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THE ROLES OF HOST EVOLUTIONARY RELATIONSHIPS (GENUS: *NASONIA*) AND DEVELOPMENT IN STRUCTURING MICROBIAL COMMUNITIES

Robert M. Brucker[1] and Seth R. Bordenstein[1,2]

[1]*Department of Biological Sciences, Vanderbilt University, Nashville, Tennessee*

[2]*E-mail: s.bordenstein@vanderbilt.edu*

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The comparative structure of bacterial communities among closely related host species remains relatively unexplored. For instance, as speciation events progress from incipient to complete stages, does divergence in the composition of the species' microbial communities parallel the divergence of host nuclear genes? To address this question, we used the recently diverged species of the parasitoid wasp genus *Nasonia* to test whether the evolutionary relationships of their bacterial microbiotas recapitulate the *Nasonia* phylogenetic history. We also assessed microbial diversity in *Nasonia* at different stages of development to determine the role that host age plays in microbiota structure. The results indicate that all three species of *Nasonia* share simple larval microbiotas dominated by the γ-proteobacteria class; however, bacterial species diversity increases as *Nasonia* develop into pupae and adults. Finally, under identical environmental conditions, the relationships of the microbial communities reflect the phylogeny of the *Nasonia* host species at multiple developmental stages, which suggests that the structure of an animal's microbial community is closely allied with divergence of host genes. These findings highlight the importance of host evolutionary relationships on microbiota composition and have broad implications for future studies of microbial symbiosis and animal speciation.

KEY WORDS: **Coevolution, genetic variation, parasitism, speciation, symbiosis**

It is increasingly apparent that microbial symbionts in eukaryotes are not transient passengers randomly acquired from the environment. Rather, their varied roles in nutrition (McCutcheon et al. 2009), immunity (Lee and Mazmanian 2010), development (McFall-Ngai 2002; Fraune and Bosch 2010), reproduction (Perlman et al. 2008; Werren et al. 2008), and speciation (Bordenstein 2003) indicate that symbiosis is a major component of eukaryotic fitness and evolution. However, it is less apparent whether the general microbial community, that is, the richness and abundance of multiple microbial species, diverges in parallel with the host during the formation of new host species. Simply put, can the relationships between microbial communities predict the evolutionary relationships of the host species and vice versa?

A recent study of wild hominids demonstrated that the relatedness of several primate species was correlated to the relationships of the microbial communities that they harbored in fecal samples (Ochman et al. 2010). Likewise, in a study focusing on wild termite populations, the relationships of the microbiota for each termite species recapitulated the phylogenetic relationships of the hosts (Hongoh et al. 2005). The findings suggest that the majority of the gut bacteria are species-specific symbionts that co-diverged as a community with their host termite species. Finally, a study comparing the gut microbiota within and between two species of scarab beetle larvae (genus: *Pachnoda*) showed that the variation in microbial community structures within species is reduced relative to the microbial variation observed between species (Andert et al. 2010). Of these studies, no attempt was made to determine if the observed microbiota variation was due to host genotype or numerous environmental factors (e.g., diet, temperature, humidity). For instance, what proportion of the

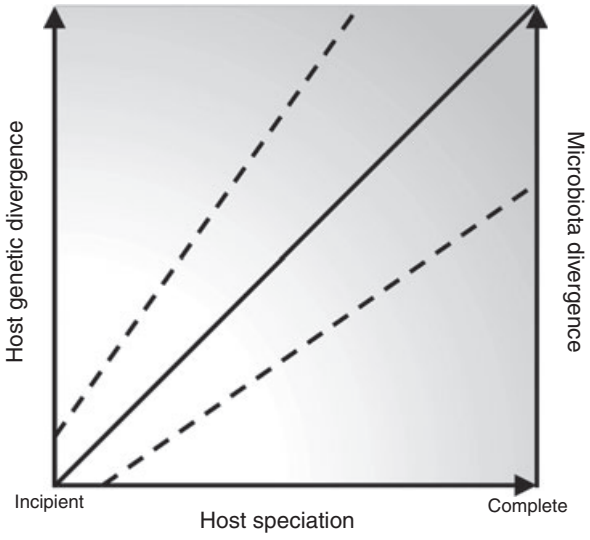

Figure 1. A hypothetical plot of the relationships between divergence in host nuclear genes, divergence in microbial communities, and host speciation. The relationship between nuclear genetic divergence and speciation is a major tenet of genetic studies of speciation. Here we hypothesize that during a host speciation event, from the incipient to complete stages (*x*-axis), divergence in host nuclear genes (*y*-axis) positively correlates with divergence in microbial communities (*z*-axis) and the timing of speciation (denoted by the solid black line). Failure to control environmental variables when sampling host microbiota can skew the correlation. Thus, a portion of the variation in the microbial community would not correlate with host genetic divergence (denoted by the upper and lower bounds in the dashed lines). The relationship of both microbiota divergence and host speciation is proposed to be linear to host genetic divergence, but may take alternative forms in practice. Microbiota divergence may occur in regards to both microbial species richness and abundance, because hosts may select for different microbial species, as well as different abundances of shared species.

variation in the microbiota is due to the environment as opposed to intrinsic, genetic factors of the host? Thus, our study aims to explore the microbial communities between closely related species to determine if the host evolutionary history is recapitulated in the structure of their microbial communities when environmental variables do not confound the analysis.

Figure 1 illustrates the basic concept of how host nuclear genes and microbial communities as a whole may diverge in parallel during the process of speciation. This conceptual model does not presume that microbial communities are stable or vertically transmitted from generation to generation; rather it only assumes that for each generation, host species will accumulate communities of microbes that are more closely related to each other than to their sibling species, and that the levels of genetic divergence between host species will also associate with the amount of divergence between their microbial communities. If true, this model would suggest that the speciation process leads to parallel divergence in both nuclear genes and microbial species composition/abundance.

To investigate the evolutionary relationships between closely related species and their bacterial microbiota, and the effects of development on these relationships in a controlled environment, we selected three young species from the *Nasonia* genus of parasitoid wasps: *N. vitripennis*, *N. giraulti*, and *N. longicornis*. *Nasonia vitripennis* diverged approximately one million years ago from the ancestor of *N. giraulti* and *N. longicornis,* which diverged from each other less than 400 thousand years ago (Raychoudhury et al. 2010). *Nasonia* is becoming a powerful model system for the biological sciences owing to the vast number of genomic resources (Werren and Loehlin 2009; Werren et al. 2010) and the benefits of haplodiploid sex determination, in which haploid males can be exploited to ubiquitously express recessive genes.

Although decades of research have characterized the role of reproductive endosymbionts in *Nasonia* such as *Wolbachia* (Saul 1961) and *Arsenophonus* (Werren et al. 1986), little attention has been given to the general bacterial community in *Nasonia*. We report six key findings: (1) The *Nasonia* microbial community diversifies as development proceeds from larva to adult. (2) Microbial communities differ between host species, despite identical rearing conditions. (3) The differences in the species' microbiotas mirror the evolutionary relationships of the host species during the pupal and adult developmental stages. (4) The primary bacterium harbored in the wasps' blow fly host (Diptera: *Sarcophaga bullata*) is prevalent in all three species, especially in larvae, suggesting an initial horizontal transmission of microbiota from fly host to *Nasonia*. (5) The dominant class of bacteria in adults colonizes the hindgut of all three *Nasonia* species. (6) Finally, of the bacterial taxa discovered in this study, 32% are novel to sequence databases, indicating *Nasonia* form symbioses with bacterial species that are new to science. Our findings demonstrate that the general microbiota of animal species can diversify in a manner that parallels host phylogenetic relationships. Further, as a consequence of identifying the microbes present in the *Nasonia* genus, this work will allow genetic studies of interspecific differences in an animal model to be combined with microbiological studies of resident bacteria in a controlled laboratory setting.

Materials and Methods

NASONIA STRAINS AND COLLECTION

Three species of *Nasonia* were used throughout the experiment: *N. vitripennis* strain 13.2 was derived from the inbred strain R511 that was collected in the Rochester, NY area. It underwent prolonged larval diapause for two years at 4°C to cause a spontaneous

loss of the *Wolbachia* infection (Perrot-Minnot et al. 1996). *Nasonia giraulti* strain RV2xU was derived from the isofemale inbred strain RV2 collected in the Rochester, NY area. This strain was created from an individual mother to son mating and cured of *Wolbachia* using rifampin for three generations in 2005. The RV2xU strain has been in the standard laboratory culture in our laboratory since October 2008 and its original description is in Werren et al. (2010). *Nasonia longicornis* strain IV7R3–1b was produced from the standard inbred strain IV7 and cured of *Wolbachia* using rifampin for three generations in 2000. The absence of *Wolbachia* from our experimental strains is important as the endosymbiont occurs in high abundance and would ultimately mask less-abundant microbial species during the cloning and sequencing procedure (see Table S1). In addition, another goal of this investigation was to characterize the microbiota of *Nasonia* strains that are regularly used in the research community. All strains were reared under identical conditions: 25°C, with constant light, on *S. bullata* pupae from the same brood. The *S. bullata* were reared on beef liver until their last larval instar (about 4-day old) under 25°C and constant light; then the larvae were transferred to a 30°C incubator under constant dark for two days before their use in experiments.

Virgin *Nasonia* females were collected and serially hosted onto two unparasitized *S. bullata* pupae from the same brood every 48 h over a period of 14 days. Three life stages from each species were collected in pools of 10 males: final instar larvae, yellow/red eye pupae, and newly emerged adults. We used male *Nasonia* for four reasons. First, there are no available techniques that easily determine the sex of the larval stages. Second, female *Nasonia* are diploid whereas males are haploid; therefore, males would represent all the potential genetic variation that might underlie recessive traits that could contribute to microbial variation between *Nasonia* species. Third, when fertile *Nasonia* mothers oviposit their eggs they do not produce a 1:1 ratio of male to female offspring. Often the number of females produced outweighs the number of males or vice versa. Eliminating this sex ratio variable reduces the likelihood that our variation in our observations is due to factors that are not associated with species' specific variation. Fourth, the mothers of the male *Nasonia* used in this study were collected and isolated as pupae to ensure virginity. This isolation circumvents interactions between *Nasonia* reducing the likely number of unknown variables such as a horizontal transfer of microbes. Before DNA extraction, all wasps were serially washed in sterile water (nucleotide-, DNAse-, RNAse-free) and molecular grade 70% ethanol to rinse the body surfaces of environmental microbial contamination. DNA was extracted from the pooled samples using the Qiagen DNeasy Blood & Tissue extraction kit according to manufacturer's protocol and prepared for PCR amplification of the bacterial 16S rRNA gene. An additional 15 adult male wasps of each species were collected for fluorescent in situ hybridizations described below. All *Nasonia* were flash frozen in liquid nitrogen and stored at –80°C until further processing, except for four adult and four larval *N. vitripennis* that were used in the identification and culturing of bacteria as described below.

CLONING AND SEQUENCING TO CHARACTERIZE BACTERIAL COMMUNITIES

16S rRNA sequences were amplified using the universal primers 5′-AGAGTTTGATCCTGGCTCAG-3′ (27F) and 5′-GGTTACCTTGTTACGACTT-3′ (1492R) (Weisburg et al. 1991) and the following PCR protocol: (1) 94°C for 10 min; (2) 30 cycles of 94°C for 1 min, 54°C for 1 min, 72°C for 2 min; (3) 72°C for 5 min. The following PCR conditions were used: 0.15 μl of Phusion® High-Fidelity DNA polymerase (Finnzymes, Thermo Scientific), 1.2 μl of each primer at 5 μM, 0.3 μl of dNTPs at 10 μM each nucleotide (New England Biolabs), 1.8 μl of 25 μM $MgCl_2$, and 3 μl of 5X Phusion® Buffer with the final reaction volume (15 μl) constituting the DNA template to be amplified. The 16S amplicon was cloned into an *E. coli* vector (pCR®-TOPO4) via the Invitrogen TOPO-TA cloning kit with TOPO10 One Shot® Chemically Competent cells. Transfomants were plated onto Luria-Bertani plates containing kanamycin monosulfate (Research Products International) and X-Gal (ChromoMax™, Fisher Scientific) at a final concentration of 50 μg/mL. Plasmid-containing bacterial colonies were isolated and sequenced by Genewiz, Inc. (South Plainfield, NJ). Sanger sequencing was conducted on ∼90 clones from each sample. A total of 882 clones were sequenced and checked for short, ambiguous, and chimeric sequences using the GreenGenes chimera checker program (DeSantis et al. 2006). The remaining 807 sequences were trimmed to approximately 550bp in length and aligned to three genomic databases (NCBI, RDPII, and Hugenholtz) using GreenGenes to identify bacterial operational taxonomic units (OTUs; DeSantis et al. 2006). All OTUs were determined using a 97% pairwise identity cutoff for each 16S rRNA sequence to a known member of the database. Sequences are available at NCBI (http://www.ncbi.nlm.nih.gov/; accession numbers: JN594818–JN595784)

STATISTICAL ANALYSIS OF THE MICROBIAL COMMUNITIES

To determine if the microbial species richness and abundance of *Nasonia* are significantly different across development or host species, we used the UniFrac metric in the program Fast UniFrac (Hamady et al. 2010). The analysis begins by making a maximum likelihood phylogeny of all OTUs. Using a phylogeny based on PhyML, which was supported by an independent RaxML predicted phylogeny, the UniFrac metric and significance is then calculated by measuring the differences between two samples in

terms of the tree branch length that is unique to one sample or the other. The metric ranges from 0.0 to 1.0, indicating that every OTU in one sample is represented in the other sample or no OTUs are shared between the two samples, respectively. Because the relative abundance of different kinds of bacteria can be critical for describing community changes, weighted UniFrac weighs the branches based on abundance information during the calculations. Therefore, the analysis detects changes in how many organisms from each lineage are present, as well as detecting changes in which organisms are present. Samples are significantly different if the UniFrac value for the real tree is greater than would be expected if the sequences were randomly distributed between the samples, using 1000 permutations. The reported *P*-value is the fraction of permuted trees that have UniFrac values greater or equal to that of the real tree. The UniFrac tree that depicts relationships between microbial communities is generated from a hierarchical clustering analysis on the samples based on the distance matrix between each pair of sequence sets from the maximum likelihood tree. For example, the length of the branch differences on the ML tree between *N. vitripennis* adults and *N. vitripennis* adults is zero, whereas it will be greater than zero for any comparisons that have OTU differences.

QUANTITATIVE PCR (QPCR) CONFIRMATION OF RELATIVE ABUNDANCE OF OTUS

As an independent means to verify the cloning and sequencing libraries, we designed bacterium-specific 16S primers for two of the dominant and one rare OTUs of the *Nasonia* microbiota (Table S2). Specifically, primers were designed to anneal to the 16S rRNA genes of *Providencia*, *Acinetobacter*, and *Brevundimonas*. Two samples consisting of pools of 10 individuals were analyzed for each of the following three sample types: *N. vitripennis* adults, *N. giraulti* adults, and *N. longicornis* pupae. All samples were run in duplicate in a BioRad CFX-96 Real-Time System, using iQ™ SYBR® Green Supermix (BioRad) after the initial template DNA concentrations were normalized between samples. Each experimental plate of samples was run under the same PCR conditions: 30 cycles with an annealing temperature of 55°C for 30 sec with a melting curve observed at the end of each run. Standards were prepared for each of the three bacterial primer sets consisting of a seven-step 1:10 dilution series that was run with each qPCR to establish a standard curve. Cycle threshold (CT) values of each sample were then compared to the standard curve to approximate the copy numbers (CN) for each bacterium in a given sample.

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

Following a modified protocol outlined in Hugenholtz (Hugenholtz et al. 2002), whole insects were flash frozen in liquid nitrogen, mounted for sectioning using tissue-freezing medium (Triangle Biomedical Sciences, Inc) and sectioned with a cryostat into 20-μm transverse cross-sections. Approximately 10 cross-sections of the mid-gut were made on nine individuals per *Nasonia* species, for a total of 27 individuals. Using the established bacterial probes for EU338 (Amann et al. 1990), γ-proteobacteria (Manz et al. 1992), and *Acinetobacter* (Wagner et al. 1994), hybridization was conducted in a 46°C humid chamber with a 35% formamide solution. For each hybridization, a control slide without the addition of a probe was made to compare sections for background autofluorescence. All sections were then conditioned with Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc) before imaging and analysis. All sections were imaged and analyzed on the Nikon Eclipse 90i with 20× and 40× objective lenses.

EVALUATION OF *S. BULLATA* HOST MICROBIOTA

Four samples of four to 10 unparasitized blow fly hosts were collected at the time the *Nasonia* were hosted on the *S. bullata* pupae. These samples were washed, flash frozen, and extracted in the same conditions as the *Nasonia* samples for cloning and sequencing analysis (see above) or kept unfrozen and used in the identification of culturable bacteria (see below).

IDENTIFICATION OF CULTURED BACTERIAL SPECIES

Four larvae and four adult *N. vitripennis,* as well as one unparasitized *S. bullata* pupae, were individually rinsed in 0.5 mL of sterile PBS, then submerged in 70% ethanol for 1 min for surface sterilization, rinsed again in sterile PBS and homogenized in 0.5 mL of sterile PBS. The homogenate solutions were subsequently diluted 1:10 in sterile PBS, and 1 mL was plated on Nutrient agar plates (standard media type composed of 0.5% peptone, 0.3% yeast extract, 1.5% agar, and 0.5% NaCl; by volume, at a pH of 6.8) in replicates of three. The plates were incubated at 25°C for 48 h. Colony counts for each plate were tabulated as colony forming units (CFUs) and five morphologically unique colonies were selected and recultured from each of the sample types onto new Nutrient agar plates for identification via sequencing and gram staining. A single discrete colony for each of the cultures was suspended in 50 μl of sterile PBS; 25 μl were streaked and fixed to sterile glass slides for gram staining and cell morphology analyses, whereas 1 μl was used as PCR template (see above). The resulting 16S rRNA amplicon was sequenced at Genewiz, Inc to assign the OTU. Additional cultures of the external surfaces of the *S. bullata* puparium, pupae, and pupae gut were also made to determine where the developing *Nasonia* acquire any host-derived microbiota.

Results

THE *NASONIA* MICROBIAL COMMUNITY

A total of 882 clones containing 16S rRNA amplicons were sequenced across the larvae, pupae, and adults of *N. vitripennis*,

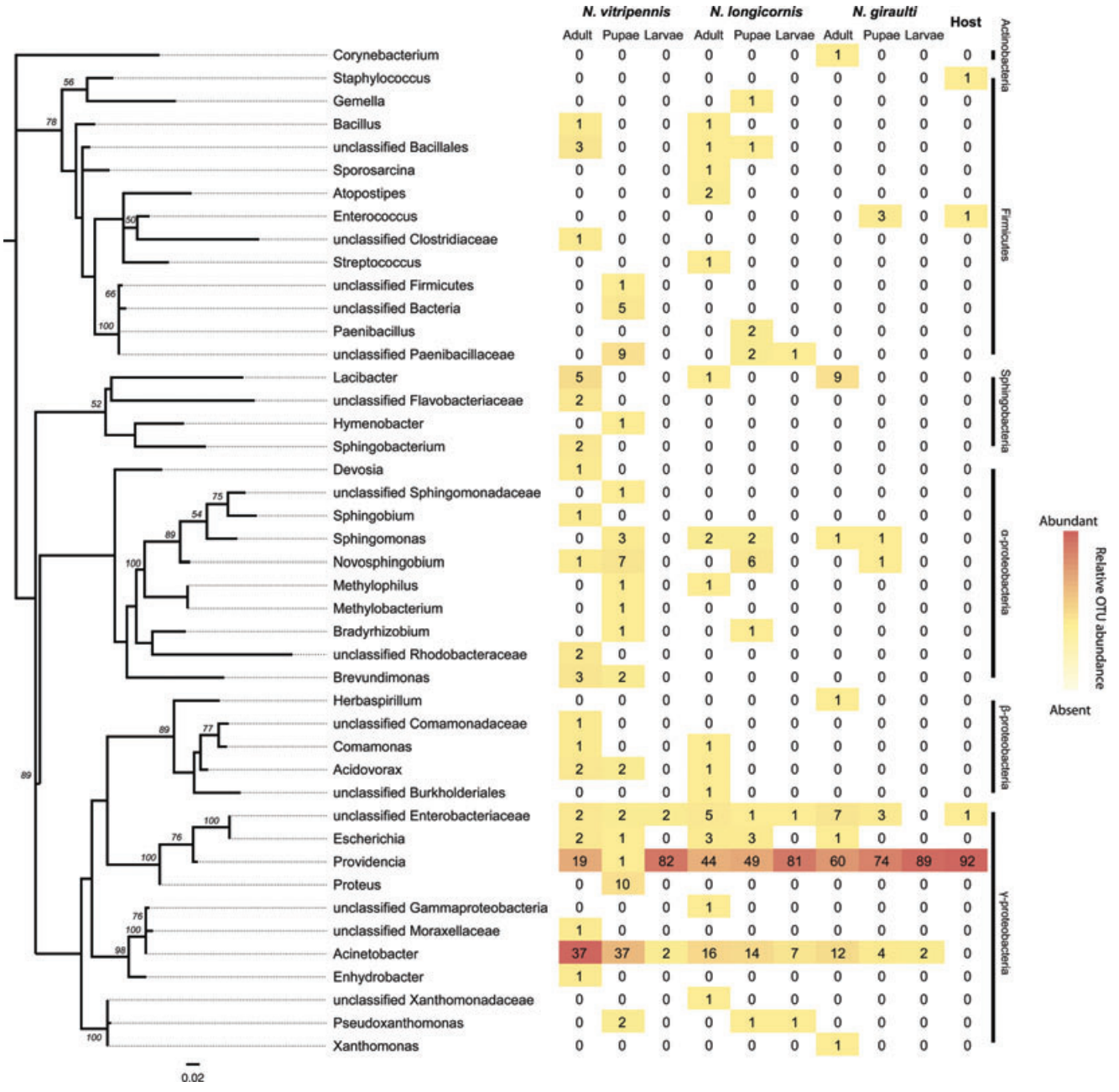

Figure 2. **Bacterial OTUs from the three *Nasonia* species and the *S. bullata* host. The Best PhyML tree is based on 16S rRNA nucleotide sequences and inferred using the General Time Reversible model. It depicts the microbial diversity across larvae, pupae, and adults of three *Nasonia* species and their pupal host, *Sarcophaga bullata*. The adjacent matrix indicates the number of incidences that each bacterial OTU occurred within any given sample. The heat map indicates the fraction of each bacterial OTU relative to the total number of OTUs within a sample's microbial community. Branch labels denote bootstrap support and unlabeled branches indicate bootstrap support below 50%.**

*N. girault*i, and *N. longicornis*, as well as the unparasitized *S. bullata* pupae. The combined clone library had 44 different bacterial OTUs based on a 97% or greater sequence identity cutoff (Fig. 2), of which 14 did not have a significant match at the species level in the Ribosomal Database Project (http://rdp.cme.msu.edu/) or NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) databases. These 14 unknown bacterial species occur at low frequencies within our dataset, yet nine are shared between at least two *Nasonia* species (Fig. 2). We have assigned these unknown bacterial species to a genus or family based on the closest match to the online databases. The bacteria in the datasets are predominantly from the γ-proteobacteria class (Fig. 2). Of the sequences, 67.0% were identified as *Providencia rettgeri*. The second most abundant OTU was that of *Acinetobacter* sp. isolate

Table 1. Microbial community analysis.

		No. of Shared OTUs[1]												
		N. vitripennis			***N.longicornis***			***N. girault***						
	Stage	Adult	Pupae	Larvae	Adult	Pupae	Larvae	Adult	Pupae	Larvae	Host	No. of bacterial OTUs[2]	Chao1 (no. of OTUs)[3]	Coverage[4]
N. vitripennis	Adult		7	3	9	6	3	5	3	2	2	21	38	0.55
	Pupae			2	6	8	5	5	5	2	2	18	25	0.72
	Larvae				3	3	3	3	3	2	2	3	3	1.00
N. longicornis	Adult					4	4	4	3	2	2	17	27	0.63
	Pupae						5	4	3	2	2	12	14	0.86
	Larvae							3	3	2	2	5	6	0.83
N. girault	Adult								3	2	2	9	14	0.64
	Pupae									2	3	6	7	0.86
	Larvae										2	3	3	1.00
	Host											4	5	0.80

[1]Number of shared operational taxonomic units (OTUs) for cloning and sequencing results based on the 16S rRNA gene clone library.
[2]Number of OTUs within a given sample, based on 3% sequence divergence.
[3]Chao1, ACE, Shannon index, and Simpson's evenness was calculated for each sample (only Chao1 species richness estimator shown).
[4]The proportion of OTUs observed/estimated in a given sample based on the Chao1 estimate of community richness.

AU3560, constituting 14.8% of the clones. Rarefaction analyses indicate that the bacterial diversity is simple in the larvae and more complex in the pupal and adult stages (Fig. S1). As each *Nasonia* species develops from larvae to adult, the microbial diversity markedly increases.

In fourth-instar larvae, the microbial diversity is simple and nearly identical in all three *Nasonia* species. *Providencia rettgeri* constitutes 95.3% of the sequences in *N. vitripennis*, 97.8% of the sequences in *N. girault*i, and 89.0% of the sequences in *N. longicornis*. The remaining sequences in both *N. vitripennis* and *N. girault*i larvae align to *Acinetobacter* sp. AU3560 and an unclassified *Enterococcus*. The *N. longicornis* larvae have the most diversity, comprised of five bacterial OTUs spanning *P. rettgeri*, *Acinetobacter* sp. AU3560, a *Pseudoxanthomonas* sp., an unknown *Paenibacillaceae* sp., and an unknown *Enterococcus* sp. Overall, the larval microbial community is very similar to that of the *S. bullata* fly host, in which the dominant species is *P. rettgeri* (96.8% of the clones sequenced), suggesting that the larvae may have acquired the dominant members of their microbial community from their food source.

A microbial succession is evident during wasp development as the microbial communities diversify from larvae to pupae. The pupal OTU diversity is more than twice that of the larval diversity in *N. girault*i and *N. longicornis* and six times greater in *N. vitripennis* (Table 1). All of the bacterial OTUs in the larval stages of the three *Nasonia* are observed in the pupal stage. The two dominant OTUs in this life stage are *P. rettgeri* and *Acinetobacter* sp. AU3560. There is, however, a *Nasonia* species-specific difference in the abundance of these two microbes. *Providencia rettgeri* remains the most dominant taxa in *N. girault*i and *N. longicornis* (87.9% and 66.2% of clone sequences, respectively), yet is a rare taxa in *N. vitripennis* (1.1% of the clone sequences). *Acinetobacter* sp. AU3560 is the most common OTU in *N. vitripennis* (representing 52.7% of the clones sequenced) followed by a *Proteus* sp. (12.3%) that is closely related to the genus *Providencia*. The bacterial OTUs shared in the larval and pupal stage are also shared in the adult stage with the exception of *N. longicornis*, which only contains three-fifth of the OTUs observed in the earlier life stages. Approximately half of the OTUs are shared from pupae to adult (7/18 for *N. vitripennis*, 6/12 for *N. longicornis*, and 3/6 for *N. girault*i). In adults, each of the three *Nasonia* species has both unique and shared taxa (Fig. 2) comprised of 21 (*N. vitripennis*), 17 (*N. longicornis*), and 9 (*N. girault*i) observed OTUs. Species richness estimators indicate that all three *Nasonia* species harbor more bacterial species than sequenced (Table 1). For example, *N. vitripennis*, *N. longicornis*, and *N. girault*i are estimated to harbor 38, 27, and 14 OTUs based on the Chao1 species richness estimator (Table 1).

To determine if the variation in OTU diversity in adults is significantly different between the three species, we compared the bacterial diversity and abundances using the program Fast UniFrac (Table S3). The sister species *N. longicornis* and *N. girault*i, which diverged approximately 400,000 years ago (Campbell et al. 1993; Werren and Loehlin 2009), have significantly different microbiotas ($P < 0.001$) and are also significantly different from *N. vitripennis* ($P < 0.001$ for *N. longicornis* and $P = 0.024$ for *N. girault*i).

RELATIONSHIPS BETWEEN THE MICROBIAL COMMUNITIES RECAPITULATE THE *NASONIA* PHYLOGENY

To test the basic prediction that when environment is controlled for, the relationships of the host's microbial communities reflect the phylogenetic relationships of the host species, we compared the OTU richness and relative abundance between each of the *Nasonia* species and displayed the relationships in two separate UniFrac generated distance trees (not weighted and weighted to abundance differences). First, when the data are analyzed with OTU diversity alone, without regard to abundance variation, we observe a notable clustering of the microbial communities at each developmental stage (Fig. 3A). As expected, the larvae and *S. bullata* host bacterial communities cluster together because they have a simple microbiota dominated by *P. rettgeri*. With the increase in microbial diversity in the later stages of development, the pupal and adult microbiotas from each of the three species each exhibit community relationships that recapitulate the evolutionary relationships of the three *Nasonia* species (Fig. 3A). For instance, the adult microbiotas of the sibling species *N. giraulti* and *N. longicornis* are more closely related to each other than either is to the microbiota of *N. vitripennis*; this same pattern holds for pupal microbiotas. The probability of observing this pattern in the distance tree for six taxa (the sum of the three pupal and adult taxa) is 0.009. Additionally, the probability of observing that each developmental stage reflects the *Nasonia* phylogeny compared to any possible or unresolved tree with three taxa is $\frac{1}{4}$ (pupal microbiota tree) $\times$ $\frac{1}{4}$ (adult microbiota tree), or 0.0625. The microbial community relationships are also evident when we compare the shared bacterial OTUs (Table 1). For instance, the two sister species *N. giraulti* and *N. longicornis* share 35% of their adult bacterial OTUs, whereas *N. vitripennis* shares only 24% and 18% with each of them, respectively. Thus, the composition of the bacterial communities in regards to OTU richness is strongly development specific and the relationships directly reflect the host's evolutionary relatedness. Next, when taking into account OTU diversity and abundance differences by weighting the Unifrac analysis to the proportion of each OTU, Figure 3B continues to show that host species has a significant effect on how much of an OTU is present. In particular, the weighted Unifrac distance tree again clusters the larvae and host microbiota because of their simple community dominated by *P. rettgeri*. However, the *N. vitripennis* adult and pupal microbiotas, dominated by *Acinetobacter* sp. AU3560, cluster independently from the *N. longicornis* adult and pupal microbiotas, as well as the *N. giraulti* adult microbiota. The *N. giraulti* pupal microbiota clusters with the simple larval microbiotas because 87.9% of the sequences in the *N. giraulti* pupae are from *P. rettgeri*. In sum, developmental stage has a profound effect on which microbes colonize and host species background has an effect on how much of each species is present.

QPCR CONFIRMATION OF INTERSPECIFIC VARIATION IN MICROBIAL DIVERSITY

To confirm the major differences in OTU presence and abundance, we used quantitative PCR (qPCR) with species-specific primers to the 16S rRNA gene in independent samples of the three *Nasonia* species. We targeted the two dominant bacterial species in the three *Nasonia* species, *P. rettgeri* and *Acinetobacter* sp. AU3560, and the bacterium *Brevundimonas diminuta* strain zjs01 that was only observed in *N. vitripennis*. Between the two samples of *N. vitripennis*, the copy number observed of *P. rettgeri* and *Acinetobacter* sp. AU3560 are 2.249 ± 90 and 6.173 ± 579, with a relative ratio of 1:2.75. This ratio is remarkably similar to the ratio determined from the cloning and sequencing results of 1:2.5. Furthermore, when we compare *P. rettgeri* to *B. diminuta* (7 ± 2 copies) we observe a ratio of 1:0.003 using qPCR, which is similar to the 1:0.03 ratio from the cloning and sequencing results. Finally, the two *N. longicornis* and two *N. giraulti* samples had average proportions of *P. rettgeri* to *Acinetobacter* sp. AU3560 that were identical to what was observed in cloning and sequencing with relative ratios of 14:1 ($1.341 \pm 354 : 95 \pm 15$) and 6:1 ($2.954 \pm 305 : 537 \pm 60$), respectively. These samples were, as expected, negative for *B. diminuta*. Taken together, the qPCR findings on multiple, independent samples indicate a high degree of repeatability for the relative OTU abundance differences.

IDENTIFICATION OF CULTURED BACTERIAL SPECIES

Culturing on nutrient agar and 16S rRNA sequencing of a subset of bacterial colonies from three *N. vitripennis* larvae and three adults, as well as three *S. bullata*, also confirm that *Providencia rettgeri* strain YL is common in the dataset (E-value = 0, 99.7% pairwise identity, Fig. S2). This species is the same taxon identified in the cloning and sequencing results. From the larvae, a colony also aligned to *Morganella morganii* strain Sam123–6 (E-value = 0, 100% pairwise identity) that was not previously detected in the cloning and sequencing results (Fig. S2C-D). The remaining bacterium detected was the gram-positive *Bacillus* sp. (strain 1P01SE) that was cultured from both larvae and adults (Fig. S2E-F). This *Bacillus* sp. was previously observed in the cloning and sequencing results of the adult *N. vitripennis* and *N. longicornis* (E-value = 0, 100% pairwise identity). The bacteria sequenced from the unparasitized *S. bullata* pupae were predominantly *P. rettgeri* and a single colony of the gram-positive *Staphylococcus lentus* strain CICCHLJQ29 (E-value = 0, 100% pairwise identity, Fig. S2H-I); both were previously observed in the cloning and sequencing results from *S. bullata*. Cultured bacteria from the exterior and interior of the

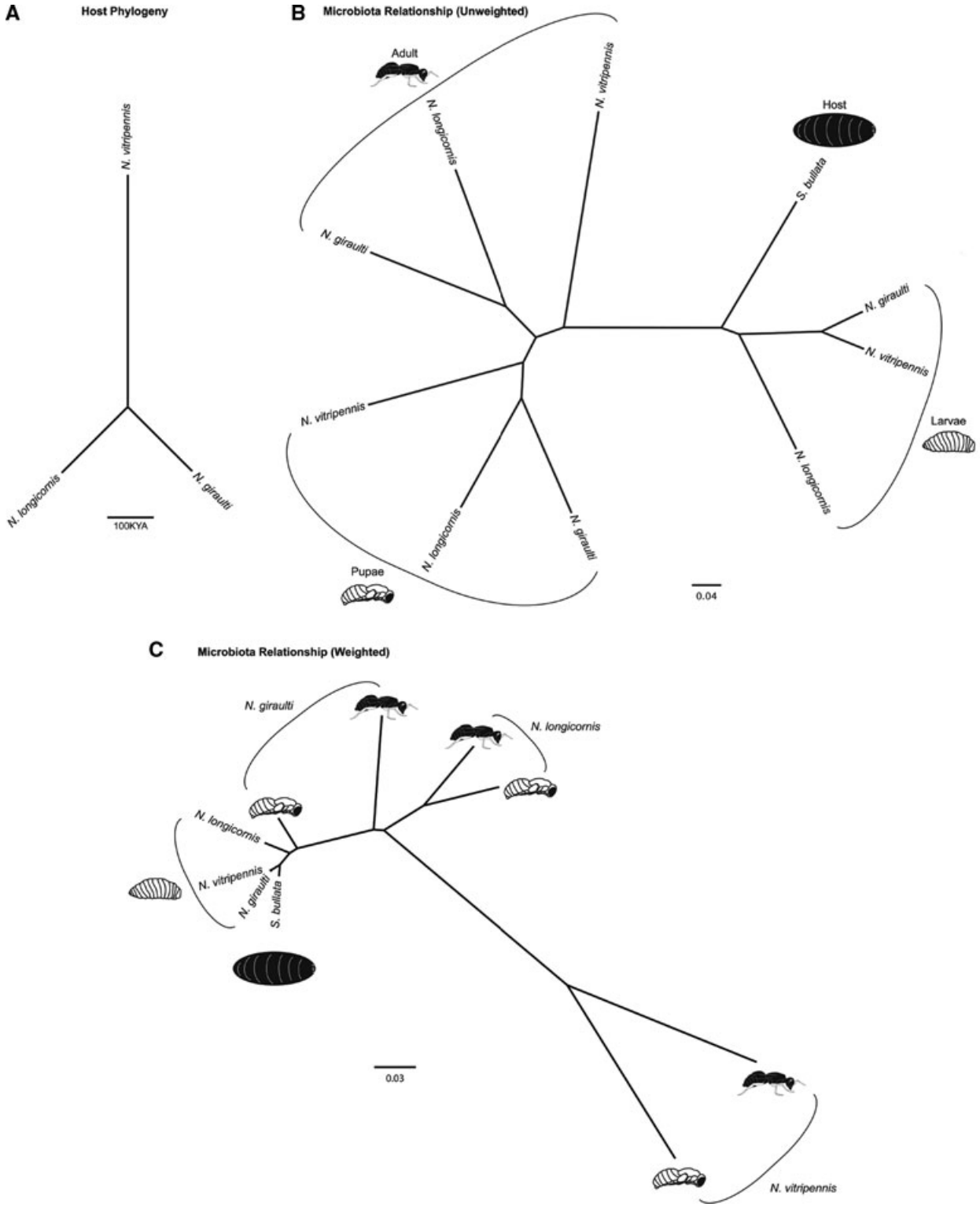

Figure 3. The phylogenetic relationship of the three *Nasonia* species compared to a cluster analysis of the microbiota across all samples. (A) Schematic of the evolutionary relationships of the three *Nasonia* species based on nuclear divergence (estimated time scale in thousands of years) (Campbell et al. 1993; Werren and Loehlin 2009). (B) Unweighted UniFrac cluster analysis depicting the relationships of the bacterial microbiotas generated from the three *Nasonia* species during larval, pupal, and adult life stages, as well as the unparasitized *Sarcophaga* host. (C) A weighted UniFrac cluster analysis.

fly puparium, in addition to the gut of the developing fly pupa, indicated a high abundance of *Providencia* in all areas of the fly host.

Despite the parallels between specific members of the bacterial cloning and culturing, we did not culture the dominant bacterium cloned in the adult *N. vitripennis* (*Acinetobacter* sp. isolate AU3560). This OTU is not known to be cultured with the growth conditions used in this study, but other *Acinetobacter* species have been cultured on clinical blood agar plates (La Scola et al. 2001).

LOCALIZATION OF BACTERIA USING FLORESCENT IN SITU HYBRIDIZATION

We conducted fluorescent in situ hybridizations (FISH) on 20-μm cross-sections of the three adult *Nasonia* species with a general probe for γ-proteobacteria and a specific probe for the *Acinetobacter* genus. The first probe targets the dominant class of bacteria that was identified in *Nasonia* and the latter probe targets a specific bacterial genus within this class. Results indicate that the hindgut is the primary organ harboring these bacteria within the mature insect (Fig. 4A). Approximately 10 cross-sections of the hindgut were made on nine individuals per *Nasonia* species, for a total of 27 samples. Total area of fluorescence was measured using the Nikon software NIS-Elements Basic Research (Nikon Instruments Inc.) to determine pixel intensity and overlay. A ratio of the average area of the two florescent markers was determined for each species. Compared to the cloning and sequencing results, these hybridizations again confirm that there is a significant difference in the abundance of *Acinetobacter* in the hindgut of *N. vitripennis* compared to *N. longicornis* or *N. giraulti* (Fig. 4C, Mann–Whitney U; $P < 0.01$ respectively).

Discussion

Nasonia parasitic wasps harbor a diverse community of bacteria that undergo several successions during the insects' development. Our results demonstrate that the three species of *Nasonia* begin their larval life with a similar, simple microbiota. However, as development progresses to adulthood, the observed species richness of the microbiota increases yielding a more complex community of bacteria. The Chao1 richness estimates for the three adult species of *Nasonia* range from 38 to 14 bacterial OTUs and are within the range of species richness in other insects: 49 OTUs in the emerald ash borer *Agrilus planipennis* (Vasanthakumar et al. 2008), 9.5–49 OTUs in the fruit fly *Drosophila melanogaster* (Corby-Harris et al. 2007; Cox and Gilmore 2007), 45 in the termite *Reticulitermes speratus* (Ohkuma and Kudo 1996), 10 in the ant lion *Myrmeleon mobilis* (Dunn and Stabb 2005), 30–46.5 OTUs in the fire ant *Solenopsis invicta* (Lee et al. 2008). The expansion of OTU diversity between the *Nasonia* larval microbiota and the later developmental stages could be due to the final meconium that is released by the larvae before pupation. Although the microbiota of the fecal matter was not explored, such a large expulsion of microbial cells from the gut would likely have a large affect on turn over and succession of the microbial community.

By comparing the microbial communities across the three species of *Nasonia* reared under the same environmental conditions, we were able to test the association of the relationships of microbiota to that of the host genotype. Of the three developmental stages observed, two (pupae and adults) harbored development-specific microbial communities that each reflect the *Nasonia* species-level divergence (Fig. 3), indicating a relationship between host genotype and the types of bacteria that persist in the latter stages of development. Of the three species, the more divergent *N. vitripennis* harbors the most divergent microbial community compared to the other two sister species, *N. longicornis* and *N. giraulti*. In particular, the dominant bacterium observed in *N. vitripennis* is from the *Acinetobacter* genus (40% and 42% in the pupae and adult, respectively) whereas the other two species of *Nasonia* were dominated by the *Providencia* genus (53% for both *N. longicornis* developmental stages, and 64% and 86% for *N. giraulti* adults and pupae, respectively).

We speculate that the differences in microbial communities among closely related species are likely due to rapidly evolving interactions between the host immune system and the microbes that it encounters. Recent studies indicate that immunity genes are subject to higher rates of adaptive evolution compared to the rest of the genome (Schlenke and Begun 2003; Obbard et al. 2009). It is likely that future systems of animals and plants will show that the divergence of the host species affects the microbial symbiont community structure. This concept supports the same basic observation in a recent analysis of the microbiotas of wild hominids (Ochman et al. 2010), indicating that host phylogeny shapes the gut microbiota.

A core of two γ-proteobacterial species is shared among all the developmental stages: *Providencia rettgeri* and *Acinetobacter* sp. AU3560 (Fig. 2). γ-proteobateria is the dominant class in a number of different insects (Dunn and Stabb 2005; Babendreier et al. 2007; Lee et al. 2008; Vasanthakumar et al. 2008; Yoshiyama and Kimura 2009). Interestingly, other insects from the order Hymenoptera, of which *Nasonia* belongs, exhibit a high prevalence of γ-proteobateria (Lee et al. 2008; Yoshiyama and Kimura 2009). Much like *Nasonia*, the larvae of the fire ant, *S. invicta*, have *Providencia* as the dominant bacteria (Lee et al. 2008).

The *Providencia* genus is implicated in many symbiotic roles in different insects such as nutrition (Sasaki-Fukatsu et al. 2006) and pathogenesis (Jackson et al. 1995), but the function of *Providencia* within *Nasonia* is not yet clear. This genus has been isolated from the gut of the honey bee and is implicated as a natural control against the insect pathogen *Paenibacillus larvae*

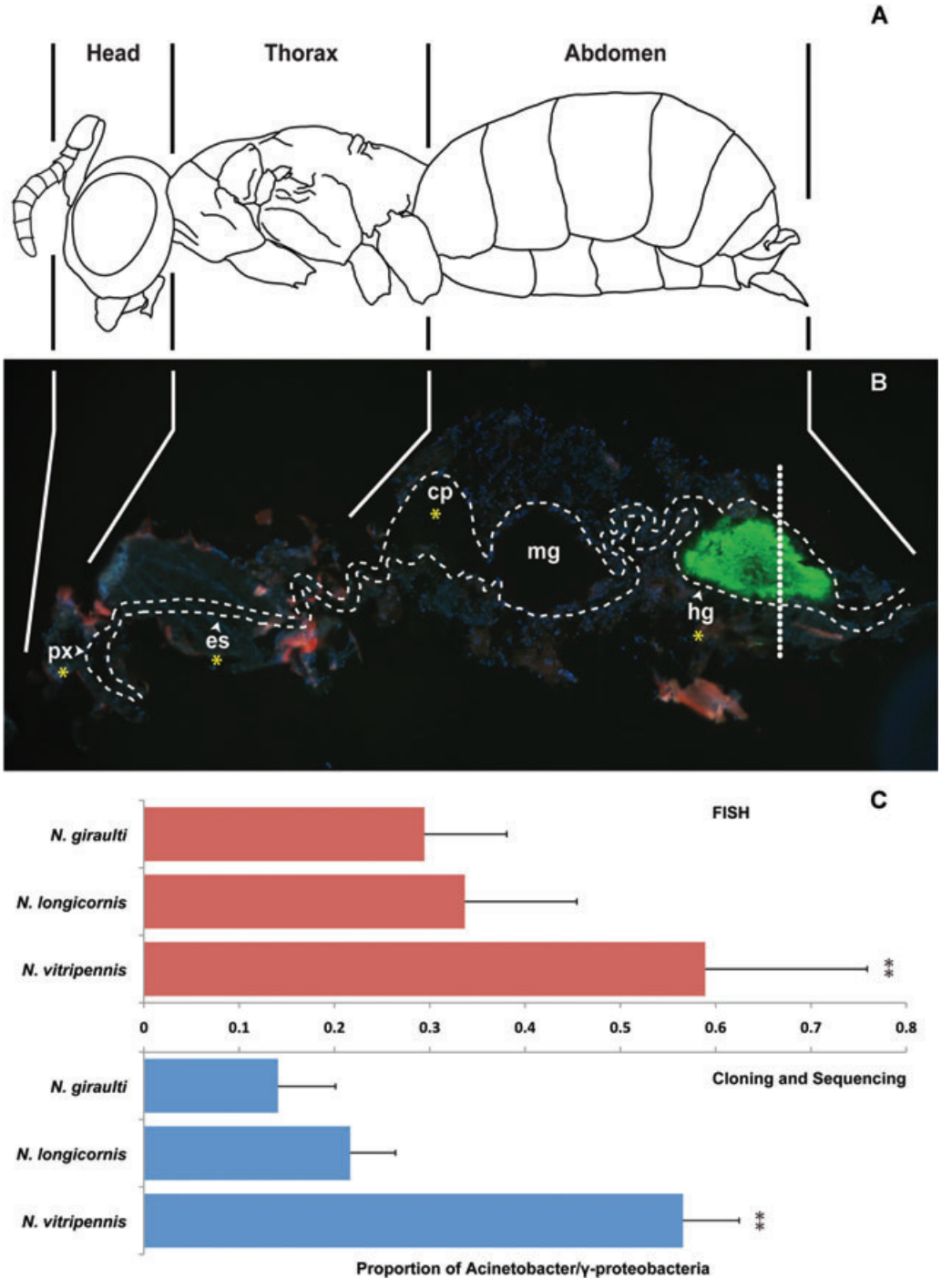

Figure 4. Fluorescent in situ hybridization (FISH) of γ-proteobacteria and *Acinetobacter* in adult *Nasonia*. (A) A schematic of the sagittal view of an adult male *Nasonia*. (B) A sagittal cross-section of an adult *N. vitripennis* with the γ-proteobacteria probe (green). The digestive tract is outlined in dashed lines with its main tissues labeled; cr-crop, es-esophagus, hg-hindgut, mg-midgut, and px-pharynx. Tissues containing γ-proteobacteria have been indicated with a yellow asterisk. All sections were co-stained with DAPI (blue), which localizes to the nucleus. To compare colocalization of γ-proteobacteria and *Acinetobacter* probes, transverse cross-sections were taken of the abdomen for all three species (approximate location indicated with the dotted line), as this is the region in which the hindgut is located. (C) Red bars indicate the proportion of *Acinetobacter to* γ-proteobacteria ± standard error within the transverse cross-sections of the hindgut for each of the three species ($n = 9$ per species; red bars). ** $P < 0.01$, Mann–Whitney U test. Blue bars indicate the proportion of *Acinetobacter* to all γ-proteobacteria ± standard error that are observed in the cloning and sequencing results.

(Yoshiyama and Kimura 2009). *Paenibacillus larvae* is a gram-positive spore-forming pathogen that is the cause of the honey bee disease, American Foul Brood (Genersch et al. 2006). It is highly contagious and destructive to the honey bee colony as it over-replicates inside the gut of young larvae, killing them before they can pupate. Recent propositions have suggested using endogenous gut bacteria, like *Providencia,* as control agents against American Foul Brood to manage the disease without the use of antibiotics (Evans and Armstrong 2006; Yoshiyama and Kimura 2009). It is possible that the bacteria within the gut of *Nasonia* are serving a similar protective function, particularly in the early stages of development. An alternative function of *Providencia* could be that it plays a behavioral or olfactory role in locating fly hosts for *Nasonia* to parasitize. An example of this is the adult screwworm fly *Cochliomyia hominivorax*, which have a rich community of bacteria including several species of *Providencia* (Caballero et al. 1996). These bacteria infect bovine wounds and provide a chemical cue for additional screwworm females to locate the wound and oviposit their eggs (Hammack et al. 1987; Chaudhury et al. 2002, 2010).

Interestingly the other dominant microbe, *Acinetobacter*, has also been widely observed in multiple insect species, including the human body louse (La Scola and Raoult 2004), Fomicidae species of ants (Lise et al. 2006), and the honey bee (Evans and Armstrong 2006). This bacterial genus is thought to be a beneficial symbiont associated with the digestive track and nutrient acquisition, although the exact mechanisms by which it benefits its host are unknown.

As indicated by the FISH analysis, a high density of bacteria occurs in the hindgut of all three species of adult *Nasonia*. A less-dense microbial community was distributed across the gastrointestinal tract with the exception of the midgut, in which no signal was observed. In other insects, the reduced microbiota in the midgut is attributed to the high levels of enzymatic activity in the organ, which limits the types of microbes that can thrive there (Terra and Ferreira 1994; Andert et al. 2010). The high density of the microbes within the hindgut, whose main function is to store fecal matter, suggests that the bacteria could be functioning in a number of different manners of which we can only speculate. In the hindgut, bacteria are not competing directly with the host for nutrient acquisition of digested compounds and the host's immune defenses are typically the weakest. The microbiota of the hindgut has been implicated in several functions in insects such as carbohydrate utilization—as seen in the cricket *Acheta domesticus* (Kaufman and Klug 1991), nitrogen fixation and prevention of pathogens—as seen in the termites *Coptotermes lacteus* (French et al. 1976; Veivers et al. 1982), and pheromone signaling—as seen in the desert locust *Schistocerca gregaria* (Dillon et al. 2000). Exploitation of the hindgut bacterial community has been attempted as a means of vector control in human disease transmitting insects. For example, a transgenic hindgut symbiont in the reduviid bug, *Rhodnius prolixus*, was generated to produce a pore-forming peptide that kills *Trypanosoma cruzi*, the causative agent of Chagas disease, (Durvasula et al. 1997).

Because the simple microbiota of the *S. bullata* blow fly that *Nasonia* parasitizes is similar to the *Nasonia* larvae, it is uncertain as to how the microbial diversity in *Nasonia* pupae and adult arises. We suggest three scenarios. (1) First, *Nasonia* foundresses could include a seed culture of bacteria by defecation or other means when she oviposits into the *S. bullata* host, thereby inoculating the developing offspring with the bacterial consortia observed in our study. This seed culture could exist at very low titers inside or on the skin of the blow fly, and it would be undetectable with the methods used in this study. Maternal deposition of symbionts is seen in other insects such as the human body louse *Pediculus humanus* (Sasaki-Fukatsu et al. 2006) or the European beewolf *Philanthus triangulum* (Kaltenpoth et al. 2005). (2) Second, the bacteria could endogenously exist within the *S. bullata* host at very low titers but were not detected in our study. However, as the *Nasonia* larvae feed on the fly pupae over time, they would be inoculated with an increasing number of bacterial OTUs. (3) Finally, there is a possibility that the bacteria within the developing *Nasonia* are derived from the laboratory environment. Because the fly pupal casings in which *Nasonia* are developing are not completely isolated from environmental microbes, new bacterial species could be acquired from the air or surfaces within the laboratory. As these possibilities are not mutually exclusive, some or all could contribute to the overall bacterial diversity within the *Nasonia*. One final consideration in the genesis of the microbiota is the fact that the *Nasonia* used in this study lacked a *Wolbachia* infection. The obligate intracellular bacterium naturally infects all three species of *Nasonia*. It has been shown in other arthropods to associate with colonization resistance against RNA viruses (Teixeira et al. 2008; Hedges et al. 2008) and eukaryotic parasites (Kambris et al. 2009; Moreira et al. 2009; Hughes et al. 2011). *Wolbachia*-free *Nasonia* were purposely used in this study for two reasons. First, the abundance of the endosymbiont would overshadow rare members of the microbial community and make the phylogenetic variation between strains more difficult to detect (see Table S1 for direct observations). Second, the goal of this study was to understand the microbiotas of *Nasonia* that are widely used in the laboratory community for studies of interspecific differences.

This research is the first investigation of the microbial community within the closely related species of the emerging *Nasonia* model system. The results observed in the four lines of experimentation independently confirm the species-specific microbiotas and their relatedness. Future sampling of additional

strains and species will extend and bolster this basic observation. The high proportion of γ-proteobacteria observed across all three species of *Nasonia* during their development is reflective of the microbial communities in other insects. Further, by comparing bacterial communities across species between 400,000 and 1,000,000 years old, we have shown that bacterial microbiotas may essentially serve as phylogenetic markers for host evolution. Because *Nasonia* is a tractable animal system with interfertile species, it is an ideal model for testing interconnected questions in development, microbial symbiosis, and speciation.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Microbial diversity in *Nasonia*.
Figure S2. Cultured bacteria from *Nasonia* wasps and blow fly host.
Table S1. Comparison of bacterial OTUs from *Wolbachia* uninfected (strain 13.2) and infected (strain 12.1) *N. vitripennis* adults.
Table S2. qPCR oligonucleotides.
Table S3. *P*-values of UniFrac test of Significance for each pair of samples with 1000 permutations.

Supporting Information may be found in the online version of this article.

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