Herrmann et al. 2006b). A 25 µl volume PCR reaction was performed using 1.5 µl of both primers (to a final concentration of 0.6 mM), 2 µl of worm lysate and 12.5 µl of Apex Taq Master Mix (Genesee Scientific, La Jolla, CA). The reaction mixture was subjected to initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 75 seconds. If the expected 900 bp product appeared on a 1% agarose gel, then the other half of the PCR reaction was purified for DNA using DNA Clean and Concentrator (Zymo Research, Irvine, CA) and sequenced using the internal sequencing primer RH5403(SSU9R)(5'-AGCTGGAATTACCGCGGCTG-3'). For species identification, ~600 nucleotides were queried against GenBank by BLASTN and aligned using Geneious 5.0 software (Biomatters, Auckland, New Zealand). Statistical significance for biased nematode associations with the three sympatric beetle species (*Amblonoxia*, *Cyclocephala pasadenae*/*hirta*, and *Serica*) was determined by Pearson's chi-squared test using R.

Chemoattraction Assay

Population chemoattraction assays were performed as described previously for *P. pacificus* (Hong and Sommer 2006). Briefly, nematodes from well-fed cultures with mostly adults were washed two times in M9 buffer and placed on 8.5-cm NGM agar plates. The chemotaxis index (CI) for each plate was calculated as the fraction of the nematodes on the

attractant area minus those on the counter-attractant (ethanol) area, divided by the total number of nematodes counted. To reduce genetic heterogeneity in each wild *P. pacificus* isolate, we established near-isogenic lines by single worm descent for ten generations before performing chemotaxis assays. For each strain, at least 10 replicate assays with >100 J3 to adult worms per assay were measured after 15 hours at ~23°C. The oriental beetle pheromone, ZTDO ((*Z*)-7-tetradecen-2-one, Bedoukian Research, Danbury, CT) was diluted in ethanol to a 10% working concentration (v/v).

DNA extraction and 16S rRNA gene sequencing

To extract bacterial DNA from the nematodes, five adult nematodes maintained on native bacteria within the first two generations (3–17 days post dissection) from individual beetles were picked into 10 µl of lysis solution (10 µl of dH₂O, 2 µg/µl of Proteinase K), incubated at 65°C for 1 hour, and heated to 95°C for 10 min. The resulting lysate was used for PCR to amplify the 16S rRNA gene. The MoBio BiOstic Bacteremia DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) was used to extract bacterial DNA from the beetle samples collected in 2014. DNA was extracted from a total of 278 beetles belonging to 8 different beetle species from greater Los Angeles, California, and Wooster, Ohio on multiple days in cohorts of 10–15 beetles. The 12-base error-correcting Golay primers (515F/806R) designed for Illumina sequencing by Caporaso et al. (2010) were used to amplify variable region 4 (V4) of the 16S rRNA gene. For each beetle and nematode sample, 25 µl DreamTaq PCR Master Mix reactions (ThermoFisher, Waltham, MA) were performed in triplicates and combined, including a negative control without template for every 96-well plate. The PCR reactions that worked (from 270 beetle and 74 nematode DNA samples) were quantified using PicoGreen dsDNA assay kit (Invitrogen, Carlsbad, CA). Quantified PCR products were pooled in equimolar concentrations and cleaned using MoBio UltraClean PCR Clean-up. Each PCR plate contained a negative control without DNA template, resulting in 12 total negative control reactions. The library was then sequenced using the MiSeq™ System v2, 2x150bp (MS-102-2002, Illumina, San Diego, CA), which allows for paired-end sequencing. We were unable to amplify 16S rRNA genes using the same protocol described above from DNA extracted from *P. pacificus* dauer juveniles or starved non-dauer nematodes, suggesting that the bacterial load is below the threshold of PCR detection in these nematode stages.

Sequence processing and analysis

Raw fastq files of 16S rRNA genes were processed using the UPARSE pipeline (Edgar 2013) with a few modifications. Briefly, a custom Python script was used to demultiplex and prepare sequence files for paired-end assembly and clustering. Paired sequences were assembled using UPARSE with the following parameters: fastq_truncqual 3, fastq_maxdiffs 1, fastq_minovlen 20, fastq_minmergegelen 200. Assembled sequences were filtered at a maximum value of 0.5 on average, only one nucleotide in every two sequences is potentially incorrect. Quality sequences were then dereplicated and singletons were removed. Operational taxonomic units (OTUs) were assigned with UPARSE at a 97% sequence identity threshold. Taxonomy was assigned to representative sequences of each OTU using the RDP classifier (Wang et al. 2012) with a confidence threshold of 0.5 against the Greengenes 13_5 database (DeSantis et al. 2006) as implemented in QIIME v. 1.6.0

(Caporaso et al. 2010). QIIME was also used to calculate various alpha diversity metrics (richness, phylogenetic diversity (Faith 1992) and the Shannon Diversity Index). Similarity in community composition between each pair of samples (beta-diversity) was determined using Bray-Curtis similarities as implemented in PRIMER v6 (PRIMER-E, UK). All metrics were calculated at an equal number of sequences per sample (n=5000) to eliminate biases introduced by sequencing depth. 257 beetle (out of 270 successful PCR) and 74 nematode (out of 77) samples passed through the rarefaction step and were used for subsequent analysis. A total of 8709 unique OTUs (range of 5-1152 per sample) were observed in the rarefied sequences. Thirty-seven percent (2761) of these OTUs that were observed only once were omitted from further analysis.

Three of the four negative pooled PCR controls showed faint bands in the gels and were sequenced. There were 549 combined OTUs present in the negative controls, which constitute 6% of the OTUs present in the beetle samples and 39% of the OTUs present in the nematode samples. We analyzed the data by removing these 549 OTUs from beetle and nematode data and we found that the trends remained the same. Since genuine sample OTUs could overlap with contaminating OTUs, the analysis of sequence data was performed without the removal of OTUs present in negative controls (Salter et al. 2014).

Statistical analyses of Microbial Community

Eight (out of 257) beetle samples were removed because of their low sampling numbers: five unidentified black beetles, two *Phyllophaga hirticulata* (OH), and one *Polyphylla decemlineata*. Two *C. longula* beetles (beetles 171 and its control 172, out of 249 beetle samples), were also taken out of analyses due to their low number in the category of *C. longula* beetle with nematode, resulting in 247 beetles belonging to five species remaining for microbiome analyses. One nematode sample (nematode 631 out of 74 nematode samples) was removed from analysis because the species could not be identified using molecular methods or mating assays, resulting in 73 nematode samples that were used for microbiome analyses. Diversity of the bacterial communities in the samples was determined by Shannon index and OTU-based richness in QIIME. The alpha diversity metrics were calculated for 10 individual sampling events and averaged for samples belonging to the same sample category. To determine if alpha diversity was significantly different between each pair of groups of samples we used the nonparametric Kruskal-Wallis test in R (Team R 2013).

To compare community composition between samples, we used taxonomy-based Bray-Curtis similarity index for each pair of samples resulting in a distance matrix. Subsequent multivariate analyses were conducted in PRIMER v6 (PRIMER-E, UK). We used two-way nested PERMANOVA design to examine whether beetle species and/or geographical site were significant predictors of variability in bacterial communities of beetles (McArdle and Anderson 2001). Geographical site was treated as a fixed factor and beetle species was treated as a random factor. To test for significant differences in community composition between the beetle species, we used one-way analysis of similarity (ANOSIM). We used two-way nested PERMANOVA to examine whether nematode species and/or beetle host species were significant predictors of variability in bacterial communities of nematodes.

Host beetle species was treated as a fixed factor and nematode species was treated as a random factor. Variation of bacterial taxonomic composition among samples was visualized using principal coordinates analyses (PCoA).

Results and Discussions

Beetle-nematode associations

The beetles from 12 locations (up to 48 km apart) can be categorized into five recognizable species, collectively referred to as June beetles (Hogue 2015): *Cyclocephala pasadenae*, *Cyclocephala hirta*, *Cyclocephala longula*, *Amblonoxia palpalis*, and *Serica alternata*. First, to test if the nematodes could be easily dislodged from live beetles, as would be the case in phoretic nematodes that reside on the surface of insect hosts (Kiontke and Sudhaus 2006; Okumura et al. 2013), we dunked 35 *C. pasadenae* and *C. hirta* beetles captured at the same location and time period into water but did not observe emergence of nematodes. However, subsequent dissection did yield nematodes in eight out of these same 35 ‘soaked beetles.’ This result suggests that the nematodes likely reside in parts of the body difficult to dislodge (endo-phoretic), or that the death of the host was a necessary trigger for nematode dispersal and resumption of development (necromenic), or both depending on the type of association (Sudhaus 2009). Such nematodes could reside in the hindgut, genital chamber, or other cavities (Richter 1993; Kuhne 1994; Kiontke 1997; Manegold and Kiontke 2001; Kiontke and Sudhaus 2006), necessitating dissection of live beetles to isolate the wild nematodes.

The isolation of nematodes from their beetle hosts was performed in two phases. The identification of nematode species in the first four years (2010–2013) from 725 beetles informed us of the beetle species that were abundant or had high infestation frequency, which guided us to mount a more comprehensive survey in 2014 using 712 beetles that included the identification of bacterial compositions of both the beetles and nematodes (Table S3). All together, we found approximately 12% (177/1437) of the five species of beetles were infested with nematodes. Species assignments were made primarily by 18S rRNA sequencing, as well as the presence of a pharyngeal grinder (Rhabditidae) and mode of reproduction (hermaphroditic or gonochoristic) (Blaxter et al. 1998; Holterman 2006; Mayer et al. 2007). In 2014, 92% (71/77) of the beetle-derived nematodes could be maintained on both the native bacteria from the beetle and *E. coli* OP50. However, three samples (samples 581, 603, 631) could not be cultured for unknown reasons, while another three obligate entomopathogenic nematode *Steinernema carpocapsae* (samples 343, 635, 649) were isolated without their own symbiotic bacteria (*Xenorhabdus*) and could not be cultured.

The nematode infestation rate varied widely among the five beetle species (Table 1). *Amblonoxia palpalis* Horn (syn. *Parathyces palpalis* Hardy) (Evans and Smith 2005) has the largest body size and was the rarest species among the beetles used for this study, although at least one *Amblonoxia* was isolated every year. Approximately 77% (13/17) of *Amblonoxia* beetles were infested with a single species of a large, gonochoristic (male-female) *Diplogasteroides* nematode, provisionally named *Diplogasteroides sp.1* (Fig. 1). In contrast, the smallest beetle we sampled, *Serica alternata*, showed a much lower infestation

rate of ~5.9% (25/422), and was the host of seven nematode species, including the exclusive host to the hermaphroditic *Diplogasteroides* sp. 2.

The most consistently abundant beetle species we captured every year were the masked chafers made up of the *Cyclocephala* species complex, *C. pasadenae* Casey and *C. hirta* LeConte (Fig. 1). To study the influence of geography, we also sampled 31 *C. hirta* beetles in 2014 from Wooster, Ohio, which were sampled during the first study on *Pristionchus* infestation patterns in the United States (Herrmann et al. 2006a). Over the five years, we found ~14% (128/907) of *C. pasadenae* and *C. hirta* in Los Angeles were infested with eight nematode species, whereas a related species, *C. longula*, showed the lowest infestation rate at 1.7% (1/60) (Table S1). Of the infesting nematodes, *P. pacificus* was the most commonly isolated nematode (36%, 46/128 infested beetles), followed by *Oscheius* sp. (29%, 37/128), and *P. maupasi* (19%, 24/128). *P. maupasi* represents a species complex consisting of at least two nematode species with different modes of reproduction that could not be resolved with the partial 18S rRNA sequence and are known to be able to cross hybridize (Herrmann et al. 2006a). However, the *P. maupasi*-like strains from Los Angeles were hermaphroditic and thus likely to be *P. maupasi*, while all the *P. maupasi*-like strains from Wooster were gonochoristic and thus likely to be either *P. aerivorous* or *P. pseud aerivorous*. The vast majority of strains from the Rhabditid family came from a single uncharacterized species, *Oscheius* sp. BW282, a member of the *Oscheius* insectivorus-group containing newly characterized entomopathogenic and necromenic nematode species (Stock et al. 2005; Zhang et al. 2008; Ye et al. 2010; Torres-Barragan et al. 2011b). The nematode-beetle infestation profile expands the known host range of *Pristionchus* and *Diplogasteroides* nematodes to include four Californian beetle species as hosts.

To assess possible behavioral adaptations important for host-finding, we measured the host-finding behavior of the oriental beetle pheromone, (z)-7-tetradecene-2-one, of wild *P. pacificus* isolates using population chemosensory assays (Hong and Sommer 2006). Because past studies suggest a correlation between the host origin of wild *P. pacificus* isolates and attraction to the pure pheromones of their corresponding hosts (Hong et al. 2008b; McGaughan et al. 2013), we expected most Los Angeles isolates to exhibit weak attraction to the oriental beetle pheromone. We found that only a single one out of 12 *Cyclocephala*-derived strains exhibited attraction to the oriental beetle pheromone (Fig. 2), suggesting that Los Angeles isolates have lost attraction to this pheromone in the absence of the oriental beetle host, and local populations may have switched to another host pheromone for host-seeking.

We next asked if infestation patterns varied by sampling year and if specific nematodes can be found on one or more beetle host species. Although the representation of most nematode species vary from year to year, *P. pacificus*, *P. maupasi*, and *Oscheius* sp. consistently showed up every year in *Cyclocephala* and *Serica* beetles (Table S1 and Table S2). In particular, three of the five most frequently isolated nematode species were likely to be found associated with only one of the three sympatric beetle species (*Amblonoxia*, *Cyclocephala pasadenae*, and *Serica*). *Diplogasteroides* sp. 1 appears to have an exclusive association with *Amblonoxia* ($P=2.2e^{-16}$); *P. pacificus* and *Oscheius* sp. 1 are more likely to be found on *C. pasadenae* beetles ($P=0.0019$ and $P=0.00059$, respectively), while *P. maupasi*

and *Rhabditolaimus* sp. 1 did not exhibit a host preference (Table 1). The infestation rate from Los Angeles nematodes are comparable with previous findings for *Pristionchus* species: 4.4% of *Exomala orientalis* beetles in Japan were infested with *P. pacificus* (compared to 5% of *C. pasadenae*); and 2.4–10% of *Melolontha* beetles in western Europe were infested with *P. maupasi* (compared to 2.4% of *C. pasadenae*) (Herrmann et al. 2006a; Herrmann et al. 2007). Unlike the 1:1 relationship between *Amblonoxia* and *Diplogasteroides* sp. 1, the hermaphroditic *Diplogasteroides* sp. 2 was only found on *Serica* beetles, which is also a host for other nematodes. Mating trials between *Diplogasteroides* sp. 1 males and *Diplogasteroides* sp. 2 hermaphrodites yielded very few cross progeny, confirming they are non-interbreeding populations (unpublished results). These exclusive host preferences of *Diplogasteroides* sp. 1 and sp. 2 on *Amblonoxia* and *Serica* beetles are the most striking examples of species-specific nematode association, although more sampling of both species in other nearby locations would be needed to determine the range of these association profiles. The family Diplogastridae was the most diverse in the number of species and isolates identified (6 species, 130 isolates)(Fig. S1), compared to species from Rhabditidae (3 species, 40 isolates) and Steinernematidae (1 species, 5 isolates).

Beetle-bacteria associations

Since several nematode species show differences in infestation rate by beetle species (e.g. *Pristionchus pacificus* and *Oscheius* sp. 1), and some nematode species (*Diplogasteroides*) infest only a single species of beetles, we were interested to know if the bacterial microbiome also differs by beetle species and if those differences correlated with the nematode infestation patterns. Specifically, we hypothesized that the bacterial microbiome of the *Amblonoxia* beetles would be different from *Cyclocephala* and *Serica* species, given the 1-1 association between *Amblonoxia* and *Diplogasteroides* sp. 1, while the bacterial microbiome of *Serica* may overlap with that of *Cyclocephala* spp., based on the observation that *Pristionchus* species are found on both species of beetles. To test this hypothesis, we characterized the bacterial communities associated with all dissected beetles from 2014 using high-throughput sequencing of the 16S rRNA gene. (Caporaso et al. 2010; Caporaso et al. 2011). Out of 78 nematode isolates from 723 beetles, bacterial DNA was extracted from 77 nematode isolates, because the remaining single *Diplogasteroides* sp. 1 male (beetle 364) yielded insufficient amount of DNA. Each sample consisted of the other half of each beetle previously frozen immediately after dissection for nematodes, representing bacteria from both nematodes and the beetle host, or from just the beetle if no nematodes were isolated.

We first took a broad perspective and looked at how bacterial community composition varied by beetle species regardless of nematode infestation. We found that each beetle species surveyed possessed a unique bacterial microbiome (247)(Figure 3A, Fig. S2). Several bacterial families were driving these differences ($P < 0.01$, Figure 3B), most notably the high abundance of obligate anaerobes including sulfate reducers (*Desulfovibrionaceae* and *Desulfobacteraceae*) and *Synergistaceae* in *Amblonoxia* and *Serica* beetles. In contrast, *Enterobacteriaceae* dominated the communities associated with *Cyclocephala* beetles. Beetle genera captured most frequently together from the same southern California sites (*Cyclocephala* and *Serica*, or *Cyclocephala* and *Amblonoxia*) were less similar in bacterial community composition than beetles of the same species captured from distant locations (*C.*

pasadenae/hirta from Los Angeles vs. *C. hirta* from Wooster, Ohio) (Table 2, Table S3). These *C. hirta* from Ohio also possessed the least diverse bacterial communities (Fig. 4A, Table 2). These results suggest that the host beetle species, rather than geographic origin, is the primary determinant of bacterial composition.

Since each beetle species possessed a unique bacterial microbiome, we next compared the microbiomes of beetles with and without nematodes within each beetle species. Surprisingly, we did not observe differences in diversity levels ($P>0.05$, Fig. 4B) between infested and uninfested beetles within each beetle species. This result suggests that the nematodes do not significantly reduce the hosts' bacterial diversity as would *Photorhabdus* or *Xenorhabdus* bacteria after infection with entomopathogenic nematodes, nor do nematode infestations increase bacterial diversity by the potential introduction of nematode-specific microbiome. Unlike EPNs and vertebrate nematode parasites that can alter the host microbiome (Walk et al. 2010), our study shows entomophilic nematodes do not impact the bacterial diversity of living adult beetles.

To further investigate the nematode microbiome, we characterized the bacterial communities associated with nematodes that emerged from beetle carcasses under laboratory culture conditions. We did not observe dauers in freshly dissected beetles and all nematodes exited the beetle carcass as non-dauer larvae onto small patches of beetle-derived bacteria. Analysis of the 73 adult nematodes from these early bacterial patches showed no consistent patterns in bacterial microbiome composition by nematode species; differences within nematode species were the same (if not greater) as differences between nematode species (Fig. 5A). It is important to note that *Escherichia* accounted for only ~0.3% of all bacteria detected from the nematodes, suggesting that the ubiquitous lab bacterium, *E. coli*, was not contributing significantly to the *Enterobacteriaceae* count (Table S4). Bacterial microbiome composition from nematodes also did not differ according to which beetle host the nematodes emerged from, but are distinct from the beetle-derived bacteria (Fig. 5B, Fig. S3). Instead, the nematode microbiome appeared random and likely reflected the bacteria that grew quickest on the NGM plates and could sustain nematode growth. Consistent with this hypothesis was the extremely low diversity of the nematode microbiomes (Fig. S4). Taxonomically, nematodes from the same species varied dramatically with some dominated by a single bacterial taxon while that same taxon was absent in nematodes of the same species (Table S5). Therefore, unlike the obligate EPNs that kill their hosts using specific bacterial symbionts (Grewal et al. 1993b; Campbell et al. 1996; Goodrich-Blair and Clarke 2007), the entomophilic nematodes in our study did not carry nematode-specific or host-specific bacteria. This result may either be due to the lack of a nematode-specific bacterial community, or because those bacteria do not proliferate in the living beetle enough to change the hosts' microbiome, both possibilities are consistent with the notion that this nematode-beetle relationship is commensal or mutualistic.

Entomophilic Nematodes Lack Core Bacteria

Nematode host preferences in similar habitats may have evolved to promote the transmission of beetle core microbiome between the above-ground adults and the under-ground larvae, because nutritious growth-promoting bacteria are found only with specific beetles, or as a

consequence of differences in beetle defense responses to various nematode species. Our findings are consistent with a previous report of cultured *Pseudomonas*, *Serratia*, *Bacillus*, and *Enterobacter* bacteria from oriental beetles and *Melolontha* cockchafers (Rae et al. 2008). Previous direct sequencing approaches to determine the bacterial census in the free-living bacterivorous nematode *Acrobeloides maximus* revealed a core-microbiome that includes several *Ochrobactrum* sp. and *Pedobacter* sp. (Baquiran et al. 2013), while the associated bacteria of the plant parasitic nematode *Meloidogyne incognita* are mostly Proteobacteria (Cao et al. 2015).

Furthermore, our data does not support the hypothesis that the entomophilic nematodes play a role in transmitting the species-specific beetle bacteria because the bacterial taxa do not drive differences between the nematodes' bacterial microbiome, *i.e.* the bacteria found with different nematodes do not exhibit signatures specific either to nematodes or their host beetles. We detected an abundance of obligate anaerobic bacteria in the beetle hosts, but not in the nematode microbiome, suggesting that entomophilic nematodes are unlikely to function as vessels for transporting *Synergistaceae* and *Desulfovibrionaceae* bacteria between adult beetles and their eggs. It is possible however, that natural deaths promote a different set of bacterial community in the beetle carcass than the one observed under laboratory conditions.

Limited conservation of beetle microbiome

While the cultivable bacteria from individual nematodes did not reflect their host origin, we noticed broad similarities for beetle-nematode-bacteria associations across distant geographic sites. In Los Angeles, the masked chafers *C. pasadenae* and *C. hirta* are the most common hosts of *P. pacificus*, which were found in 36% (46/128) of the nematode infested masked chafers, compared to 20% of the nematodes from *Serica* (5/25), a single nematode from *C. longula* (1/60), and no nematode from *Amblonoxia* (0/13) (Table 1). Interestingly, *C. pasadenae* are native to continental US, particularly in the southwest, and are similar in size and abundance to the oriental beetle *Exomala orientalis*, which is the primary host of *P. pacificus* in Japan (Herrmann et al. 2007). Our chemosensory assays on wild *P. pacificus* isolates from Los Angeles differ significantly from the isolates from Japan (Hong 2008b), likely as a consequence of differences in regionally available hosts.

Host-switching may have occurred between more closely related beetles from the same family. The primary beetle hosts of *Diplogasteriodes magnus* and *Diplogasteriodes nasuensis* nematodes in central Germany are the cockchafers *Melolontha melolontha* and *Melolontha hippocastani* (Manegold and Kiontke 2001; Hong et al. 2008a), which are similar in size to *Amblonoxia palpalis* in California. The related hermaphroditic *Diplogasteriodes* sp. 2 was found only on another local beetle, *Serica alternata*, which also belongs to the Melolonthinae family. Based on their mode of reproduction and 18S sequences, it is possible that *Diplogasteriodes* sp. 1 and *Diplogasteriodes* sp. 2 are the same species as *Diplogasteriodes nasuensis* and *Diplogasteriodes magnus* found in central Europe, respectively (Manegold and Kiontke 2001; Kiontke et al. 2001; Herrmann et al. 2006b). Taken together, these two Melolonthinae beetle species have strong associations with *Diplogasteriodes* species on separate continents. However, whereas both *Diplogasteriodes*

magnus and *Diplogasteriodes nasuensis* nematodes were found in 95% of the *Melolontha* beetles in Europe, and *Diplogasteriodes sp. 1* was found in 77% of *Amblonoxia* beetles in this study, only 0.7% of *Serica* beetles (3/422) were infested with *Diplogasteriodes sp. 2*. This low infestation rate in *Serica* may indicate a more recent utilization of *Serica* as a host (Schulte 1989). More intriguingly, our study supports a previous study that found *Desulfovibrionaceae* in *Melolontha* beetles, suggesting that sulfate-reducing bacteria could represent a hallmark bacterial family of Melolonthinae beetles (Egert et al. 2005). Further work is required to describe sulfur cycling inside *Amblonoxia* and *Melolontha* beetles, and to determine if sulfate-reducing bacteria can influence species of nematode association in favor of *Diplogasteriodes* spp.

Conclusion

Entomophilic nematodes differ in beetle hosts associations according to geographical locations but their impact on the bacterial microbiome of their hosts have not been investigated (Herrmann et al. 2006b; Herrmann et al. 2006a; Herrmann et al. 2007; Morgan et al. 2012). Several members of the *Pristionchus* genus (Diplogastridae) show distinct association profiles for scarab beetles in western Europe, Japan, La Reunion Islands, and eastern United States. This study provides another example of the cosmopolitan pattern of nematode-beetle associations in *Pristionchus* (in *C. pasadenae*), as well as to other members of a closely related genus, *Diplogasteriodes* spp., and their high fidelity to Melolonthinae beetles (*Amblonoxia* and *Serica*). Since the entomophilic nematodes in this study neither altered host microbial diversity of their living hosts, nor associated specifically with certain bacteria after the death of their hosts, our results suggest that entomophilic nematodes do not carry their own bacterial communities during host infestations. However, it is also possible that our analysis could not detect a cadre of several taxonomically distant bacteria species that make up the nematode specific associations.

Another aim of this study was to detect the possible existence of ‘transitional’ necromenic nematodes that alter the microbiome of their host insects without symptoms of pathogenicity by sequencing the entire bacterial community of each host with and without nematodes. Entomopathogenic nematodes have been initially defined by their symbiotic relationship with internal bacteria (Kaya and Gaugler 1993), but recent studies have widened the spectrum of EPNs by including opportunistic surface associated bacteria that enable facultative entomopathogenicity through the association of soil bacteria with entomophilic nematodes (Ye et al. 2010; Dillman et al. 2012). Despite looking into the microbiome of the five beetle species, the nematodes of Los Angeles did not exert an effect on the bacterial community of their adult hosts, either because such ‘transitional’ necromeny is transient and/or rare. Other studies on entomophilic nematodes suggest that certain transitional necromenic nematodes with surface-associated bacteria may be pathogenic only on new host species, because long-term hosts usually evolve antibacterial defenses until a détente is reached that still benefits the nematodes without killing the host, *i.e.* by resuming reproductive development only when the host is already dead (Schulte 1989; Sudhaus 2009). In this framework, higher host fidelity in necromeny could confer stability to the tripartite beetle-nematode-bacteria relationship that would decrease the chance of such associations radicalizing into entomopathogenicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Five-year sampling summary of southern California scarab beetles and their associated nematodes

Only nematode infested beetles are represented. Proportion of nematode infestations on three major species of beetles. *Diplogasteroides sp. 1* shows the strongest host preference for *Amblonoxia palpalis* beetles while the closely related species *Diplogasteroides sp. 2* was isolated only from *Serica alternata*. *P. pacificus* was the most abundant nematode species isolated from the most commonly captured *Cyclocephala pasadenae* and *Cyclocephala hirta* beetles. The three beetle species are shown in relative size proportions, with the *Cyclocephala spp.* body length representing ~1 cm.

Figure 2. Most *P. pacificus* strains from Los Angeles show neutral or negative chemotaxis towards the oriental beetle pheromone

All but one out of twelve *P. pacificus* strains obtained from *Cyclocephala* beetles in 2012 show neutral (*rlh54-rlh56*, *** $P < 0.001$) or repulsive (*rlh59-rlh71*, **** $P < 0.0001$) chemotaxis response to the oriental beetle pheromone (z)-7-tetradec-2-one (Zhang et al. 1994; Herrmann et al. 2007). 10–21 trials of greater than 10 nematodes each were assayed for each strain. Dunnett's multiple comparisons test was performed against the reference Washington strain, PS1843.

Figure 3. The beetle bacterial microbiome is driven by differences between beetle genera (A) Principle coordinates plot of pair-wise Bray-Curtis similarities shows that the bacterial microbiomes differ according to beetle species (P=0.001). (B) A heat map of the average relative abundance of bacterial taxa occurring at 1% in the whole beetle microbiome, as arranged by abundance in the *Amblonoxia* microbiome. *Amblonoxia palpalis* and *Serica alternata* had higher abundance of anaerobes from the family *Synergistaceae*, *Desulfovibrionaceae* and *Desulfobacteraceae*. Taxon assignment is at the family level unless otherwise indicated and darker shades denote higher abundance. Significant difference in corrected P values for FDR * <0.05 , ** <0.01 (Kruskal-Wallis). [^]Includes *Cyclocephala hirta* and hybrids from Los Angeles.

Figure 4. Entomophilic nematode infestation does not alter the diversity of the beetle bacterial microbiome

(A) Globally, diversity differed across beetle species. Results of a Nemenyi post-hoc test in the PMCMR package of R found that diversity was different between *C. hirta* from Ohio and *C. pasadenae/hirta* from California ($P < 0.01$) and between *C. hirta* and *S. alternata*. ($P < 0.05$). (B) However, within each beetle species, there was no difference in bacterial diversity in beetles infested with nematodes(+) compared to those not infested(-). The comparison was not possible in *C. longula* due to only a single case of nematode infestation. ($P > 0.05$, Kruskal-Wallis nonparametric test).

Figure 5. The cultivable nematode bacterial microbiome does not differ by nematode species or beetle host

(A) The beetle-derived nematode microbiome do not differ according to nematode species ($P=0.61$), nor (B) by which beetles the nematodes were isolated from ($P=0.18$).

Table 1

Frequencies of beetles that carried nematodes (2010–2014).

Beetle species	<i>Amblyonoxia palpalis</i>	<i>Cyclocephala pasadenae</i> ^a	<i>Cyclocephala longula</i>	<i>Serica alternata</i>	<i>Cyclocephala hirta</i> (OH)
Number collected	17	907	60	422	31
Nematode species; Accession number					
<i>Diplogasteroides</i> sp. 1 (g) ^a (JX649209.1)	13 (76%)	-	-	-	-
<i>Diplogasteroides</i> sp. 2 (h) (JQ005869.1)	-	-	-	4 (0.95%)	-
<i>Oscheius</i> sp. 1 BW282 ^b (JX649214.1)	-	37 (4.1%)	-	2 (0.47%)	1 (3.2%)
<i>Oscheius chongminensis</i> (EU273597.1)	-	1 (0.11%)	-	-	-
<i>Pristionchus entomophagus</i> (JX649217.1)	-	4 (0.44%)	-	2 (0.47%)	-
<i>Pristionchus marianneae</i> (KT188836.1)	-	-	-	-	1 (3.2%)
<i>Pristionchus aerivorius-like</i> (g) (JX649211.1)	-	-	-	-	6 (19%)
<i>Pristionchus maupasi</i> (h) ^c (JX649211.1)	-	24 (2.6%)	-	10 (2.4%)	-
<i>Pristionchus pacificus</i> ^d (JX649213.1)	-	46 (5.1%)	1 (1.7%)	5 (1.2%)	-
<i>Rhabditinae</i> sp. 1 TMG-2010 (HQ332391.1)	-	1 (0.11%)	-	-	-
<i>Rhabditolaimus</i> sp. 1 RLH2013 ^e (JX649208.1)	-	10 (1.8%)	-	1 (0.24%)	-
<i>Steinernema carpocapsae</i> (JX649221.1)	-	-	-	1 (0.24%)	2 (6.4%)
Total beetles isolated with nematodes (infestation rate)	13 (76%)	123 (14%)	1 (1.7%)	25 (5.9%)	10 (32%)

Numbers in parenthesis denote the percent of individual beetles infested. (h) hermaphroditic; (g) gonochoristic;

* Includes *Cyclocephala hirta* and hybrids. Significant difference in nematode association with a sympatric beetle host (*Amblyonoxia palpalis*, *Cyclocephala pasadenae*, and *Serica alternata*) was detected using Pearson's Chi-squared test:

^a $P < 2.2 \times 10^{-16}$;^b $P < 5.9 \times 10^{-4}$;^c Not significant;^d $P < 1.9 \times 10^{-3}$;^e Not significant.

Bacterial community composition differed more between beetle species from Los Angeles than between the same species from distant geographic sites (e.g. Wooster, Ohio)

Table 2

All pairwise comparisons between beetle species were significant ($P < 0.01$) as determined by a one-way analysis of similarity (ANOSIM) in PRIMER v6. R statistics are shown and values closest to 1,000 denote the strongest statistical significance

	A. palpalis	C. hirta (Ohio)	C. longula	C. pasadenae/hirta
A. palpalis				
C. hirta (Ohio)	0.860			
C. longula	0.806	0.648		
C. pasadenae/hirta	0.703	0.286	0.177	
S. alternata	0.546	0.445	0.488	0.399