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Tissue Localization and Variation of Major Symbionts in *Haemaphysalis longicornis*, *Rhipicephalus haemaphysaloides*, and *Dermacentor silvarum* in China

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ABSTRACT Ticks are important disease vectors, as they transmit a variety of human and animal pathogens worldwide. Symbionts that coevolved with ticks confer crucial benefits to their host in nutrition metabolism, fecundity, and vector competence. Although over 100 tick species have been identified in China, general information on tick symbiosis is limited. Here, we visualized the tissue distribution of *Coxiella* sp. and *Rickettsia* sp. in lab-reared *Haemaphysalis longicornis* and *Rhipicephalus haemaphysaloides* by fluorescent *in situ* hybridization. We found that *Coxiella* sp. colonized exclusively the Malpighian tubules and ovaries of *H. longicornis*, while *Rickettsia* sp. additionally colonized the midgut of *R. haemaphysaloides*. We also investigated the population structure of microbiota in *Dermacentor silvarum* ticks collected from Inner Mongolia, China, and found that *Coxiella*, *Rickettsia*, and *Pseudomonas* are the three dominant genera. No significant difference in microbiota composition was found between male and female *D. silvarum* ticks. We again analyzed the tissue localization of *Coxiella* sp. and *Rickettsia* sp. and found that they displayed tissue tropisms similar to those in *R. haemaphysaloides*, except that *Rickettsia* sp. colonized the nuclei of spermatids instead of ovaries in *D. silvarum*. Altogether, our results suggest that *Coxiella* sp. and *Rickettsia* sp. are the main symbionts in the three ticks and reside primarily in midgut, Malpighian tubules, and reproductive tissues, but their tissue distribution varies in association with species and sexes.

IMPORTANCE Tick-borne diseases constitute a major public health burden, as they are increasing in frequency and severity worldwide. The presence of symbionts helps ticks to metabolize nutrients, promotes fecundity, and influences pathogen infections. Increasing numbers of tick-borne pathogens have been identified in China; however, knowledge of native ticks, especially tick symbiosis, is limited. In this study, we analyze the distribution of *Coxiella* sp. and *Rickettsia* sp. in tissues of laboratory-reared *Haemaphysalis longicornis* and *Rhipicephalus haemaphysaloides* and field-collected *Dermacentor silvarum*. We found that the localization patterns of *Coxiella* sp. in three Chinese tick species were similar to those of other tick species. We also found a previously undefined intracellular localization of *Rickettsia* sp. in tick midgut and spermatids. In addition, we demonstrate that tissue tropisms of symbionts vary between species and sexes. Our findings provide new insights into the tissue localization of symbionts in native Chinese ticks and pave the way for further understanding of their functional capabilities and symbiotic interactions with ticks.

KEYWORDS tissue localization, ticks, *Coxiella*, *Rickettsia*, microbiota

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Ticks are obligate bloodsucking invertebrates transmitting a variety of diseases all over the world. WHO estimates that 532,125 cases of Lyme disease in the United States and Western Europe and 10,000 to 12,000 cases of tick-borne encephalitis occurred globally in 2017 (1). In China alone, 33 tick-borne pathogens have been identified since 1982. This number, added to that of traditional tick-borne diseases, indicates that tick-borne diseases have emerged as a public health concern (2, 3). Human cases of Lyme borreliosis have been confirmed in almost all provinces except Tibet and Shanghai (2). Patients from 8 provinces have been diagnosed with babesiosis, with *Babesia microti*, *Babesia divergens*, and *Babesia venatorum* as the major disease-causing agents (2, 4). Severe fever with thrombocytopenia syndrome virus (SFTSV) is an emerging pathogen that has infected 2,543 humans, causing 154 deaths in China in 2013 (2). However, tick-borne diseases are still underestimated because of the complex distribution and the lack of effective diagnostic methods in China.

There are a total of 117 species of ticks in China, divided into 2 families, the Argasidae and the Ixodidae (5). Thirteen species belong to Argasidae, of which 4 species are able to transmit pathogens causing human illness (6). A total of 104 species in Ixodidae have been found in China, of which 32 species are confirmed to be disease-transmitting vectors (6). Of these 32 species, *Haemaphysalis longicornis* and *Ixodes persulcatus* are considered to be the most competent vectors, as they transmit at least 15 pathogens, including *Borrelia burgdorferi*, *Theileria* spp., *Coxiella burnetii*, *Babesia* spp., *Anaplasma phagocytophilum*, *Ehrlichia*, *Bartonella*, spotted-fever group rickettsiae (SFGR), Huaiyangshan virus, and the recently identified New bunyavirus, among others. The tick *Dermacentor silvarum* is another major vector associated with 13 emerging pathogens, including SFGR, *Anaplasmataceae*, *Borrelia* spp., and *Babesia* spp. (2).

In addition to pathogens, ticks also harbor microbiota that are critical for their physiology (7, 8). *Coxiella*, as an obligate symbiont, is present in at least two-thirds of tick species in the world (8, 9). This symbiont preferentially colonizes the ovaries and Malpighian tubules, suggesting its role in fecundity, metabolism, and osmoregulation (10–14). Its genome encodes almost complete pathways for the biosynthesis of major B vitamins and cofactors that are essential in tick physiology (15). Elimination of *Coxiella* by antibiotic treatment reduces the fecundity of *Amblyomma americanum* and *H. longicornis* and prevents *Rhipicephalus microplus* from developing into its adult form (16–18). *Rickettsia* is another obligate tick symbiont found in multiple tick species (8, 13, 14, 19). Its genome contains all the genes for folate *de novo* biosynthesis, suggesting its role in nutrition provision to ticks (20). Ticks also harbor other maternally transmitted symbionts, including *Francisella*, *Arsenophonus*, *Rickettsiella*, *Cardinium*, *Spiroplasma*, *Lariskella*, *Midichloria*, and *Wolbachia*, although their functions need to be further investigated (8). In addition to symbionts, other microbiota are present across several hard tick species, with 5 bacterial genera frequently observed. They are *Pseudomonas*, *Sphingobacterium*, *Acinetobacter*, *Enterobacter*, and *Stenotrophomonas* (7). The presence of these microbiota is essential in determining the infection outcome of different tick species (21). Increasing the quantities of the endosymbiont *Rickettsia* in the tick *Dermacentor andersoni* results in the reduction of *Anaplasma marginale* infection, while decreasing the quantities of *Francisella* leads to a reduction in infections by the pathogen *Francisella novicida* (22). Disturbing the homeostasis of gut microbiota in the tick *Ixodes scapularis* by antibiotic treatment reduces colonization by *Borrelia burgdorferi* (23). The presence of rickettsial endosymbionts protects *I. scapularis* from *B. burgdorferi* infection (24). Native ticks in China also harbor a variety of microbiota. *Coxiella* and *Rickettsia* are present in *H. longicornis* (Hebei), *D. silvarum* (Hebei), and *R. microplus* (13, 14, 25). *Arsenophonus* has been detected in *H. longicornis* (Hebei) and *D. silvarum* (Hebei) (13). In *I. persulcatus*, the genera *Acinetobacter*, *Rickettsia*, *Pseudomonas*, *Chryseobacterium*, and *Sphingobacterium* are the most abundant bacteria in unfed ticks (26). Studies of native Chinese ticks focus mainly on the detection of microbes using PCR and 16S rRNA sequencing techniques, while further information about their tissue distribution is limited.

In this study, we have investigated the localization of microbiota in 3 native Chinese

ticks, *H. longicornis*, *R. haemaphysaloides*, and *D. silvarum*, using fluorescent *in situ* hybridization (FISH), and examined the microbial population structure of field-collected *D. silvarum* (from Inner Mongolia). We found that midgut, reproductive tissues, and Malpighian tubules are the three primary tissues in which microbes reside. Field-collected *D. silvarum* ticks have a complex microbiota, with *Pseudomonas*, *Coxiella*, and *Rickettsia* as the dominant genera.

RESULTS

Localization of microbiota in *H. longicornis* and *R. haemaphysaloides*. We first examined the tissue localization of microbiota in two tick species, *H. longicornis* and *R. haemaphysaloides*, originally from Yunnan Province, China, that had been maintained in the insectary for at least 2 years. To visualize the distribution of microbes in tissues of *H. longicornis*, whole bodies as well as dissected organs of female ticks were analyzed by FISH using a fluorescently labeled universal 16S rRNA probe (Fig. 1A to C). Intense staining was observed in Malpighian tubules and ovaries of whole-body sections (Fig. 1A to C). To further confirm the spatial distribution in the two organs, tissues, including midgut, salivary glands, Malpighian tubules, and ovaries were dissected 48 h following a blood meal and analyzed in the same manner as the whole-body sections. Similarly, strong fluorescent signals were detected in Malpighian tubules and ovaries (Fig. 1E1 and G1). No detectable signal was observed in midgut or salivary glands (Fig. 1D1 and F1). No signal was detected in controls with noneubacterial probe or without probe (Fig. 1D2 to G2 and D3 to G3). There was a high density of symbionts in the cytosol of Malpighian tubule cells but not in the tubule lumen (Fig. 1E1). Asynchronously developed oocytes were observed in the ovaries (Fig. 1G1). A large number of egg chambers at different developmental stages were attached to the ovarian wall. Symbionts were clustered around the nuclei in young oocytes but were concentrated on one side of the oocytes once matured (Fig. 1G1). This staining pattern indicates that Malpighian tubules and ovaries are the two major tissues harboring microbiota in female *H. longicornis*.

The tissue distribution of microbiota in female *R. haemaphysaloides* ticks was also investigated in the same way as that described above. We again detected intense fluorescent signals in Malpighian tubules and ovaries, with the same subcellular localization pattern as in *H. longicornis* (Fig. 2B1 and D1). In addition, we observed that midgut was the third organ that harbors microbiota (Fig. 2A1). Here the microbes clustered within a few midgut cells, leaving the midgut lumen free of bacteria. Again, no bacteria were detected in salivary glands (Fig. 2C1). Our results indicate that the *R. haemaphysaloides* microbiota shows a broader tissue tropism than does that of *H. longicornis*.

Localization of *Coxiella* sp. and *Rickettsia* sp. symbionts in *H. longicornis* and *R. haemaphysaloides*. *Coxiella* sp. and *Rickettsia* sp. (species were not identified here; see Materials and Methods) are two of the main symbionts present in multiple tick species in China, including *Haemaphysalis tibetensis*, *H. longicornis*, *D. silvarum*, and *R. microplus* (13, 14, 25, 27, 28). To examine the presence and abundance of these species in lab-reared *H. longicornis* and *R. haemaphysaloides*, we quantified their relative abundance by real-time PCR using genus-specific primers (Fig. 3). *Coxiella* sp. was the dominant symbiont in both *H. longicornis* and *R. haemaphysaloides*. *Rickettsia* sp. was barely detected in *H. longicornis*, but it was present in both male and female *R. haemaphysaloides* ticks, although its relative abundance was significantly lower than that of *Coxiella* sp. (Fig. 3A and B).

We next examined the tissue distribution of these two symbionts in *H. longicornis* and *R. haemaphysaloides* colonies. As no *Rickettsia* sp. was detected in *H. longicornis*, sections of the same four tissues were hybridized only with a *Coxiella*-specific 23S rRNA probe (Fig. 4A1 to A4). We again found the same localization pattern as the one revealed by the universal 16S rRNA probe. Strong signals were detected exclusively in Malpighian tubules and ovaries (Fig. 4A2 and A4).

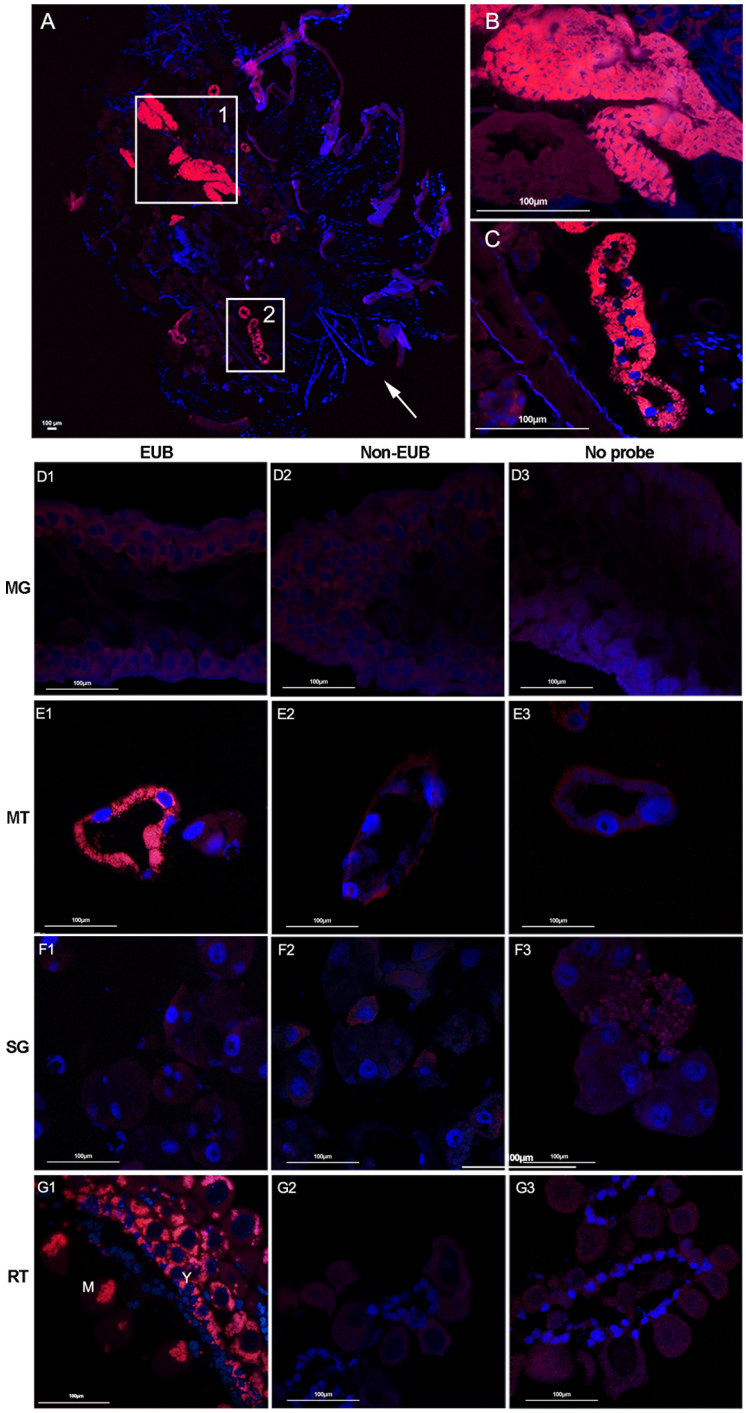

FIG 1 Localization of microbiota in whole body (A to C) and different tissues (D to G) of female *H. longicornis* by FISH analysis with universal 16S rRNA probe (red). Nuclei were stained with DAPI (blue). (A) Signals detected in whole body sections; the arrow denotes the direction from head to bottom; (B) close-up view of box 1 showing the ovary filled with microbiota; (C) close-up view of box 2 showing that the microbiota colonized the Malpighian tubules. Images are representative of at least 5 individual tick sections. Different tissues, including midgut (D), Malpighian tubules (E), salivary gland (F), and ovaries (G) were hybridized with universal 16S rRNA probe (EUB) (D1, E1, F1, and G1), noneubacterial probe (Non-EUB) (D2, E2, F2, and G2), and no probe (D3, E3, F3, and G3). MG, midgut; MT, Malpighian tubules; SG, salivary glands; RT, ovaries; Y, young oocytes; M, mature oocytes. Tissues from at least 5 individual ticks were pooled and used for FISH analysis. Images are representative of three independent experiments.

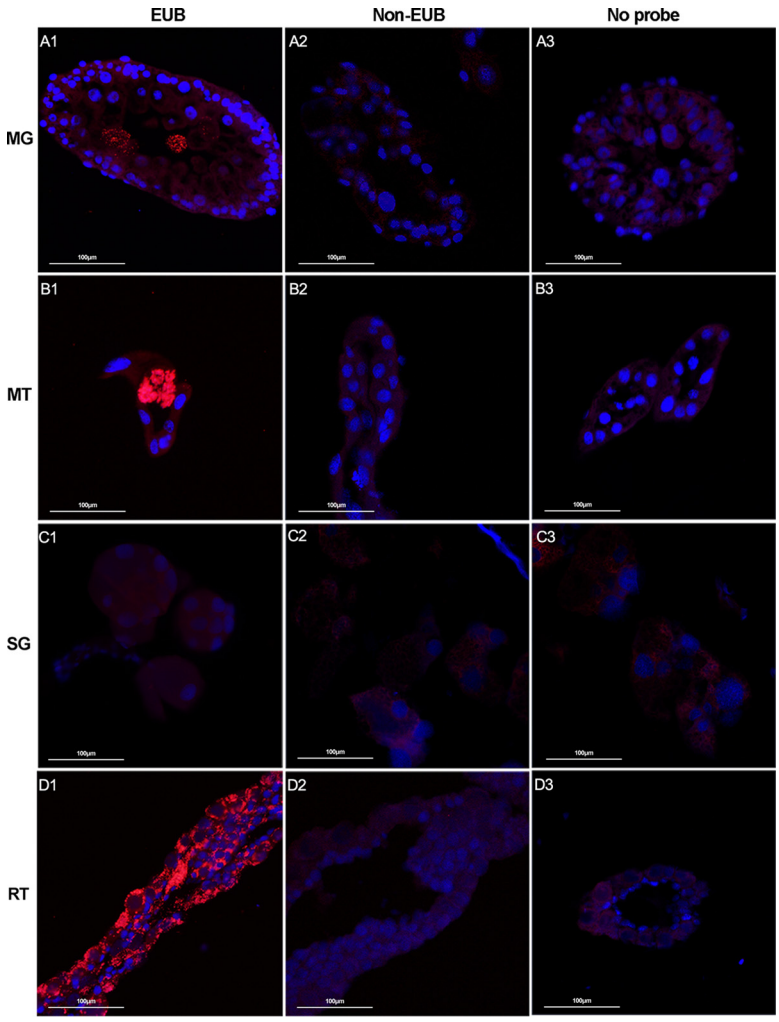

FIG 2 Localization of microbiota in different tissues of female *R. haemaphysaloides* by FISH analysis with universal 16S rRNA probe (red). Nuclei were stained with DAPI (blue). Fluorescent signals were examined in midgut (A), Malpighian tubules (B), salivary glands (C), and ovaries (D). Hybridizations with noneubacterial probe (Non-EUB) and without probe were used as negative controls. Tissues from at least 5 individual ticks were pooled and used for FISH analysis. Images are representative of three independent experiments.

As the two symbionts were detected in *R. haemaphysaloides*, costaining of *Coxiella* sp. and *Rickettsia* sp. was performed in both female and males (Fig. 4B1 to C4). Again, intense signals of *Coxiella* sp. were observed in ovaries (Fig. 4B4 and B5) of female ticks and in Malpighian tubules of both male and female ticks (Fig. 4B2, C2, and C5). *Rickettsia* sp. was extensively distributed in midgut, Malpighian tubules, and ovaries (Fig. 4B1, C1, B2, C2, B4, and B5). The midgut was colonized by *Rickettsia* sp. exclusively. This bacterium clustered intracellularly in certain gut cells, leaving major parts of gut free of bacteria (Fig. 4B1 and C1). *Rickettsia* sp. in Malpighian tubule and ovary displayed a subcellular localization pattern similar to that of *Coxiella* sp., as the two bacteria reside intracellularly in Malpighian tubule cells, in interstitial cells between primary oocytes, and in cytoplasm of developing oocytes. As fluorescent signals of *Coxiella* sp. were detected only in a few sections of female Malpighian tubules while *Rickettsia* sp. was easily observed, we next analyzed the localization of the two symbionts in Malpighian tubules using whole-mount staining. We found that *Coxiella* sp. colonized only the distal region of Malpighian tubules, while *Rickettsia* sp. was widely spread all over the tissue (Fig. 4D1 to D3). Colocalization of these two symbionts is observed in ovaries and Malpighian tubules, indicating that a mutually beneficial

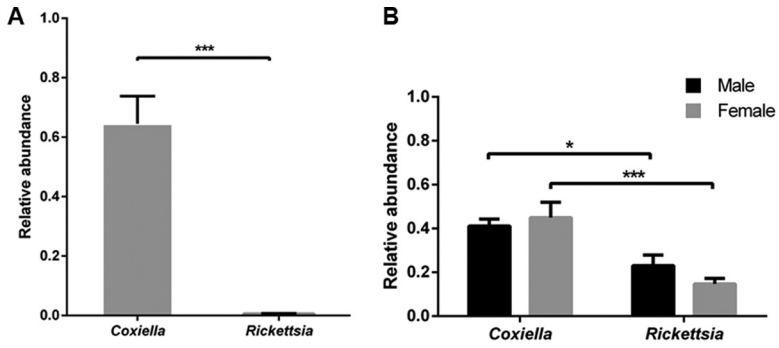

FIG 3 Relative abundance of *Coxiella* sp. and *Rickettsia* sp. in *H. longicornis* (A) and *R. haemaphysaloides* (B) determined by real-time quantitative PCR. Error bars indicate standard errors ($n = 15$). Significance was determined by Mann-Whitney test. *, $P < 0.05$; ***, $P < 0.001$.

relationship possibly exists between these two microbes (Fig. 4B2, B4, and C2). Species-specific labeling techniques confirm the different tissue tropism patterns of *Coxiella* sp. and *Rickettsia* sp. and the colocalization of these two symbionts in the same tissue.

Population structure of microbiota in *D. silvarum*. To understand the diversity of microbiota in field ticks, *D. silvarum* ticks, an important disease vector in the northern part of China, were collected from Inner Mongolia in March 2017. The population structure of microbiota in unfed male and female *D. silvarum* was analyzed using high-throughput sequencing of the 16S rRNA gene (V3-V4 region). The phylum *Proteobacteria* was dominant in this species, with relative abundance ranging from 83.3% to 97.5% (Fig. 5A). Ten bacterial genera, including *Coxiella*, *Pseudomonas*, *Rickettsia*, *Acinetobacter*, *Sphingomonas*, *Empedobacter*, *Chryseobacterium*, *Methylobacterium*, *Sphingobacterium*, and *Janthinobacterium*, were identified in all experimental groups but at different relative levels of abundance (Fig. 5B). *Pseudomonas*, *Coxiella*, and *Rickettsia* were the most abundant genera in *D. silvarum*. The rarefaction curves of operational taxonomic unit (OTU) numbers performed with male and female ticks confirmed that sequencing coverage was sufficiently extensive to fully evaluate the microbially diverse populations (Fig. 5C). Female and male ticks share most taxa. To examined if there was a difference in population structure of microbiota between male and female *D. silvarum* ticks, alpha diversity was calculated. There was no significant difference between male and female ticks as depicted by the Observed-species, Chao1, Shannon, and Simpson indices (Table 1). To further confirm the relative abundance of *Pseudomonas*, *Coxiella*, and *Rickettsia* estimated by 16S rRNA deep sequencing, real-time quantitative PCR (qPCR) was performed using universal 16S rRNA primers and genus-specific primers. In agreement with sequencing data, *Pseudomonas* sp., *Coxiella* sp., *and Rickettsia* sp. abundantly colonized *D. silvarum*, with relative abundances similar to those determined by 16S rRNA deep sequencing (Fig. 5D). To further confirm that *Pseudomonas* sp. resided within tick organs instead of being merely a cuticle contaminant, different tissues, including Malpighian tubules, salivary glands, midgut, and reproductive organs (ovaries/testes) were used to determine the presence and abundance of this bacterium (Fig. 5E). It was detected in all four tissues, with higher abundance in males than females. Our data show that *Coxiella* sp. and *Rickettsia* sp. are the main symbionts in *D. silvarum* and that *Pseudomonas* sp. is another dominant commensal with broad tissue distribution. There is no significant difference of microbiota structure between males and females.

Localization of symbionts in *D. silvarum*. We next analyzed the tissue distribution of microbiota in *D. silvarum*. As in *R. haemaphysaloides*, where they are widely spread all over the tissues, high densities of microbes were detected intracellularly in the midgut, Malpighian tubules, and ovaries (Fig. 6A1 and A4, B1 and B4, and D1). Interestingly, fluorescent signals were also observed in the nuclei of spermatids (Fig. 6D4 and D7), suggesting the presence of symbionts in male testes.

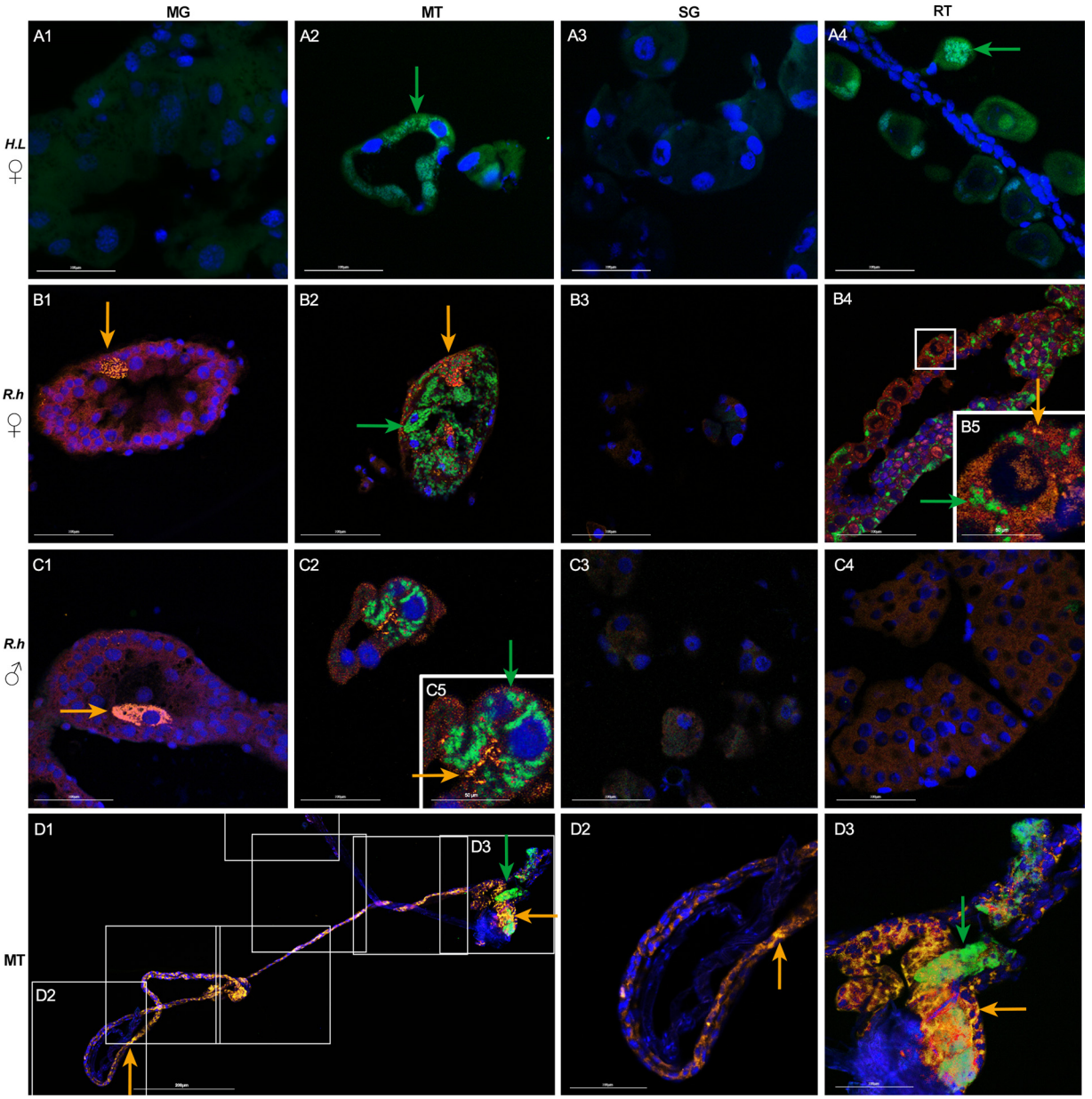

FIG 4 Localization of *Coxiella* sp. (green) and *Rickettsia* sp. (yellow) in *H. longicornis* (A) and *R. haemaphysaloides* (B to D) using species specific probes. Nuclei were stained with DAPI (blue). Merged images of DAPI and *Coxiella* sp. staining in female *H. longicornis* (A1 to A4). Merged images of DAPI, *Coxiella* sp., and *Rickettsia* sp. staining in female (B1 to B4) and male (C1 to C4) *R. haemaphysaloides*. Spliced view of whole-mount *in situ* hybridization in female Malpighian tubules of *R. haemaphysaloides* (D1 to D3). (A1, B1, C1), midgut (MG); (A2, B2, C2), Malpighian tubules (MT); (A3, B3, C3), salivary glands (SG); (A4), ovaries of *H. longicornis* (reproductive tissues [RT]); (B4), ovaries of *R. haemaphysaloides*; (C4), testes of *R. haemaphysaloides*; (B5) close-up view of boxed region, showing colocalization of *Rickettsia* sp. and *Coxiella* sp. in ovaries; (C5) close-up view of panel C2, colocalization of *Rickettsia* sp. and *Coxiella* sp. in Malpighian tubules. (D2, D3) Close-up view of boxed regions in D1. Green arrows denote *Coxiella* sp. and yellow arrows denote *Rickettsia* sp. Tissues from at least 5 individual ticks were pooled and used for FISH analysis. Each image shows a single focal plane. Images are representative of three independent experiments. Bars, 100 μm.

We next localized *Coxiella* sp. and *Rickettsia* sp. in the same four tissues, including midgut, Malpighian tubules, salivary glands, and ovaries/testes. In females, *Coxiella* sp. was detected in Malpighian tubules and ovaries (Fig. 7B1 and D1), while *Rickettsia* sp. was detected in midgut and Malpighian tubules (Fig. 7A2 and B2). In males, Malpighian tubules were the only tissues that were heavily infected with *Coxiella* sp. (Fig. 7B4). *Rickettsia* sp. was distributed in midgut, Malpighian tubules, and testes (Fig. 7A5, B5, and D5). It was interesting that each spermatid contained multiple *Rickettsia* sp. cells and that this bacterium preferentially localized in the nucleus of spermatids (Fig. 7D5).

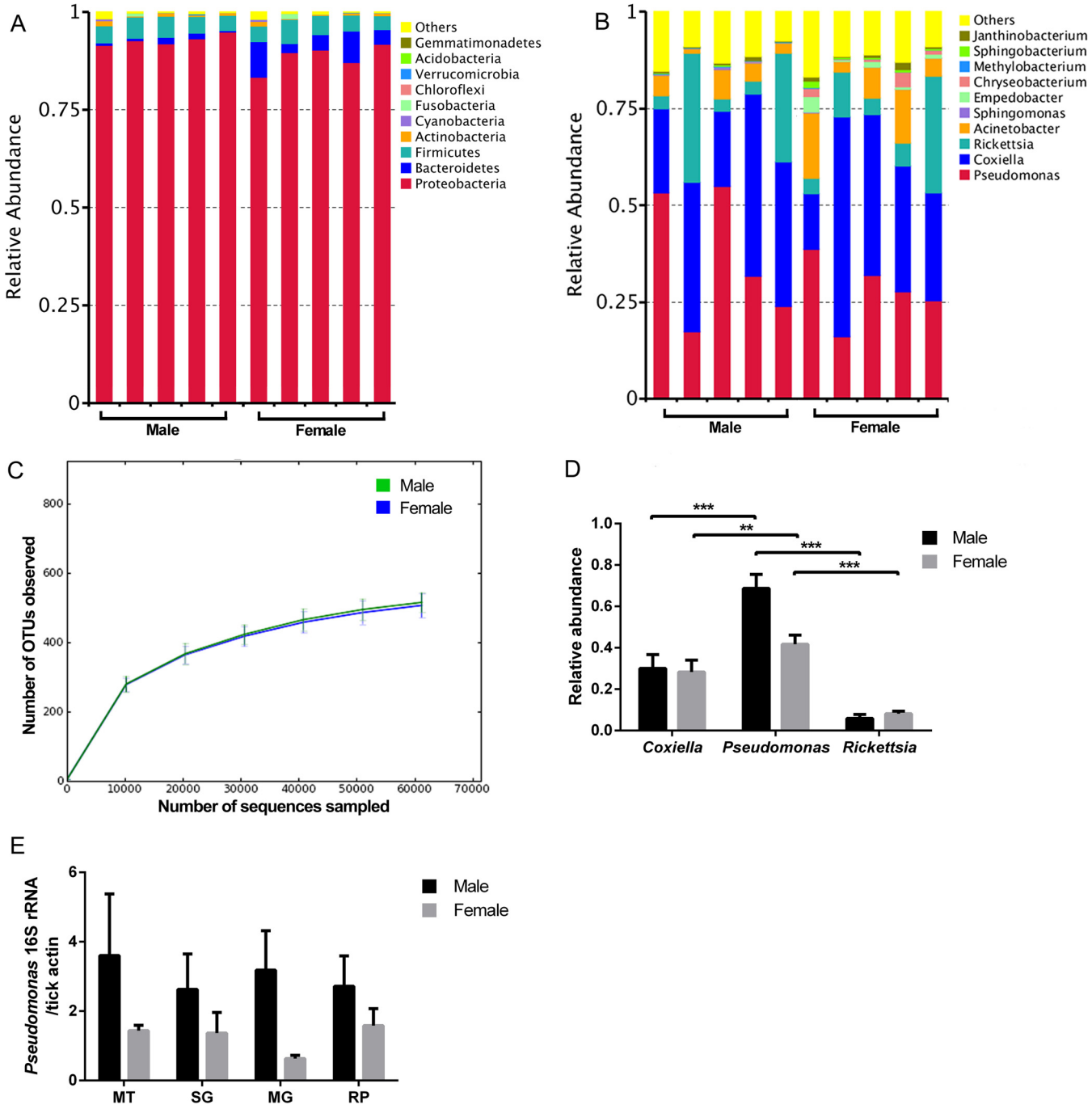

FIG 5 Population community of *D. silvarum* by 16S rRNA pyrosequencing. Phylum level (A) and genus level (B) abundance profiles for individual *D. silvarum* males and females prior to a blood meal. Each column represents one tick. (C) Rarefaction curve of the 16S rRNA gene sequences in male and female *D. silvarum* ticks based on OTUs determined at 97% similarity. (D) Relative abundance of *Coxiella* sp., *Pseudomonas* sp., and *Rickettsia* sp. in *D. silvarum* analyzed by qPCR. (E) Quantification of *Pseudomonas* sp. in different tick tissues by qPCR. Tissues from five individual tick were pooled for one biological replicate. Five biological replicates were used for qPCR analysis. Error bars indicate standard errors ($n = 5$). Statistical significance was determined using the Mann-Whitney test. **, $P < 0.01$; ***, $P < 0.001$.

This is in agreement with results obtained by using a universal 16S rRNA probe, showing that bacteria colonized nuclei of spermatids. To further confirm the presence of *Pseudomonas* sp. in *D. silvarum*, we performed whole-mount *in situ* hybridization in midgut, which contained a relatively high abundance of *Pseudomonas* sp. (Fig. 7E1 to E3). Strong signals were observed in the midgut. Our results reveal that colocalization of *Coxiella* sp. and *Rickettsia* sp. is observed in Malpighian tubules in a manner similar

TABLE 1 Alpha diversity analysis of bacterial population structure in *D. silvarum*

Tick sex or *P* value	Value (mean ± SD)			
	Observed-species index	Chao1 index	Shannon index	Simpson index
Male ticks	515.4 ± 40.7	585.1 ± 35.6	2.95 ± 0.33	0.71 ± 0.02
Female ticks	506.6 ± 48.8	563.7 ± 55.4	3.42 ± 0.66	0.76 ± 0.09
P value[a]	1.0000	0.6586	0.1061	0.1136

[a]No significant difference was found between male and female ticks for any index of diversity.

to that seen in *R. haemaphysaloides*. Although female and male *D. silvarum* ticks share the same bacterial taxa, their localization in reproductive tissues is different.

In summary, our results from *H. longicornis*, *R. haemaphysaloides*, and *D. silvarum* suggest that Malpighian tubules and ovaries are two major organs harboring *Coxiella* sp. and *Rickettsia* sp. and that the midgut is colonized with *Rickettsia* sp. The tissue localization pattern of the same symbionts in these three tick species varies. Additionally, the two symbionts in the same tick species also display different tissue tropisms between sexes.

DISCUSSION

Emerging evidence shows that ticks harbor multiple symbionts that are important for their fecundity and vector competence (7, 8). However, information regarding tissue

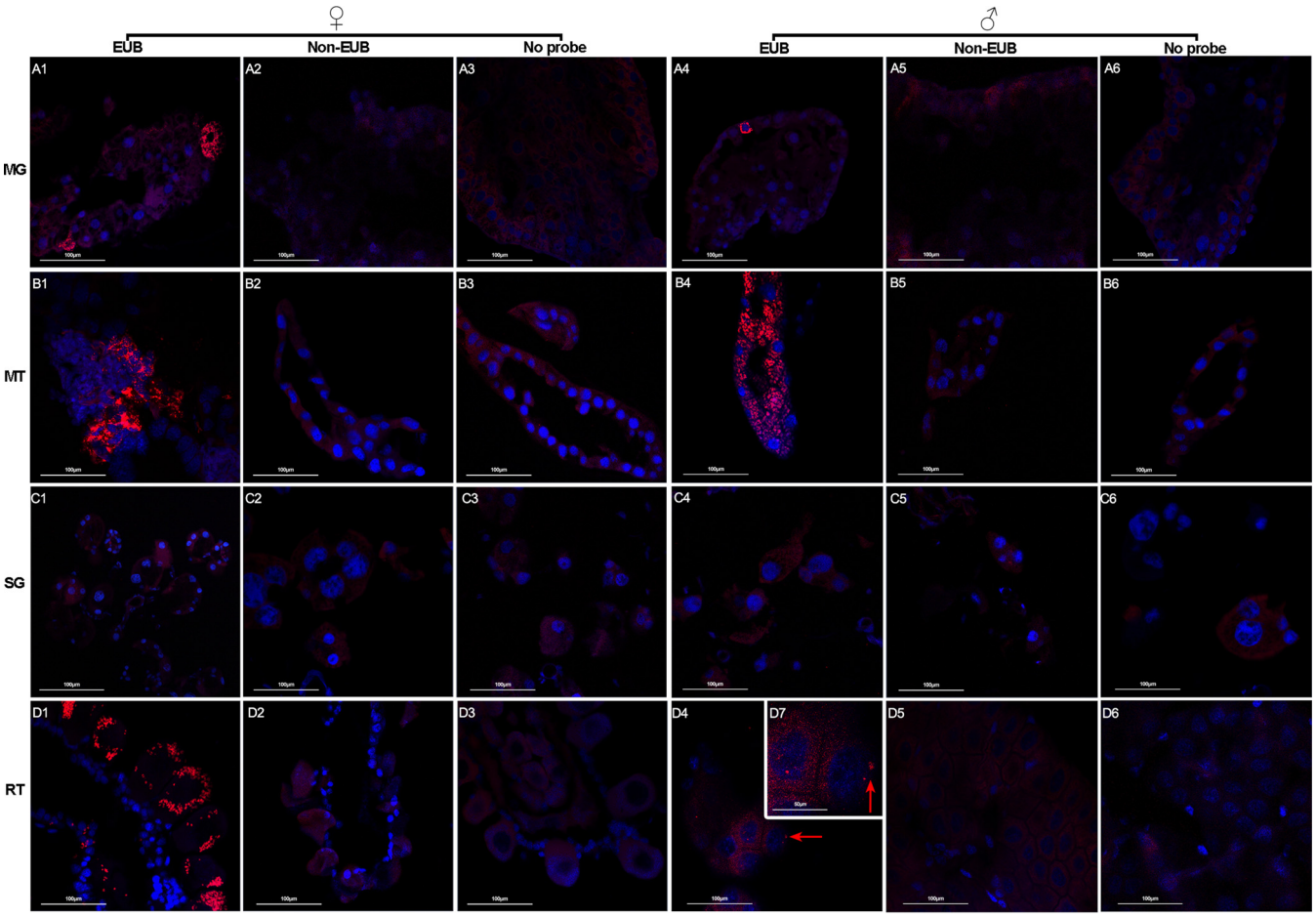

FIG 6 Localization of microbiota in different tissues of female and male *D. silvarum* ticks by FISH analysis with universal 16S rRNA probe (red). Nuclei were stained with DAPI (blue). Fluorescent signals were examined in midgut (A), Malpighian tubules (B), salivary glands (C), ovaries (D1 to D3), and testes (D4 to D7). Hybridizations with noneubacterial probe (Non-EUB) and without probe were used as negative controls. Red arrows denote residential bacteria. MG, midgut; MT, Malpighian tubules; SG, salivary glands; RT, reproductive tissue. Tissues from at least 5 individual ticks were pooled and used for FISH analysis. Images are representative of three independent experiments.

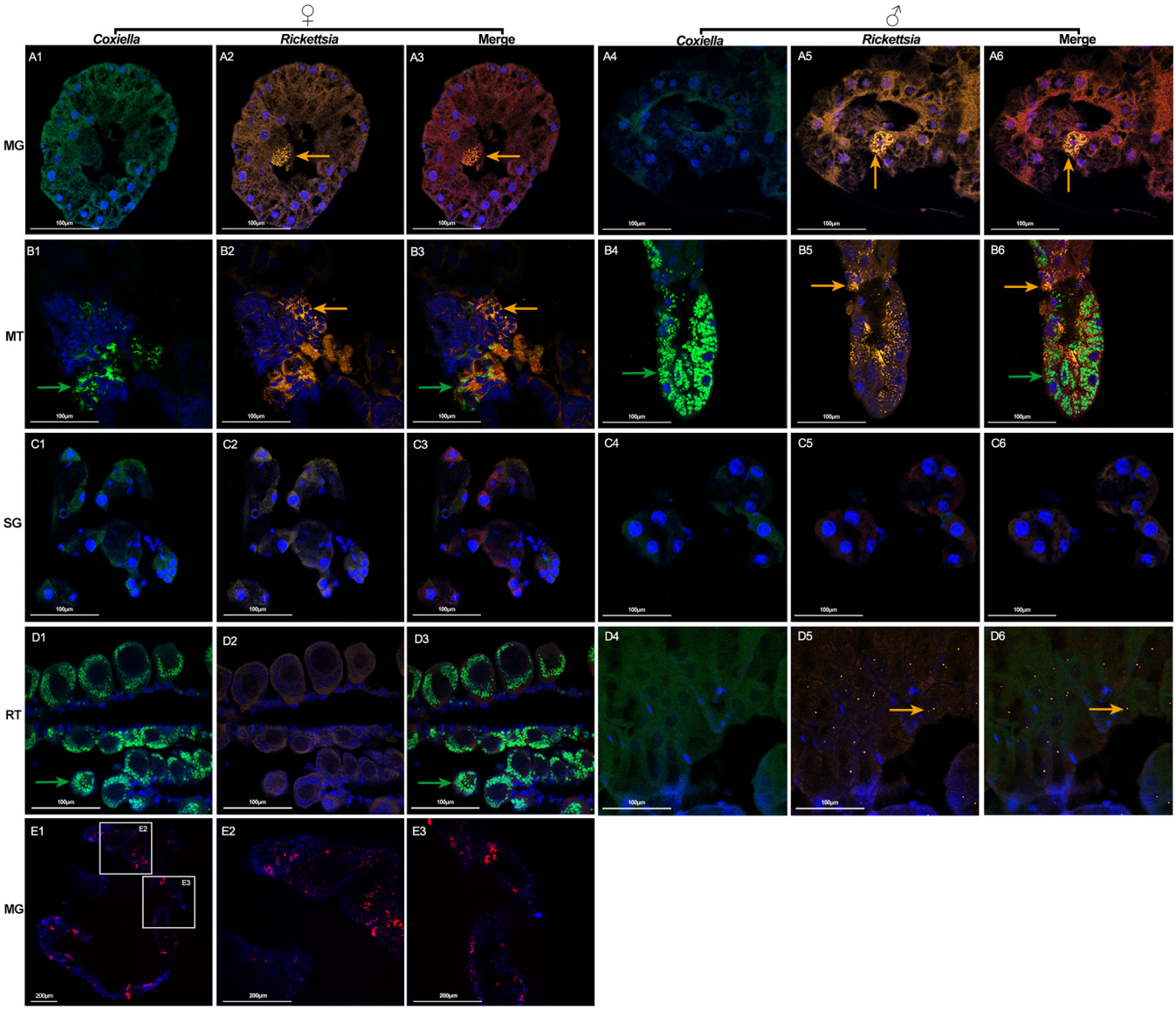

FIG 7 Costaining of *Coxiella* sp. (green), *Rickettsia* sp. (yellow), and *Pseudomonas* sp. (red) in *D. silvarum*. Nuclei were stained with DAPI (blue). Sections of midgut (A1 to A6), Malpighian tubules (B1 to B6), salivary glands (C1 to C6), ovaries (D1 to D3), and testes (D4 to D6) were hybridized with *Coxiella* sp.-specific 23S rRNA probe (A1 and A4, B1 and B4, C1 and C4, and D1 and D4) and *Rickettsia* sp.-specific 16S rRNA probe (A2 and A5, B2 and B5, C2 and C5, and D2 and D5) simultaneously. (A3 and A6, B3 and B6, C3 and C6, and D3 and D6) Merged images of DAPI, *Coxiella* sp., and *Rickettsia* sp. staining. Green arrows denote *Coxiella* sp. and yellow arrows denote *Rickettsia* sp. MG, midgut; MT, Malpighian tubules; SG, salivary glands; RT, reproductive tissue. (E1 to E3) Whole-mount *in situ* hybridization of *D. silvarum* midgut using *Pseudomonas* sp.-specific 16S rRNA probe. (E2, E3) Close-up view of boxed regions in panel E1. Tissues from at least 5 individual ticks were pooled and used for FISH analysis. Each image shows a single focal plane. Images are representative of three independent experiments.

distribution and population diversity of the microbiota in most native ticks in China is far from complete. Here we visualized and compared the localization patterns of symbionts in three Chinese ticks, *H. longicornis*, *R. haemaphysaloides*, and *D. silvarum*, and analyzed the bacterial population structure in *D. silvarum* ticks collected from Inner Mongolia, China.

The presence of microorganisms in ticks was first described by Cowdry in 1925 (29). Multiple symbionts have been detected in different hard tick organs, with *Coxiella*, *Rickettsia*, *Rickettsiella*, *Francisella*, and *Wolbachia* as the most common ones that can be maternally transmitted (8, 21). In addition to symbionts, other microbiota belonging to the phyla *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* have been identified in various tick species (7). In our study, *Coxiella* sp. and *Rickettsia* sp. are the two major symbionts in *H. longicornis*, *R. haemaphysaloides*, and *D. silvarum*. Malpighian

tubules and ovaries are the two tissues that were massively colonized with symbionts in all three tick species. Malpighian tubules are responsible for the excretion of insoluble nitrogenous waste, the detoxification of metabolic wastes and xenobiotics in the hemolymph, and osmoregulation (30, 31). They are retained in both hemimetabolous and holometabolous arthropods throughout metamorphosis (32, 33). It is highly possible that *Coxiella* sp. and *Rickettsia* sp., by hiding in relatively stable Malpighian tubule cells, facilitate their transstadial transmission. Ovaries are the organs that are frequently colonized with symbionts, as the cytoplasm of the eggs provides ample space for symbionts to survive (34). The residence of *Coxiella* sp. or *Rickettsia* sp. in ovaries may facilitate its vertical transmission and ensure the fitness of the next generation. Interestingly, we also observed the intranuclear localization of *Rickettsia* sp. in spermatid of *D. silvarum*, suggesting that this bacterium may be paternally transmitted to the offspring during mating. Symbiont-like microorganisms are associated with the sperm cells and transmitted from male to female through copulation in *Hyalomma marginatum*, *Hyalomma dromedarii*, and *Amblyomma hebraeum* (35). In addition to ticks, *Rickettsia* sp. also infects sperm cells of multiple arthropods (36). It colonizes the spherical coenospermia of a kind of large bird spider (*Pamphobeteus* sp., Araneae) (36). As a facultative symbiont of a leafhopper, *Nephotettix cincticeps*, it also resides inside the nuclei of sperms without influencing male fecundity (37). Paternal transmission of beneficial symbionts is also observed in aphids (38). *Regiella insecticola* and *Hamiltonella defensa* are two male-borne symbionts that localize in testes and accessory glands in aphids. Both of them can be transferred to females during mating (38). Although it is still unclear how *Rickettsia* sp. infects the nucleus of a tick spermatid and what its influence on male fecundity is, the preferential colonization of this bacterium in *D. silvarum* spermatid nucleus indicates the distinct strategies that symbionts use to facilitate their vertical transmission. Such paternal transmission results in coinfections in the offspring that may have broad implications for symbiont-symbiont and symbiont-host interactions. The midgut is the third organ that is colonized with *Rickettsia* sp. in *R. haemaphysaloides* and *D. silvarum*. It is still unclear what type of midgut cells are colonized with this bacterium, and its influence on the hosts is not known. As the midgut is the principal digestive organ and the first tissue that encounters invading pathogens, this close association between *Rickettsia* sp. and midgut might be important for a tick's metabolism and vector competence.

The tissue localization pattern of symbionts differs in the three tick species. *Coxiella* sp. specifically colonizes Malpighian tubules and ovaries of all three tick species. This symbiont is widely distributed in multiple hard tick species collected from different countries, including Brazil, Colombia, Kenya, and China (8, 9, 39). In addition to colonizing Malpighian tubules and ovaries, it also infects the salivary glands of *Amblyomma americanum* and *Amblyomma cajennense* (11, 12). However, it is still unclear if and how the colonization of salivary gland in *Coxiella* sp. influences ticks. *Rickettsia* sp. is present in *R. haemaphysaloides* and *D. silvarum* but absent in *H. longicornis*. *Rickettsia* sp. resides preferentially in ovaries of *R. haemaphysaloides* but is absent in *D. silvarum* ovaries. Instead, it localizes in testes. The differential spatial localization patterns of these symbionts may result from various levels of interactions that these different ticks have with symbionts and from interactions between different symbionts during their long coevolution (8).

The population structure of native microbes also differs in the three tick species. *H. longicornis* in this study is colonized primarily with *Coxiella* sp., while the same tick species collected from Xiaowutai National Natural Reserve Area, in the northeast of China, harbors both *Coxiella* sp. and *Rickettsia* sp. (14). One possible reason is that *H. longicornis* in this study is parthenogenetic, with offspring developing from unfertilized eggs. The unique reproductive strategy that prevents the symbionts from paternally transmitting to offspring possibly leads to the absence of *Rickettsia* sp. in this tick strain (40). Both *Coxiella* sp. and *Rickettsia* sp. are detected in *R. haemaphysaloides* just as in *Rhipicephalus microplus* ticks collected in multiple places in China (25). Field-collected *D. silvarum* harbors a diverse microbiota, with *Pseudomonas*, *Rickettsia*, and *Coxiella* as

TABLE 2 Primers used in this study

Primer	Sequence	Gene	Organism(s)	Reference
RicCS-AF	5′-GAGTGTAGTAGGGGATGATG-3′	*gltA*	*Rickettsia*	46
RicCS-AR	5′-CCACCATGTCAAGGGTTGGT-3′			
Cox sp434f	5′-CCTTTTGAGCGTTGACGTTA-3′	16S rRNA	*Coxiella*	47
Cox sp1004r	5′-CCAAAGGCACCAAGTCATTT-3′			
Pse435F	5′-ACTTTAAGTTGGGAGGAAGGG-3′	16S rRNA	*Pseudomonas*	48
Pse686R	5′-ACACAGGAAATTCCACCACCC-3′			
Eub27F	5′-AGAGTTTGATCCTGGCTCAG-3′	16S rRNA	*Eubacteria*	45
Eub338R	5′-CATGCTGCCTCCCGTAGGAGT-3′			
DsacF	5′-TTCCAGCCCTCGTTCCTGGGTAT-3′	*actin*	*D. silvarum*	27
DsacR	5′-AATGATCTTGATCTTCATGGT-3′			

the three dominant bacterial genera. The diversity and composition of tick microbiota are influenced by multiple factors, including environmental factors and host factors. The former factors include geographical location, habitat type, seasonal weather, and the vertebrates that they feed on. The latter factors include tick species, development stage, sex, and reproduction strategy (39). It is highly possible that the natural *D. silvarum* population is exposed to a more-complex environment and a greater variety of vertebrates during their development in the field.

Overall, our experiments demonstrate localization patterns of *Coxiella* sp. in three native tick species similar to those observed in other studies (8). We also show a specific intracellular localization pattern of *Rickettsia* sp. in midguts and spermatids, of which little is known in most of the tick species. Although emerging evidence shows that symbionts contribute to many aspects of host development, physiology, fecundity, and immunity, the molecular mechanisms through which the symbionts exert their beneficial influence are still largely undefined. Our work paves the way for future studies focusing on the interactions between coexisting symbionts and tripartite interaction between ticks, native microbes, and pathogens.

MATERIALS AND METHODS

Tick collecting and rearing. Laboratory-reared *H. longicornis* (parthenogenesis strain) and *R. haemaphysaloides* ticks originally collected from Yunnan Province in 2015 and 2013, respectively, were maintained at the laboratory of National Institute of Parasitic Diseases in Shanghai as described previously (41). Adult *D. silvarum* ticks were collected from the Jiagedaqi forest, Inner Mongolia, in March 2017 and maintained in the same place as *H. longicornis* and *R. haemaphysaloides*. Tick species were identified by both morphological characterization and cytochrome *c* oxidase I (COI) genes as previously described (42). The work has been done according to guidelines for scientific ethical practices of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, based in Shanghai.

Tick dissection. Ticks were dissected as previously described with some modifications (43). Briefly, ticks were surface sterilized by 75% ethanol twice, followed by 1× phosphate-buffered saline (PBS) twice. Forty-eight hours following blood feeding, individual ticks were placed dorsal side up on a drop of glue in the petri dish. Ticks were dissected in drops of 1× PBS. After removing upper cuticle, different tissues, including Malpighian tubules, midgut, ovaries/testes, and salivary glands, were collected and pooled for fixation.

Quantification of microbiota in ticks. Ticks were surface sterilized by 75% ethanol twice, followed by 1× PBS twice. Total DNA from whole ticks or dissected tissues was extracted by the method of Holmes and Bonner as described previously (44). Fifteen individual unfed whole adults of each sample group were used for microbial quantification. Tissues from five individual adults 48 h following blood feeding were pooled for one biological replicate. Five biological replicates were used for qPCR analysis. Total bacterial density was quantified by universal 16S rRNA primers (45). The species of *Coxiella* and *Rickettsia* were unable to be identified using the primers in this study, so we refer to the two symbionts as *Coxiella* sp. and *Rickettsia* sp. Bacterial densities of *Coxiella* sp., *Rickettsia* sp., and *Pseudomonas* sp. were quantified using genus-specific primers (Table 2) (46–48). The copy number of each species and total microbiota was determined by standard curve. The relative abundance of each symbiont was normalized with total bacterial density. Quantitative real-time PCR was performed by the Roche LightCycler 96 real-time PCR detection system with the SYBR green qPCR master mix (Biomake, China) using the following conditions: 95°C for 5 min and 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. Fluorescence readings were taken at 72°C after each cycle, followed by a melting curve (60°C to 95°C) to confirm the identity of the PCR product. Significance was determined using the Mann-Whitney test.

Fluorescent *in situ* hybridization. Samples, including whole tick bodies with their upper cuticles removed as well as dissected tissues, were fixed in 4% paraformaldehyde for 1 day. Sample preparation

and FISH were performed as described previously (49). Five whole bodies of unfed *H. longicornis* and tissues from at least five partially engorged ticks of all three species were used for fluorescent *in situ* hybridization. Briefly, after fixation, samples were washed by 1× PBS 4 or 5 times for 2 to 3 h. After washing, samples were dehydrated in an ethanol series (70%, 95%, and 100%), followed by 100% butanol for 10 min, twice, and then stored in 100% butanol at 4°C for 1 day. Tissues were embedded in paraffin and sectioned at a thickness of 5 μm. Slides were hybridized with 10 ng/μl universal 16S rRNA probe (5′-GCTGCCTCCCGTAGGAGT-3′) labeled with Alexa Fluor 555 (Life Technology) (50). The probe for *Coxiella* sp. was *Coxiella*-specific 23S rRNA (5′-GACTTCCCACATCGTTT-3′) labeled with Alexa Fluor 488 (Life Technology) (10). The *Rickettsia* sp.-specific 16S rRNA probe, 5′-TCCACGTCACCGTCTTGC-3′, was labeled with Alexa Fluor 647 (Life Technology) (46). Costaining of *Coxiella* sp. and *Rickettsia* sp. was performed by hybridization with *Coxiella*-specific 23S rRNA and *Rickettsia*-specific 16S rRNA probes simultaneously. Noneubacterial probe (5′-CCGTCAATTCMTTTGAGTTT-3′) labeled with Alexa Fluor 555 (Life Technology) and no probe were used as negative controls (50). Whole tick bodies of *H. longicornis* hybridized with 10 ng/μl universal 16S rRNA probe were visualized using a Nikon Eclipse IVi microscope connected to a Nikon Digital Sight DS-U3 digital camera. Different tissues of all three species were visualized using a Zeiss LSM710 confocal microscope connected to a Nikon Digital Sight DS-U3 digital camera. One focal plane was taken of each image.

Whole-mount *in situ* hybridization was performed on dissected Malpighian tubules from female *R. haemaphysaloides* and midgut from male *D. silvarum*. Tissues were fixed in 4% paraformaldehyde for 1 day at 4°C. Sample preparation and hybridization were performed as described above with some modifications. Briefly, after fixation, samples were washed by 1× PBS 4 times for 20 min. Tissues were dehydrated in an ethanol series (70%, 95%, and 100%). Malpighian tubules were subjected to hybridization with 10 ng/μl *Coxiella* sp.- and *Rickettsia* sp.-specific probes simultaneously at 45°C for 6 h. Midgut was hybridized with 10 ng/μl *Pseudomonas* sp.-specific 16S rRNA probe, 5′-AATCCGACCTAGGCTCATC-3′, labeled with Alexa Fluor 555 (Life Technology) (51). Finally, tissues were mounted with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen). Tissues were visualized using a Zeiss LSM710 confocal microscope connected to a Nikon Digital Sight DS-U3 digital camera. One focal plane was taken for each image.

Composition of microbiota in *D. silvarum*. Field-collected *D. silvarum* ticks were surface sterilized with 70% ethanol twice and 1× PBS twice. Total DNA of 5 unfed individuals of each sex was extracted by the method of Holmes and Bonner as described previously (44). The composition of the microbiota of *D. silvarum* was analyzed using an Illumina HiSeq2500 platform in Novogene (Novogene, China) by primers targeting the V3-V4 region of bacterial 16S rRNA. No-template controls were included as controls. A total of 2,453,452 reads were detected. Raw sequence data were filtered according to the QIIME (V1.7.0,) quality-controlled process (52, 53). The clean tags were compared with the reference database (Gold database), and chimeric sequences were detected using the UCHIME algorithm and removed (54, 55). Operational taxonomic unit (OTU) analyses were performed by Uparse software (Uparse v7.0.1001) (56). Sequences with ≥97% similarity were assigned to the same OTUs. A total of 1,691 OTUs were obtained. For each representative sequence, the GreenGene Database 3 was used based on the RDP classifier (v2.2) algorithm to annotate taxonomic information (57, 58). Alpha diversity was applied in analyzing the complexity of species diversity for one sample through Observed-species, Chao1, Shannon, and Simpson indices. All these indices in our samples were calculated with QIIME (version 1.7.0) and displayed with R software (version 2.15.3). The Mann-Whitney test was used to assess significant differences.

Availability of data. The raw 16S rRNA gene sequences have been uploaded to the National Center for Biotechnology Information's Sequence Read Archive (accession numbers SAMN07607842 to SAMN07607860).

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We declare that we have no conflict of interest.

REFERENCES

1. WHO. 2017. Global vector control response 2017–2030. World Health Organization, Geneva, Switzerland.
2. Fang LQ, Liu K, Li XL, Liang S, Yang Y, Yao HW, Sun RX, Sun Y, Chen WJ, Zuo SQ, Ma MJ, Li H, Jiang JF, Liu W, Yang XF, Gray GC, Krause PJ, Cao WC. 2015. Emerging tick-borne infections in mainland China: an increasing public health threat. Lancet Infect Dis 15:1467–1479. https://doi.org/10.1016/S1473-3099(15)00177-2.
3. Wu XB, Na RH, Wei SS, Zhu JS, Peng HJ. 2013. Distribution of tick-borne diseases in China. Parasit Vectors 6:119. https://doi.org/10.1186/1756-3305-6-119.
4. Zhou X, Xia S, Huang JL, Tambo E, Zhuge HX, Zhou XN. 2014. Human babesiosis, an emerging tick-borne disease in the People's Republic of China. Parasit Vectors 7:509. https://doi.org/10.1186/s13071-014-0509-3.
5. Chen Z, Yang X, Bu F, Yang X, Yang X, Liu J. 2010. Ticks (Acari: Ixodoidea: Argasidae, Ixodidae) of China. Exp Appl Acarol 51:393–404. https://doi.org/10.1007/s10493-010-9335-2.

6. Yu Z, Wang H, Wang T, Sun W, Yang X, Liu J. 2015. Tick-borne pathogens and the vector potential of ticks in China. Parasit Vectors 8:24. https://doi.org/10.1186/s13071-014-0628-x.
7. Narasimhan S, Fikrig E. 2015. Tick microbiome: the force within. Trends Parasitol 31:315–323. https://doi.org/10.1016/j.pt.2015.03.010.
8. Bonnet SI, Binetruy F, Hernandez-Jarguin AM, Duron O. 2017. The tick microbiome: why non-pathogenic microorganisms matter in tick biology and pathogen transmission. Front Cell Infect Microbiol 7:236. https://doi.org/10.3389/fcimb.2017.00236.
9. Machado-Ferreira E, Vizzoni VF, Balsemao-Pires E, Moerbeck L, Gazeta GS, Piesman J, Voloch CM, Soares CA. 2016. *Coxiella* symbionts are widespread into hard ticks. Parasitol Res 115:4691–4699. https://doi.org/10.1007/s00436-016-5230-z.
10. Lalzar I, Friedmann Y, Gottlieb Y. 2014. Tissue tropism and vertical transmission of *Coxiella* in *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* ticks. Environ Microbiol 16:3657–3668. https://doi.org/10.1111/1462-2920.12455.
11. Klyachko O, Stein BD, Grindle N, Clay K, Fuqua C. 2007. Localization and visualization of a *Coxiella*-type symbiont within the lone star tick, *Amblyomma americanum*. Appl Environ Microbiol 73:6584–6594. https://doi.org/10.1128/AEM.00537-07.
12. Machado-Ferreira E, Dietrich G, Hojgaard A, Levin M, Piesman J, Zeidner NS, Soares CA. 2011. *Coxiella* symbionts in the Cayenne tick *Amblyomma cajennense*. Microb Ecol 62:134–142. https://doi.org/10.1007/s00248-011-9868-x.
13. Liu L, Li L, Liu J, Hu Y, Liu Z, Guo L, Liu J. 2013. Coinfection of *Dermacentor silvarum* Olenev (Acari: Ixodidae) by *Coxiella*-like, Arsenophonus-like, and Rickettsia-like symbionts. Appl Environ Microbiol 79:2450–2454. https://doi.org/10.1128/AEM.03575-12.
14. Liu LM, Liu JN, Liu Z, Yu ZJ, Xu SQ, Yang XH, Li T, Li SS, Guo LD, Liu JZ. 2013. Microbial communities and symbionts in the hard tick *Haemaphysalis longicornis* (Acari: Ixodidae) from north China. Parasit Vectors 6:310. https://doi.org/10.1186/1756-3305-6-310.
15. Smith TA, Driscoll T, Gillespie JJ, Raghavan R. 2015. A *Coxiella*-like endosymbiont is a potential vitamin source for the Lone Star tick. Genome Biol Evol 7:831–838. https://doi.org/10.1093/gbe/evv016.
16. Zhong J, Jasinskas A, Barbour AG. 2007. Antibiotic treatment of the tick vector *Amblyomma americanum* reduced reproductive fitness. PLoS One 2:e405. https://doi.org/10.1371/journal.pone.0000405.
17. Zhang CM, Li NX, Zhang TT, Qiu ZX, Li Y, Li LW, Liu JZ. 2017. Endosymbiont CLS-HI plays a role in reproduction and development of *Haemaphysalis longicornis*. Exp Appl Acarol 73:429–438. https://doi.org/10.1007/s10493-017-0194-y.
18. Guizzo MG, Parizi LF, Nunes RD, Schama R, Albano RM, Tirloni L, Oldiges DP, Vieira RP, Oliveira WHC, Leite MS, Gonzales SA, Farber M, Martins O, Vaz IDS, Jr, Oliveira PL. 2017. A *Coxiella* mutualist symbiont is essential to the development of *Rhipicephalus microplus*. Sci Rep 7:17554. https://doi.org/10.1038/s41598-017-17309-x.
19. Van Treuren W, Ponnusamy L, Brinkerhoff RJ, Gonzalez A, Parobek CM, Juliano JJ, Andreadis TG, Falco RC, Ziegler LB, Hathaway N, Keeler C, Emch M, Bailey JA, Roe RM, Apperson CS, Knight R, Meshnick SR. 2015. Variation in the microbiota of *Ixodes* ticks with regard to geography, species, and sex. Appl Environ Microbiol 81:6200–6209. https://doi.org/10.1128/AEM.01562-15.
20. Hunter DJ, Torkelson JL, Bodnar J, Mortazavi B, Laurent T, Deason J, Thephavongsa K, Zhong J. 2015. The *Rickettsia* endosymbiont of *Ixodes pacificus* contains all the genes of de novo folate biosynthesis. PLoS One 10:e0144552. https://doi.org/10.1371/journal.pone.0144552.
21. de la Fuente J, Antunes S, Bonnet S, Cabezas-Cruz A, Domingos AG, Estrada-Pena A, Johnson N, Kocan KM, Mansfield KL, Nijhof AM, Papa A, Rudenko N, Villar M, Alberdi P, Torina A, Ayllon N, Vancova M, Golovchenko M, Grubhoffer L, Caracappa S, Fooks AR, Gortazar C, Rego ROM. 2017. Tick-pathogen interactions and vector competence: identification of molecular drivers for tick-borne diseases. Front Cell Infect Microbiol 7:114. https://doi.org/10.3389/fcimb.2017.00114.
22. Gall CA, Reif KE, Scoles GA, Mason KL, Mousel M, Noh SM, Brayton KA. 2016. The bacterial microbiome of *Dermacentor andersoni* ticks influences pathogen susceptibility. ISME J 10:1846–1855. https://doi.org/10.1038/ismej.2015.266.
23. Narasimhan S, Rajeevan N, Liu L, Zhao YO, Heisig J, Pan J, Eppler-Epstein R, Deponte K, Fish D, Fikrig E. 2014. Gut microbiota of the tick vector *Ixodes scapularis* modulate colonization of the Lyme disease spirochete. Cell Host Microbe 15:58–71. https://doi.org/10.1016/j.chom.2013.12.001.
24. Steiner FE, Pinger RR, Vann CN, Grindle N, Civitello D, Clay K, Fuqua C. 2008. Infection and co-infection rates of *Anaplasma phagocytophilum variants*, *Babesia* spp., Borrelia burgdorferi, and the rickettsial endosymbiont in *Ixodes scapularis* (Acari: Ixodidae) from sites in Indiana, Maine, Pennsylvania, and Wisconsin. J Med Entomol 45:289–297.
25. Xu XL, Cheng TY, Yang H, Yan F. 2015. Identification of intestinal bacterial flora in *Rhipicephalus microplus* ticks by conventional methods and PCR-DGGE analysis. Exp Appl Acarol 66:257–268. https://doi.org/10.1007/s10493-015-9896-1.
26. Zhang XC, Yang ZN, Lu B, Ma XF, Zhang CX, Xu HJ. 2014. The composition and transmission of microbiome in hard tick, Ixodes persulcatus, during blood meal. Ticks Tick-Borne Dis 5:864–870. https://doi.org/10.1016/j.ttbdis.2014.07.009.
27. Liu L, Li L, Liu J, Yu Z, Yang X, Liu J. 2016. Population dynamics of multiple symbionts in the hard tick, Dermacentor silvarum Olenev (Acari: Ixodidae). Ticks Tick-Borne Dis 7:188–192. https://doi.org/10.1016/j.ttbdis.2015.10.002.
28. Wang R, Li N, Liu J, Li T, Liu M, Yu Z, Liu J. 2017. Symbiont dynamics of the Tibetan tick *Haemaphysalis tibetensis* (Acari: Ixodidae). Parasit Vectors 10:259. https://doi.org/10.1186/s13071-017-2199-0.
29. Cowdry EV. 1925. A group of microorganisms transmitted hereditarily in ticks and apparently unassociated with disease. J Exp Med 41:817–830. https://doi.org/10.1084/jem.41.6.817.
30. Hsu MH, Sauer JR. 1975. Ion and water-balance in feeding Lone Star tick. Comp Biochem Phys A 52:269–276. https://doi.org/10.1016/S0300-9629(75)80085-5.
31. Dow JA, Davies SA. 2006. The Malpighian tubule: rapid insights from post-genomic biology. J Insect Physiol 52:365–378. https://doi.org/10.1016/j.jinsphys.2005.10.007.
32. Gautam NK, Verma P, Tapadia MG. 2017. *Drosophila* Malpighian tubules: a model for understanding kidney development, function, and disease. Results Probl Cell Differ 60:3–25. https://doi.org/10.1007/978-3-319-51436-9_1.
33. Faria VG, Sucena E. 2013. *Wolbachia* in the Malpighian tubules: evolutionary dead-end or adaptation? J Exp Zool B Mol Dev Evol 320:195–199. https://doi.org/10.1002/jez.b.22498.
34. Bright M, Bulgheresi S. 2010. A complex journey: transmission of microbial symbionts. Nat Rev Microbiol 8:218–230. https://doi.org/10.1038/nrmicro2262.
35. el Said A. 1992. Ultrastructure of symbiont-like microorganisms associated with the sperm of ixodid ticks. J Egypt Soc Parasitol 22:293–297.
36. Afzelius BA, Alberti G, Dallai R, Godula J, Witalinski W. 1989. Virus-infected and *Rickettsia*-infected sperm cells in arthropods. J Invertebrate Pathol 53:365–377. https://doi.org/10.1016/0022-2011(89)90102-X.
37. Watanabe K, Yukuhiro F, Matsuura Y, Fukatsu T, Noda H. 2014. Intrasperm vertical symbiont transmission. Proc Natl Acad Sci U S A 111:7433–7437. https://doi.org/10.1073/pnas.1402476111.
38. Moran NA, Dunbar HE. 2006. Sexual acquisition of beneficial symbionts in aphids. Proc Natl Acad Sci U S A 103:12803–12806. https://doi.org/10.1073/pnas.0605772103.
39. Greay TL, Gofton AW, Paparini A, Ryan UM, Oskam CL, Irwin PJ. 2018. Recent insights into the tick microbiome gained through next-generation sequencing. Parasit Vectors 11:12. https://doi.org/10.1186/s13071-017-2550-5.
40. Duron O, Binetruy F, Noel V, Cremaschi J, McCoy KD, Arnathau C, Plantard O, Goolsby J, Perez de Leon AA, Heylen DJA, Van Oosten AR, Gottlieb Y, Baneth G, Guglielmone AA, Estrada-Pena A, Opara MN, Zenner L, Vavre F, Chevillon C. 2017. Evolutionary changes in symbiont community structure in ticks. Mol Ecol 26:2905–2921. https://doi.org/10.1111/mec.14094.
41. Li LH, Zhu D, Zhang CC, Zhang Y, Zhou XN. 2016. Experimental transmission of *Babesia microti* by *Rhipicephalus haemaphysaloides*. Parasit Vectors 9:231. https://doi.org/10.1186/s13071-016-1517-2.
42. Chitimia L, Lin RQ, Cosoroaba I, Wu XY, Song HQ, Yuan ZG, Zhu XQ. 2010. Genetic characterization of ticks from southwestern Romania by sequences of mitochondrial *cox1* and *nad5* genes. Exp Appl Acarol 52:305–311. https://doi.org/10.1007/s10493-010-9365-9.
43. Edwards KGJ, Varela-Stokes A. 2009. Examination of the internal morphology of the Ixodid tick, Amblyomma maculatum Koch (Acari: Ixodidae); a "how-to" pictorial dissection guide. Midsouth Entomol 2:28–39.
44. Holmes DS, Bonner J. 1973. Preparation, molecular weight, base composition, and secondary structure of giant nuclear ribonucleic acid. Biochemistry 12:2330–2338. https://doi.org/10.1021/bi00736a023.
45. Armougom F, Raoult D. 2009. Exploring microbial diversity using 16S

rRNA high-throughput methods. J Comput Sci Syst Biol 2:74–92. https://doi.org/10.4172/jcsb.1000019.
46. Sakurai M, Koga R, Tsuchida T, Meng XY, Fukatsu T. 2005. *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential symbiont *Buchnera*. Appl Environ Microbiol 71:4069–4075. https://doi.org/10.1128/AEM.71.7.4069-4075.2005.
47. Lalzar I, Harrus S, Mumcuoglu KY, Gottlieb Y. 2012. Composition and seasonal variation of *Rhipicephalus turanicus* and *Rhipicephalus sanguineus* bacterial communities. Appl Environ Microbiol 78:4110–4116. https://doi.org/10.1128/AEM.00323-12.
48. Roosa S, Wauven CV, Billon G, Matthijs S, Wattiez R, Gillan DC. 2014. The *Pseudomonas* community in metal-contaminated sediments as revealed by quantitative PCR: a link with metal bioavailability. Res Microbiol 165:647–656. https://doi.org/10.1016/j.resmic.2014.07.011.
49. Attardo GM, Lohs C, Heddi A, Alam UH, Yildirim S, Aksoy S. 2008. Analysis of milk gland structure and function in *Glossina morsitans*: milk protein production, symbiont populations and fecundity. J Insect Physiol 54:1236–1242. https://doi.org/10.1016/j.jinsphys.2008.06.008.
50. Amann RI, Krumholz L, Stahl DA. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770. https://doi.org/10.1128/jb.172.2.762-770.1990.
51. Watt M, Hugenholtz P, White R, Vinall K. 2006. Numbers and locations of native bacteria on field-grown wheat roots quantified by fluorescence in situ hybridization (FISH). Environ Microbiol 8:871–884. https://doi.org/10.1111/j.1462-2920.2005.00973.x.
52. Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods 10:57–59. https://doi.org/10.1038/nmeth.2276.
53. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6:1621–1624. https://doi.org/10.1038/ismej.2012.8.
54. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200. https://doi.org/10.1093/bioinformatics/btr381.
55. Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, Ciulla D, Tabbaa D, Highlander SK, Sodergren E, Methe B, DeSantis TZ, Human Microbiome C, Petrosino JF, Knight R, Birren BW. 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 21:494–504. https://doi.org/10.1101/gr.112730.110.
56. Edgar RC. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods 10:996–998. https://doi.org/10.1038/nmeth.2604.
57. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72:5069–5072. https://doi.org/10.1128/AEM.03006-05.
58. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267. https://doi.org/10.1128/AEM.00062-07.