RESEARCH ARTICLE

Gender-specific bacterial composition of black flies (Diptera: Simuliidae)

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Abstract

Although hematophagous black flies are well-known socioeconomic pests and vectors of disease agents, their associated bacteria are poorly known. A systematic analysis of the bacterial community associated with freshly emerged adult black flies of four North American species, using cultivation-independent molecular techniques, revealed 75 nonsingleton bacterial phylotypes. Although 17 cosmopolitan phylotypes were shared among host species, each fly species had a distinct bacterial profile. The bacterial composition, however, did not correlate strongly with the host phylogeny but differed between male and female flies of the same species from the same habitat, demonstrating that a group of insects have a gender-dependent bacterial community. In general, female flies harbor a less diverse bacterial community than do males. The anatomical locations of selected bacteria were revealed using fluorescence *in situ* hybridization. Understanding the physiological function of the associated bacterial community could provide clues for developing novel pest-management strategies.

Introduction

Bacteria, as commensals, mutualists, parasites, and pathogens, play important roles in shaping the ecology and evolution of their multicellular hosts (Moran, 2007; Noda *et al.*, 2007), colonizing any tissue with accessible surfaces (Berg, 1996). Recent evidence indicates that the bacterial diversity associated with insect hosts is far from complex. A culture-independent survey of bacteria associated with wild-caught fruit flies (*Drosophila melanogaster*) revealed 23 phylotypes in 5 phyla, whereas two laboratory-reared stains had 9 and 13 phylotypes (Cox & Gilmore, 2007). One of the major bacterial colonization sites in insects is the gut. A recent study of gut-associated bacteria, using 454 sequencing, revealed five dominant operational taxonomic units (OTUs) with $\leq$ 97% sequence identity in laboratory-reared fruit flies (Wong *et al.*, 2011).

Indigenous bacteria are actively involved in insect host physiology. *Rhizobiales* bacteria in the guts of ants, for example, possibly facilitated the evolution of herbivory (Russell *et al.*, 2009). Gut bacteria also aid food digestion and immunity development in insects (Dillon & Dillon, 2004; Freitak *et al.*, 2009). Orally supplying indigenous bacteria in the first week of adult life can enhance the lifespan of axenic fruit flies, whereas introducing them later reduces the lifespan (Brummel *et al.*, 2004). Eliminating the gut bacteria from Mediterranean fruit flies (*Ceratitis capitata*), however, only increases longevity under poor nutritional conditions (Ben-Yosef *et al.*, 2008).

Autochthonous bacteria also influence insect behavior and ecology. Facultative bacterial symbionts protect aphids from attack by parasitic wasps (Oliver *et al.*, 2003, 2010), and *Spiroplasma* plays the same role in the fruit fly *Drosophila hydei* (Xie *et al.*, 2010). Orally re-introducing indigenous gut bacteria can enhance the copulation performance of sterilized males of the Mediterranean fruit fly (Ami *et al.*, 2009), and gut bacteria influence the mating preference of *D. melanogaster* by changing the cuticular hydrocarbon pheromones (Sharon *et al.*, 2010). Gut bacteria also might be involved in maintaining homeostasis of the plant-defense elicitor secreted by lepidopteran larvae (Ping *et al.*, 2007) and in formation of the locust-aggregation pheromone (Dillon *et al.*, 2000).

More than 2000 species of black flies in the family Simuliidae have been described, of which the females of about 98% feed on vertebrate blood and 1.5% transmit

agents of human diseases such as onchocerciasis (Adler *et al.*, 2010). Biting and swarming behavior, as well as transmission of disease agents, can necessitate control programs (Adler *et al.*, 2004, 2010). Black flies also contribute to ecosystem processes and stability (Adler *et al.*, 2004; Malmqvist *et al.*, 2004). The microbes associated with black flies, however, are inadequately studied, other than certain fungi and protists (Kim & Adler, 2005; McCreadie *et al.*, 2005, 2011). Yet, at least some insects that feed on blood throughout their life cycle require bacterial symbionts to provide essential nutrients (Lehane, 2005).

To gain insights into the microbial biodiversity associated with black flies, we conducted the first culture-independent survey of the bacteria of four species precinctive to eastern North America – *Simulium innoxium*, *Simulium dixiense*, *Simulium krebsorum*, and *Simulium slossonae*. Black flies, like many blood-sucking insects, are sexually dimorphic (Adler *et al.*, 2004). Particular attention, therefore, was given to the potential influence of host gender on the composition of the associated bacterial community. Gender-determined bacterial colonization is not well documented. The microbial community on the hands of women is significantly more diverse than that on the hands of men (Fierer *et al.*, 2008), and the gut microbiota of mice is influenced by gender (Ge *et al.*, 2006). To our knowledge, our study is the first to demonstrate that the associated bacterial community is gender-dependent in insects.

Materials and methods

Collection of host specimens

Pupal black flies were collected from two streams in South Carolina, USA. *Simulium innoxium* was collected from bedrock in Oconee County, Little River, State Road S-37-24, Tanyard Bridge, 34°50′13.45″N 82°58′51.88″W (elev. 245 m), on 23 October 2008. *Simulium dixiense*, *S. krebsorum*, and *S. slossonae* were collected from trailing vegetation in Richland County, Harmon's Mill Pond outlfow, County Road 86, 3.2 km NNE of Horrell Hill, 33°58′32.5″ N 80°49′41.34″W (elev. 68 m), on 22 May 2009. Pupae were transported on ice to the laboratory and held in Petri dishes with moist filter paper at 22 °C until adults emerged. Within 8 h of emergence, males and females were fixed separately in 80% ethanol, which was refreshed three times in 24 h. Adults were not fed but had initial access to moisture in the Petri dishes in which they emerged. Larvae of *S. innoxium* were collected in South Carolina, Pickens County, Todd Creek, 34°45′1.45″'N 82° 48′50.27″W (elev. 224 m), on 3 February 2010, ca. 18 km from where the pupae of this species were obtained. They were fixed in four changes of 80% ethanol. To determine the gender of each larva, the ventral cuticle of the abdomen was slit with fine needles, and the tissues pushed slightly aside to see the shape of the gonads against the body wall of segment VI; male gonads are spherical and those of females are elongated (Adler *et al.*, 2004).

Construction of 16S rRNA gene libraries

Ten male and 10 female flies of *S. innoxium*, 12 males and 12 females of each of the other three species, and five male and five female larvae of *S. innoxium* were selected from the fixed stock. All insects were pooled by gender. Insects were dried at 45 °C for about 1.5 h in a speedvac (Concentrator 5301; Eppendorf) until absolutely dry. The dried samples were crushed with a plastic pestle in a 1.5-mL tube. DNA was extracted with a PowerSoil™ DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA), according to the protocol provided by the manufacturer.

We used 240 ng of purified DNA as the template for a two-step temperature gradient PCR to amplify the 16S rRNA gene of eubacteria. Briefly, the PCR of each sample was performed with eight tubes. Every tube contained 0.4 mM of each primer, 30 ng of template, 300 mM of dNTP, 2.5 units of Taq polymerase (Invitrogen), and buffer from the manufacturer. The annealing temperatures for each tube were 47.5, 49.0, 50.5, 52.0, 53.5, 55.0, 56.5, and 58.0 °C, respectively. Denaturation was achieved by heating at 94 °C for 3 min, followed by 25 cycles: 94 °C for 45 s, annealing for 30 s, and 72 °C for 1.5 min. The final elongation was carried out at 72 °C for 4 min. The first PCR reaction was performed with primer pairs 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). The PCR product was combined and concentrated using the QIAquick PCR Purification kit (QIAGEN GmbH, Hilden, Germany) and further cleaned by running 0.8% agarose gels and cutting out bands of the correct size. Gel slices were purified using the QIAquick Gel Extraction kit (QIAGEN). We used 1 μL of the purified PCR product as the template in the second PCR, which was performed with primer pairs 357f (5′-CTCCTACGGGAGGCAGCAG-3′) and 1392r [5′-ACGGGCGGTGTGT(A/G)C-3′]. The PCR product was combined and purified. The purified PCR product was cloned with pCR2.1 TOPO TA Cloning kit (Invitrogen). Colonies were picked and sequenced as described previously (Ping *et al.*, 2008). The bacterial partial 16S rRNA gene sequences have been deposited at the National Center for Biotechnology Information (NCBI), with accession numbers JF732997–JF733465.

Sequence analysis

DNA sequences were cleaned and assembled with the DNASTAR Lasergene software package (DNASTAR, Inc.,

Madison, WI). Low-quality and chimeric sequences (16.7%) were discarded. Consensus sequences of groups and singletons were used for BLAST searches in databases at the NCBI (http://www.ncbi.nlm.nih.gov) and Greengenes (http://greengenes.lbl.gov). If the input sequence had greater than 99.5% similarity to a database sequence, it was regarded as a strain of that known species; if the similarity was lower, it was assigned as an unknown species of the most closely related genus. To determine the classification of low-similarity sequences, the BLAST results were compared with their position on phylogenetic trees. Primary sequence alignments were achieved with the neighbor-joining algorithm of CLUSTAL W2 (Larkin *et al.*, 2007). Phylogenetic trees were constructed using the dnamlk program in software package PHYLIP 3.67 (http://evolution.genetics.washington.edu/phylip.html), which incorporates the strict molecular clock hypothesis into the maximum-likelihood algorithm (ML). These trees then were compared with trees constructed with Bayesian Inference (BI), using the BEAST v1.6.1 package (Drummond & Rambaut, 2007). Parameters were adjusted for both algorithms until the bootstrap values of ML and the posterior probabilities of BI were stationary and identical tree topologies were generated. Finally, the trees were edited in DENDROSCOPE 2 (Huson *et al.*, 2007) and TREEVIEW 1.6.6 (Page, 1996). Rarefaction curves were calculated using the software RAREFACTION 1.3 (http://www.uga.edu/strata/software/index.html) at 99% sequence similarity for the species level and at 97% similarity for the generic level. Sequence numbers at the group level (family for Gram-negative bacteria and phylum for Gram-positive bacteria) were retrieved from the phylogenetic trees (Supporting Information, Figs S1–S6). The parsimony test of TREECLIMBER (Schloss & Handelsman, 2006) was performed with the software package mothur (Schloss *et al.*, 2009). OTUs were grouped with 0.05 distance cutoff. The weighted UniFrac value and principal coordinates analysis were performed with the on-line UniFrac platform (Lozupone & Knight, 2005).

Fluorescence *in situ* hybridization

Ethanol-fixed specimens were washed three times alternately with water and 70% ethanol. After overnight rehydration in water at 4 °C, the insects were surface-dried with tissue paper and fixed with 4% paraformaldehyde overnight. After washing three times with 1× phosphate buffered saline (NaCl 8 g L^{-1}, KCl 0.2 g L^{-1}, Na_2HPO_4 1.44 g L^{-1}, KH_2PO_4 0.24 g L^{-1}, and pH 7.4), the samples were embedded with Technovit 8100, according to the protocol provided by the manufacturer (Heraeus Kulzer GmbH, Wehrheim, Germany). Embedded samples were cut into 5-μm thin sections, with a rotary microtome (Microm HM355S; Thermo Scientific, Kalamazoo, MI). The thin sections were mounted on SuperFrost Ultra Plus glass slides (Thermo Scientific) and treated with 5 mg mL^{-1} of lysozyme for 15 min at 37 °C. After the lysozyme was washed away with running water, the slides were dried with blowing air. The slides were hybridized with 1.5 μM of fluorescence-labeled probe in hybridization buffer containing 900 mM NaCl, 0.02 M Tris-HCl (pH 8.0), 20% Formamide, and 1% SDS. Hybridization was performed at 46 °C for 4 h on an Advalytix slide booster (Beckman Coulter Biomedical GmbH, Munich, Germany). The slides were washed in 50 mL of washing buffer containing 0.02 M Tris-HCl (pH8.0), 0.2 M NaCl, 0.05 M EDTA, and 1% SDS at 48 °C for 20 min. They then were washed with running water for 30 s and dried with blowing air. Images were taken with an Axio Imager Z1 microscope (Carl Zeiss) equipped with an AxioCam MRM camera. The universal probe for eubacteria EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′) labeled with Cy3 was used as a control. The fluorescent images of the bacterium-specific probes labeled with Fluorescein (Table 2) were overlain with those generated with the EUB338 probe, and the superimposed foci were regarded as a true bacterial signal.

Results and discussion

Bacterial classification and nomenclature

The gut lumen and body surface of insects are routinely colonized by environmental bacteria (Ren *et al.*, 2007). However, the guts of insects are purged before pupation and are nearly microbe-free upon eclosion from the pupae (Russell *et al.*, 2009). Emerging adults also shed the pupal cuticle. We, therefore, analyzed newly emerged, unfed flies, to minimize the chances of detecting transient bacterial colonizers. We retrieved 186 and 148 high-quality bacterial partial 16S rRNA gene sequences from males and females, respectively, of *S. innoxium* (Fig. 1) and an additional 475 sequences from the other three host species (Fig. 2). Overall, 396 phylotypes were detected in the four host species; 75 phylotypes were detected more than once (Table 1). The microbial diversity associated with these black flies is high compared with the 23 phylotypes associated with wild-caught fruit flies (Cox & Gilmore, 2007). Seventeen phylotypes, with numbers following the letters 'BF' (for 'black fly'), were shared among fly species. Among the cosmopolitan phylotypes, seven showed high similarity (> 99.5%) with known species in databases. Host-specific bacterial phylotypes were represented with numbers following the letters 'SD,' 'SI,' 'SK,' and 'SS' to reflect the abbreviation of the Latin binomial of each species. Only 4 of the 58 fly-specific phylotypes had > 99.5% similarity with known species.

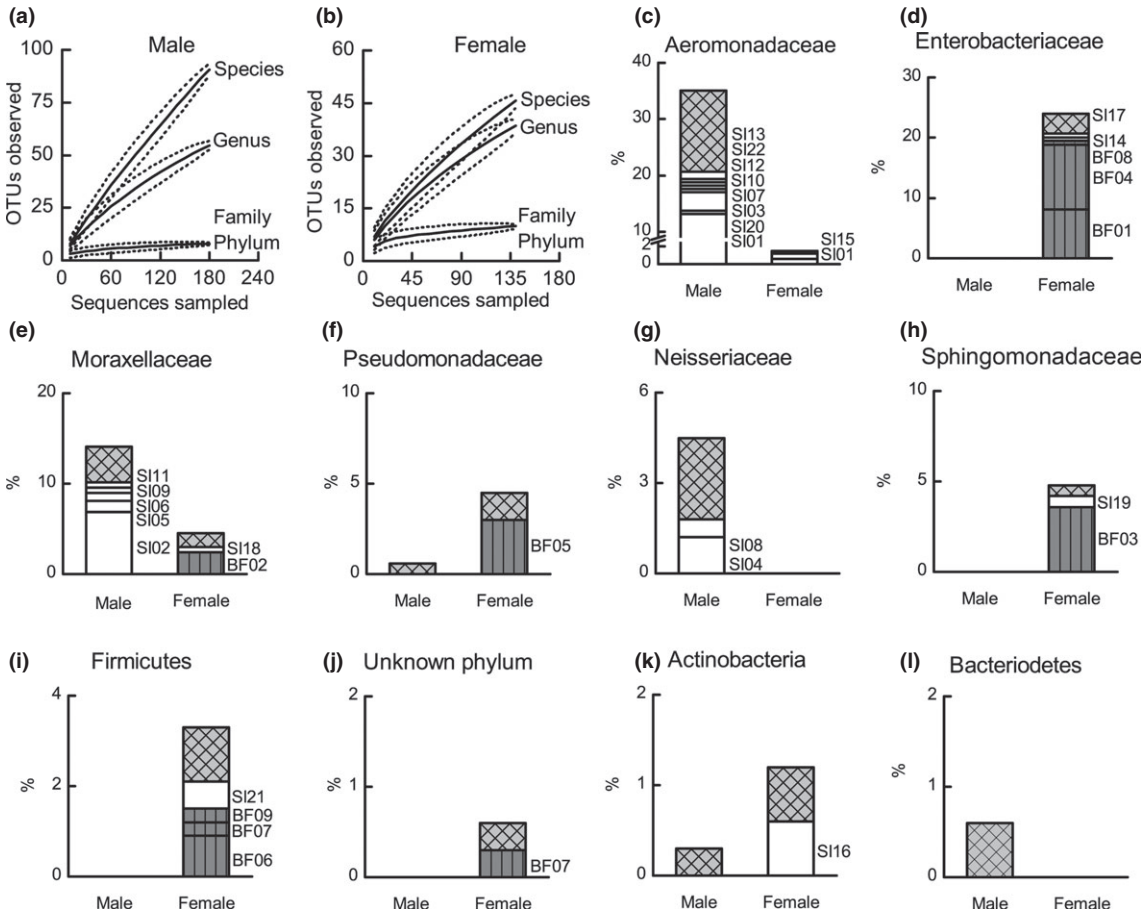

Fig. 1. Bacterial 16S rRNA gene sequences detected in adult flies of *Simulium innoxium*. The rarefaction curves of sequences from males (n = 186) and females (n = 148) are shown in a and b. OTUs are plotted at the species level, genus level, and group level (family for Gram-negative bacteria and phylum for Gram-positive bacteria). Dotted lines are 95% confidence limits. (c–l) The relative abundances of bacterial groups detected in male and female flies. Cosmopolitan phylotypes shared by different fly species are shown with gray boxes with vertical lines. Phylotypes specific to *S. innoxium* are shown with white boxes. Singletons are shown with pale gray boxes with crosshatching. Sequence numbers are listed beside each column. For simplicity, four male-specific singleton sequences of an unknown family related to Neisseriaceae are not shown.

Some of the genera and species of bacteria in our study are known associates of flies. Among them, *Pantoea* sp. BF01, *Moraxella* sp. BF02, *Sphingomonas melonis* BF03, *Klebsiella pneumoniae* BF04, *Pseudomonas* sp. BF05, *Enterococcus mundtii* BF06, *Escherichia coli* BF08, and *Sphingomonas* sp. BF12 were cosmopolitan. *Acinetobacter haemolyticus* SI02, *Propionibacterium acnes* SI16, and *Bacillus cereus* SI21 were found only in *S. innoxium*. *Bacillus cereus* SS01 was found in *S. slossonae*. Previously, *Pantoea agglomerans*, *Klebsiella* sp., and *Pseudomonas* sp. have been detected in the alimentary canals of fruit flies in the family Tephritidae (Prabhakar *et al.*, 2009); *Pantoea stewartii* has been found in the midguts of mosquitoes (Lindh *et al.*, 2005); and *Enterococcus* and *Acinetobacter* are the dominant bacteria isolated from the midguts of tsetse flies (Geiger *et al.*, 2009). The genus *Wolbachia* has been detected in many insects, such as the horn fly *Haematobia irritans* (Zhang *et al.*, 2009). It also has been found in the filarial worm *Onchocerca volvulus*, the causal agent of onchocerciasis borne by black flies (Saint André *et al.*, 2002), as well as in a limited number of larval black flies (Crainey *et al.*, 2010). However, we found no *Wolbachia* sequences in our black flies.

Gender-specific colonization

The bacterial phylotypes and their relative abundances differed significantly between male and female flies of *S. innoxium* (Fig. 1). All sequence groups in the phylum Firmicutes, the families Enterobacteriaceae and Sphingomonadaceae, and the genera *Pseudomonas* and *Moraxella* were detected only in females. Bacteria in the family Neis-

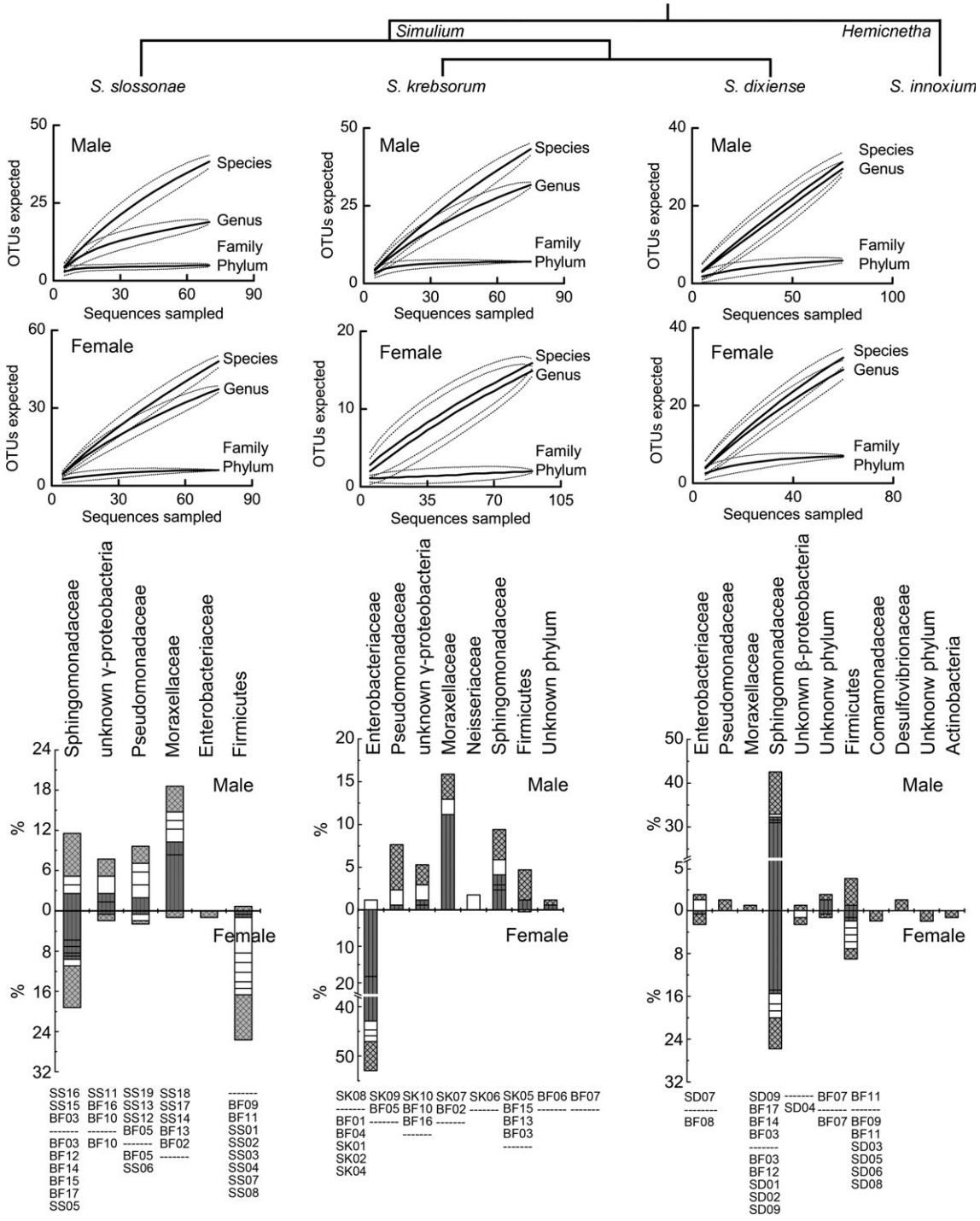

Fig. 2. Bacterial 16S rRNA gene sequences retrieved from different fly hosts in the subgenus *Simulium*. Phylogenetic relationships of the four fly species studied are at the top. The rarefaction curves of bacteria detected in *Simulium slossonae* (male 71 and female 77), *Simulium krebsorum* (male 79 and female 93), and *Simulium dixiense* (male 87 and female 68) are shown in the middle. The relative abundances of bacterial groups (family for Gram-negative bacteria and phylum for Gram-positive bacteria) are shown as cumulative bars on the bottom. Corresponding sequence numbers are listed below the graphs. Bacteria shared by different fly species are indicated by gray boxes with vertical lines, species-specific groups by open boxes, and singletons by pale gray boxes with crosshatching.

Table 1. Nonsingleton phylotypes detected from adult and larval black flies (Simuliidae) of four species (*Simulium dixiense*, *Simulium innoxium*, *Simulium krebsorum*, and *Simulium slossonae*) from South Carolina, USA

Sequence	Similar sequence/accession no.	Identity (%)	Sequence	Similar sequence/accession no.	Identity (%)
BF01	*Pantoea* sp./AM421978.1	99.60	SK10	*Sphingomonas* sp./AF409011.1	90.74
BF02	*Moraxella* sp./AY162144.1	99.81	SD01	*Sphingomonas* sp./AB434710.1	90.70
BF03	*Sphingomonas melonis*/AB334774.1	100	SD02	*Sphingomonas* sp./EF103215.1	91.31
BF04	*Klebsiella pneumoniae*/AY043391.1	99.53	SD03	*Enterococcus casseliflavus*/DQ395284.1	97.19
BF05	*Pseudomonas* sp./AY599703.1	100	SD04	*Methylophilus* sp./FJ872109.1	88.14
BF06	*Enterococcus mundtii*/EF428252.2	100	SD05	*Bacillus cereus*/EF488169.1	92.73
BF07	Desert soils clone/EF016847.1	100	SD06	*Bacillus* sp./AY572476.1	90.66
BF08	*Escherichia coli*/CU928161.2	100	SD07	*Escherichia coli*/CP001969.1	94.38
BF09	*Bacillus mycoides*/EF210306.1	93.00	SD08	*Enterococcus faecium*/EU717956.1	91.58
BF10	Sphingomonadaceae clone/GQ871733.1	98.56	SD09	*Sphingomonas melonis*/AB334774.1	96.55
BF11	*Bacillus mycoides*/EF210306.1	90.66	SD10	*Sphingomonas aquatilis*/AF131295.2	93.69
BF12	*Sphingomonas* sp./AB434710.1	95.50	SS01	*Bacillus cereus*/HM771661.1	100
BF13	*Moraxella osloensis*/EU400648.1	89.53	SS02	*Bacillus cereus*/FJ937875.1	92.42
BF14	Sphingomonadaceae bacterium/AY695722.1	89.47	SS03	*Staphylococcus kloosii*/DQ093351.1	95.24
BF15	*Sphingomonas melonis*/AB334774.1	99.24	SS04	*Bacillus cereus*/FJ937875.1	93.17
BF16	*Erythrobacter* sp./DQ312361.1	89.78	SS05	*Sphingomonas* sp./EF103215.1	92.44
BF17	*Sphingomonas aquatilis*/AF131295.2	93.69	SS06	*Pseudomonas* sp./FJ937929.1	92.32
SI01	*Aeromonas* sp./AB473005.1	99.44	SS07	*Bacillus cereus*/FJ937875.1	91.39
SI02	*Acinetobacter haemolyticus*/AY047216.1	100	SS08	*Bacillus cereus*/FJ937875.1	94.39
SI03	*Aeromonas* sp./DQ315383.1	95.42	SS11	*Sphingomonas insulae*/EF363714.1	91.20
SI04	*Iodobacter* sp./FM161451.1	98.41	SS12	*Pseudomonas* sp./EF157292.1	97.47
SI05	*Acinetobacter haemolyticus*/AY047216.1	94.21	SS13	*Pseudomonas* sp./DQ777633.1	92.80
SI06	*Acinetobacter* sp./EF103559.1	97.38	SS14	*Moraxella osloensis*/EU400648.1	91.60
SI07	*Aeromonas* sp./EF119840.1	96.26	SS15	*Sphingomonas aquatilis*/AF131295.2	95.50
SI08	*Aeromonas* sp./DQ504430.1	91.21	SS16	*Sphingomonas melonis*/FJ605424.1	97.32
SI09	*Acinetobacter haemolyticus*/AM184255.1	92.43	SS17	*Pseudomonas gingeri*/EU414476.1	93.33
SI10	*Aeromonas* sp./EF119840.1	90.53	SS18	*Moraxella* sp./DQ192213.1	97.38
SI11	*Acinetobacter* sp./EF103559.1	97.20	SS19	*Pseudomonas fulva*/FJ418772.1	98.97
SI12	*Aeromonas molluscorum*/AY532691.1	93.92	LI01*	*Microcoleus vaginatus*/EF654079.1	98.75
SI13	*Aeromonas* sp./AB472997.1	96.45	LI02	*Rhodoferax ferrireducens*/CP000267.1	97.13
SI14	*Enterobacter* sp./EU855204.1	93.64	LI03	*Solibium* sp./AM990455.1	99.32
SI15	*Iodobacter* sp./FM161451.1	92.51	LI04	*Steroidobacter denitrificans*/EF605262.1	89.91
SI16	*Propionibacterium acnes*/ABZW01000012.1	99.62	LI05	*Elizabethkingia meningoseptica*/EF426425.1	91.88
SI17	*Enterobacter cancerogenus*/FJ009375.1	92.98	LI06*	*Leptolyngbya* sp./FJ933259.1	92.28
SI18	*Acinetobacter haemolyticus*/AY047216.1	93.93	LI07*	*Bacillariophyta* sp./GQ243436.1	98.58
SI19	*Sphingomonas melonis*/AB334774.1	91.70	LI08*	*Vaucheria litorea*/EU912438.1	95.03
SI20	*Aeromonas* sp./DQ315383.1	96.54	LI09	*Thiothrix* sp./EU642573.1	94.75
SI21	*Bacillus cereus*/FJ603035.1	99.72	LI10	*Bacillus* sp./AJ544784.1	98.13
SI22	*Aeromonas molluscorum*/AY532690.1	95.88	LI11	*Flavobacterium* sp./AM934652.1	96.93
SK01	*Enterobacter kobei*/AJ508301.1	96.43	LC01	*Ralstonia* sp./AY864081.1	90.23
SK02	*Enterobacter aerogenes*/HM480361.1	98.78	LC02	*Pseudomonas* sp./AM409368.1	99.80
SK04	*Enterobacter* sp./AB244430.1	94.86	LC03*	*Porphyra yezoensis*/AP006715.1	92.99
SK05	*Moraxella osloensis*/EU400648.1	94.11	LC04	*Desulfomonile tiedjei*/AM086646.1	95.90
SK06	*Aquitalea* sp./EU287928.1	92.35	LC05*	*Synechococcus* sp./AF448080.1	98.38
SK07	*Moraxella osloensis*/EU400648.1	97.85	LC06	*Clostridium lituseburense*/EU887828.1	97.79
SK08	*Moraxella* sp./AY162144.1	92.99	LS01	*Spironema culicis*/AF166259.1	96.25
SK09	*Pseudomonas* sp./AM410625.1	96.63	LS02	*Cryobacterium* sp./EF423331.1	96.52

*Potential cyanobacteria and algae.

seriaceae were absolutely male specific. Almost all sequences showing similarity to the Aeromonadaceae bacteria – the family comprising 36% of the total sequences retrieved from *S. innoxium* – were isolated from males, as were the sequences belonging to the genus *Acinetobacter*. Gender dependence also was detected in the other three black fly species (Fig. 2). The family Enterobacteriaceae, represented by *Pantoea* sp. BF01 and *K. pneumoniae* BF04, was female specific in *S. krebsorum*. However, *Moraxella*, *Sphingomonas*, and *Enterococcus* were male specific in *S. krebsorum*. The genus *Sphingomonas* was found in both males and females of *S. dixiense*. Most of the phylotypes

specific to *S. dixiense* were found only in female flies. For *S. slossonae*, *Moraxella* was male specific, whereas *Sphingomonas* and *Pseudomonas* were detected in both sexes. The Gram-positive clade, except for one singleton, was found in females of *S. slossonae*.

The reliability of the observed specificity depends on the probability of detecting a bacterial phylotype in the sample, which follows a hypergeometric distribution (Feiler, 1968). If n denotes the total number of bacteria, n_1 the number of a particular bacterial phylotype in the pool, r the number of sequences cloned, and k the number of detected sequences belonging to the particular bacterial group, the probability of detecting the bacterial phylotype (q) is given by

$$q = \frac{\binom{r}{k}\binom{n-r}{n_1-k}}{\binom{n}{n_1}}.$$

Because r is small compared with n, q can be approximated using the normal distribution:

$$\text{Letting} \qquad h = 1\Big/\sqrt{n_1 r(n-n_1)(n-r)/n^3}$$

$$h\{k - r(n_1/n)\} \to x,$$

$$q \sim h\Phi(x)$$

Detailed discussion is given in the Data S1. If we assume male flies of *S. innoxium* carry the same amount of bacteria belonging to the Enterobacteriaceae as do the females, the probability of not detecting the bacteria in males is infinitesimal. When bacterial abundance is lower, the reliability decreases. For example, the probability of not detecting *Moraxella* sp. BF02 in males is 0.00023 and of not detecting *P. acnes* SI16 is 0.03855. We suggest that when the relative abundance of a bacterial group in one of the sexes is higher than 10%, the gender-specific observation is highly reliable. The differences between male and female bacterial communities in all four species were further confirmed using two computer algorithms, the parsimony test of TreeClimber (Schloss & Handelsman, 2006) (data not shown), and the weighted UniFrac estimator (Lozupone & Knight, 2005) (Table S2).

Different anatomies and life histories of male and female flies (Adler *et al.*, 2004) could provide differential opportunities for bacterial colonization. Bacteria in the genera *Janibacter* and *Asaia* have been detected in the guts and reproductive organs of mosquitoes and are proposed as symbionts (Ricci *et al.*, 2011), although whether they differentially colonize males and females is not known. Like mosquitoes, female black flies feed on vertebrate blood, and males take only water and plant sugars or insect (e.g. aphid) honeydew (Adler *et al.*, 2004). Insects also launch innate and systematic immune responses to cope with invasions by microorganisms (Leulier & Royet, 2009), and females are more immune-competent than are males (Kurtz *et al.*, 2000).

Food bacteria in the larval gut

The larvae of all four fly species are filter feeders and ingest a wide range of particles, including bacteria, but putatively do not select the bacterial species they ingest (Hershey *et al.*, 1996). We, therefore, used bacteria in the larval gut as a control for potential variation caused by randomly encountered bacteria. We also compared the potential selectivity of the two primer pairs employed in our research. The primer pair amplifying the inner fragment detected all major groups amplified by the primer pair for the larger fragment (Fig. 3). As expected, some of the food bacteria were cyanobacteria and algae (Table 1). The two primer pairs, nevertheless, did not differ significantly in selectivity, except *Elizabethkingia* sp. LI05 and *Leptolyngbya* sp. LI06, which were found only in males by the outer and inner primers, respectively. However, each was detected in both sexes by the other primer pair. Statistical analysis indicates that the probabilities of not detecting vs. detecting, them were 0.01763 vs. 0.29418 and 0.05638 vs. 0.36380, respectively (Table S1). Although rarefaction analysis suggests that a significant number of new bacterial species will be discovered if the sample sizes are enlarged, the data indicate that randomly caught bacteria are equally detectable in both sexes of larval black flies.

Host-specific bacterial composition

The black fly *S. innoxium* belongs to the subgenus *Hemicnetha*, whereas *S. dixiense*, *S. krebsorum*, and *S. slossonae* belong to the subgenus *Simulium* (Fig. 2). The larvae of *S. innoxium* form clusters on the crests and faces of waterfalls, dams, and rocks in clear, swift rivers (Adler *et al.*, 2004). The other three species often are found together in acidic streams, as in our study, where their larvae attach to trailing green vegetation. Adult females of *S. dixiense*, *S. innoxium*, and *S. krebsorum* feed on mammalian blood, whereas those of *S. slossonae* typically feed on avian blood (Adler *et al.*, 2004).

Each fly species harbors a distinct bacterial community, but no correlation between the bacterial composition and the host phylogeny is apparent (Figs 1 and 2). *Simulium dixiense* and *S. krebsorum* are not only closely related, but also similar in body size and anatomy (Adler *et al.*, 2004). The bacterial composition of *S. krebsorum*, however, was more similar to that of *S. innoxium*, except that some male-specific groups in *S. innoxium* were female specific in *S. krebsorum*, even though the two host species were collected from different habitats and geographic

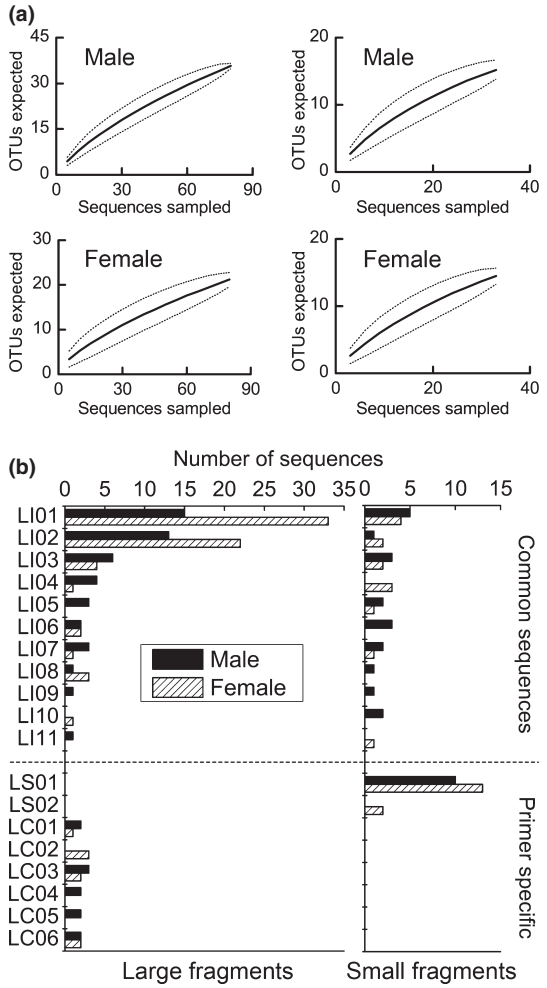

Fig. 3. Comparison of bacterial sequences from larvae of *Simulium innoxium* detected by two nested PCR primer pairs. Sequences detected with the large amplicant are on the left (male 81 and female 85) and those of the inner primer pair are on the right (male 36 and female 35). (a) Rarefaction curves of the OTUs detected against sample sizes. (b) Number of sequences detected in male and female larvae. Bacteria detected by both primer pairs are at the top and represented by numbers following 'LI' for larvae of *S. innoxium*. Sequences specific to the inner pair and outer pair are shown at the bottom, followed by the sequence number following 'LS' for larval small amplicants and with 'LC' for larval common-size amplicants, respectively.

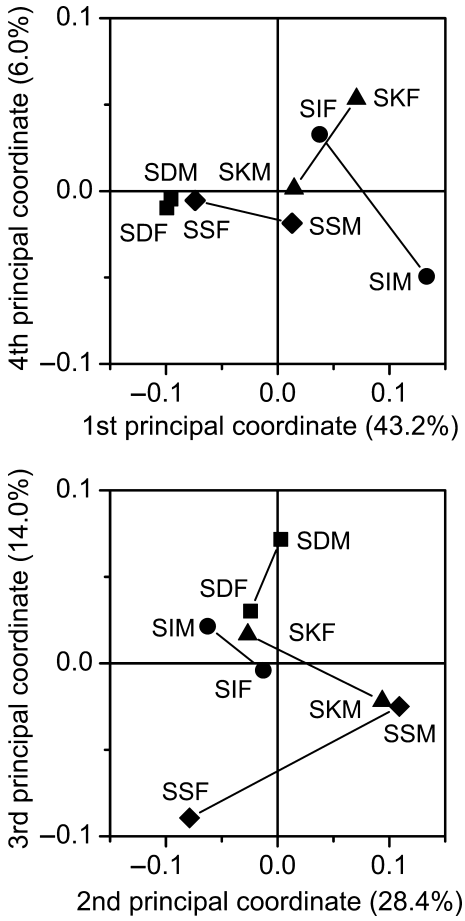

Fig. 4. First four principal coordinates from a principal coordinates analysis, explaining 91.7% of the variation observed in host flies. The percentage of variation explained by each principal component is shown in brackets. Different fly species are represented by different shapes, with a line connecting male and female data points. SIF, *Simulium innoxium* females; SIM, *S. innoxium* males; SSF, *Simulium slossonae* females; SSM, *S. slossonae* males; SKF, *Simulium krebsorum* females; SKM, *S. krebsorum* males; SDF, *Simulium dixiense* females, SDM, *S. dixiense* males.

locations. However, similar sequences in opposite genders of the two fly species might represent different bacterial species or strains. The bacterial communities also differed among all three fly species collected from the same habitat. The highest bacterial diversity was found in *S. dixiense*; some branches, such as those of *Curvibacter* and *Desulfovibrio*, were detected only in this species (Fig. S4). The smallest of the four host species, *S. slossonae*, had the simplest bacterial composition at the generic level, including *Sphingomonas*, *Pseudomonas*, *Moraxella*, and *Bacillus* (Fig. 2). Enterococci, which were widely distributed in the other three fly species, were not found in this species.

The bacterial components determining interspecific and intergender variation were not identical. The bacteria associated with male flies were often more diverse than those associated with the females (Table S2). Of the observed variation, 91.7% could be transformed mathematically into four principal coordinates (Fig. 4). The first and fourth principal coordinates primarily explained interspecific variation. The samples of *S. krebsorum* and *S. dixiense* clustered on both dimensions, whereas *S. innoxium* and *S. slossonae* clustered solely with the first and fourth coordinates, respectively. Clustering of the males of these two fly species in quadrant IV suggests that their species-specific bacteria also contributed significantly to the diversity

associated with males. The gender-dependent bacterial composition could be explained largely by the second and third coordinates. The values often decreased from males to females, except for *S. innoxium*, for which only the third coordinate produced separation. The females of *S. slossonae* were separated from all other samples by the third coordinate, in agreement with their avian blood-feeding behavior and small body size.

The composition of bacteria associated with black flies apparently is determined by complex factors. The distribution of the putative gut symbiont *Rhizobiales* is largely independent of the phylogeny of the host ants but is correlated with dietary preference (Russell *et al.*, 2009). Both diet and phylogeny are important in shaping the gut microbial community in mammals, with diversity increasing from carnivory to omnivory to herbivory (Ley *et al.*, 2008). Some cosmopolitan species that we detected might be potential symbionts or probiotic bacteria. Dependence on diet, phylogeny, or even anatomy and immunity, might be revealed when further studies are conducted on some of the detected bacteria.

Localization of bacteria in flies

Our cloning-and-sequencing analysis gives no clue about bacterial localization. Fluorescence *in situ* hybridization (FISH), therefore, was used to assess the distribution and prevalence of selected bacterial species. Probes were designed based on cloned sequences (Table 2). Because the bacterial composition of *S. krebsorum* was more similar to that of *S. innoxium*, *S. krebsorum* was excluded from the analysis. The female-specific localization of *Pantoea* sp. BF01, *Moraxella* sp. BF02, *S. melonis* BF03, and *K. pneumoniae* BF04 in *S. innoxium* was confirmed by FISH (Fig. S7 and summarized in Table 2). *Pantoea* sp. BF01 was the only bacterium among these taxa occasionally detected in males of *S. innoxium*. Foci in males likely resulted from cross-hybridization against unidentified low-abundance bacteria. In both sexes, bacteria exist in the hemocoel and were found even in the leg cavity of female flies.

Each bacterium had a specific niche. *Moraxella* sp. BF02 was found mainly in gut tissue and occasionally in the gut lumen (Table 2). The probe also detected related bacterial species in the hemolymph of *S. slossonae*. Sequencing suggests that these bacteria were *Moraxella* sp. BF13 and *Sphingomonas* sp. BF15. *Sphingomonas melonis* BF03 was the only bacterium attached to the outer cuticular surface. This bacterium was found in both sexes of *S. dixiense* and *S. slossonae*, and even on the outer surface of the larvae of *S. innoxium*, though, not on the cuticle of adult males of *S. innoxium*. The probe also revealed that other *Sphingomonas* phylotypes exist in the body cavity. *Klebsiella pneumoniae* BF04 was found in the hemolymph of adult females of *S. innoxium*, but not in males. It attached to the epithelium and other noncuticular structures. We also designed a probe against *A. haemolyticus* SI02, which was male specific in *S. innoxium*. This bacterium was found in the hemolymph, and signals of the probe also were detected in *S. slossonae* and *S. dixiense*, where similar sequences were found by sequencing (Table 1).

The gut tissue is the most bacteria-rich location in the flies. Several morphotypes were revealed in the gut tissue by the nonspecific eubactertia probe, but only *Moraxella* sp. BF02 was detected in the gut lumen. The inner surface of the peritrophic matrix had a rod-shaped bacterium in one of four females of *S. innoxium* examined by

Table 2. Localization of bacteria with FISH in three species of black flies

		S. innoxium		*S. dixiense*		*S. slossonae*		
Target	Probe sequence	Male	Female	Male	Female	Male	Female	Location
BF01	5′-TTGCTGAGGTTATTAACCTCA-3′	+*	++	–	–	–	–	Body cavity
BF02[†]	5′-CAGGTAACGTCTAATCTAATGGG-3′	–	++	–	–	++	–	Within gut wall and occasionally in gut lumen
BF03[‡]	5′-CCCGGTACTGTCATTATCATC-3′	–	++	++	++	++	++	Outer cuticular surface
BF04	5′-CGACACGGTTATTAACCGTA-3′	–	++	–	–	–	–	Inner epithelium and other tissue surfaces in hemolymph
SI02	5′-CACTCACTATAGGTATTATCTATAG-3′	++	–	++	–	–	++	Body cavity

++, abundant; +, occasionally detected; –, not detected.

*Instead of being solitary cells, these bacteria in males form small clusters.

[†]Recognized subgroup including *Moraxella* sp. BF13 and *Sphingomonas* sp. BF15.

[‡]Recognized subgroup belonging to the genus *Sphingomonas*, including BF10, BF12, SD09, BF16, SD10, and BF17, with the bacterial size and shape are distinguishable.

scanning electron microscopy (unpublished). The gut symbiont 'Candidatus *Erwinia dacicola*' in the olive fly can persist both intracellularly in the gastric caeca of the larvae and extracellularly in the lumen of the gut and ovipositor diverticula of the adult (Estes *et al.*, 2009). *Moraxella* sp. BF02 and some other bacteria might persist in the gut wall when the insects purge their guts before pupation and then recolonize the gut lumen after adult eclosion. How *S. melonis* BF03 persists on the fly when the old cuticle is shed is unknown. Differences between males and females of *S. innoxium* apparently prevent this bacterium from recolonizing and surviving in male pupae.

Conclusions

A systematic survey of associated bacteria was performed on black flies, one of the most important medicoveterinary groups of arthropods. Each species of fly had a unique bacterial profile, with clear gender dependency. The majority of detected bacteria are still-unknown species. However, 17 cosmopolitan phylotypes were found, and they are potential candidates for symbionts, probionts, or pathogens. The community of bacteria associated with black flies is remarkably rich, including bacteria that are species specific and gender specific. This bacterial colonization pattern is potentially applicable to all blood-sucking insects with similar life histories. The implication for insects generally is that pest and vector control, as well as conservation of threatened and endangered species, should consider the larger community of organisms interacting on and in both genders of each host species. Understanding the physiological roles of the associated bacteria also might pave the way for developing novel pest and vector-control strategies. Control and conservation, thus, would take on a community perspective, with the host as both an organism and a habitat supporting a diverse microbiota.

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Supporting Information

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

Fig. S1. Phylogram of bacterial 16S rDNA sequences detected in the adult fly of *S. innoxium*.

Fig. S2. Clustering of bacterial 16S rDNA sequences retrieved from *S. slossonae*.
Fig. S3. Clustering of bacterial 16S rDNA sequences retrieved from *S. krebsorum*.
Fig. S4. Clustering of bacterial 16S rDNA sequences retrieved from *S. dixiense*.
Fig. S5. Phylogram of bacterial sequences detected with the inner pair of PCR primers from larvae of *S. innoxium*.
Fig. S6. Phylogram of bacterial sequences detected with the outer pair of PCR primers from larvae of *S. innoxium*.
Fig. S7. Localization of bacterial species in black flies.
Table S1. The probability of detecting the number of bacterial phylotypes shown in Figs 1 and 3, with the normal approximation.
Table S2. Beta-diversity values between male and female black flies of four species calculated using the tree-based estimator weighted UniFrac index (all significance < 0.01).
Data S1. The probability of detecting a bacterial phylotype in a PCR sample.

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