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Coinfection of *Dermacentor silvarum* Olenev (Acari: Ixodidae) by *Coxiella*-Like, *Arsenophonus*-Like, and *Rickettsia*-Like Symbionts

Limeng Liu,[a] Lingxia Li,[a] Jiannan Liu,[a] Yonghong Hu,[a] Zhao Liu,[a] Lida Guo,[b] Jingze Liu[a]

Key Laboratory of Animal Physiology, Biochemistry and Molecular Biology of Hebei Province, College of Life Sciences, Hebei Normal University, Shijiazhuang, Hebei, People's Republic of China[a]; Department of Environment and Chemical Engineering, Hebei College of Industry and Technology, Hebei, People's Republic of China[b]

We report that multiple symbionts coexist in *Dermacentor silvarum*. Based on 16S rRNA gene sequence analyses, we prove that *Coxiella*-like and *Arsenophonus*-like symbionts, with 95.6% and 96.7% sequence similarity to symbionts in the closest taxon, respectively, are novel. Moreover, we also provide evidence that the *Coxiella*-like symbiont appears to be the primary symbiont.

Symbionts are ubiquitous in a diverse group of insect hosts. They confer crucial and diverse benefits to their hosts, affecting development (1), nutrition (2), reproduction and speciation (3), defense against natural enemies and environment stress (4, 5), and immunity (6). Symbionts usually are classified as obligate (or primary) symbionts and facultative (or secondary) symbionts according to the extent of the mutual dependence between the host and the symbiont (7). Primary symbionts, restricted to specialized tissues or cells, usually are obligate and essential for the survival of the host and their own vertical transmission. Secondary symbionts, not restricted to specific tissues, usually are facultative and unessential for the host and transmitted either vertically or horizontally between the same or different species (8, 9, 10). Many insect species are usually simultaneously infected by multiple symbionts, including one primary symbiont and various secondary symbionts (11), or two obligate mutual symbionts (12). In general, various symbionts coexist in the same host, interact with each other, and coregulate the biological processes of the host.

Like most insects, ticks exhibit close relationships with symbionts. To date, a wide range of symbionts, such as *Coxiella*-like (13), *Francisella*-like (14), *Wolbachia*-like (15), *Rickettsia*-like (16), *Arsenophonus*-like (17), "*Candidatus* Midichloria mitochondrii" (18), and *Rickettsia peacockii* (19) symbionts, have been detected in several tick species. However, little attention has been given to coinfection with multiple symbionts of ticks. Hence, we focused the present study on coinfection of the multiple symbionts in tick hosts. The new knowledge gained from this study could be meaningful for a deep understanding of the biology and ecology of ticks.

We first report that three symbionts, namely, *Coxiella*-like, *Arsenophonus*-like, and *Rickettsia*-like symbionts, coexist in *Dermacentor silvarum*. Interestingly, *Coxiella*-like and *Arsenophonus*-like symbionts are different from those in the taxon described previously, with 95.6% and 96.7% similarity to the closest taxon, respectively. Moreover, the *Coxiella*-like symbiont appears to be the primary symbiont of *D. silvarum*.

D. silvarum samples were collected in Xiaowutai National Natural Reserve Area in China by flag dragging. Several collected ticks were stored under −80°C conditions. Others were reared on rabbits as described by Liu et al. (20). Before DNA extraction, all tick samples were sterilized as described by Clay et al. (21) and dissected tissue samples were washed three times in sterile phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4). All DNA samples, including the genomic DNA from a group of adults (10 females and 10 males, respectively), from every individual field-collected adult, and from a group of ticks at different developmental stages (500 eggs, 200 larvae, and 50 nymphs) and from different tissues (ovaries, salivary glands, Malpighian tubes, and midguts), were extracted using a DNeasy tissue kit (Qiagen, Germany) according to the protocol of the manufacturer. The eubacterial 16S rRNA

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Address correspondence to Jingze Liu, jzliu21@heinfo.net.

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TABLE 1 Oligonucleotide primers used for PCR amplification and sequencing

Primer name	Species	Target gene	Nucleotide sequence (5′–3′)	Annealing temp (°C)	Approx product size (bp)	Source or reference
CLS-Ds110F	CLS-Ds	16S rRNA	CACGTAGGAATCTACCTTGTAG	55	90	This study
CLS-Ds170R			CGTTTTGTTCCGAAGAAATTAT			
ALS-Ds82F	ALS-Ds	16S rRNA	AGGGAGCTTGCTTCCTGGCCGG	59	130	This study
ALS-Ds198R			CGAAGGTGTGAGGCCTAATGG			
*Rickettsia*354F	*Rickettsia*	16S rRNA	CAGCAATACCGAGTGAGTGATGAAG	56	350	23
*Rickettsia*647R			AGCGTCAGTTGTAGCCCAGATG			
*Rp*CS.877p	*Rickettsia*	*gltA*	GGGGACCTGCTCACGGCGG	46	380	24
*Rp*CS.1258n			CATAACCAGTGTAAAGCTG			
*Rr*190.70p	*Rickettsia*	*rompA*	GGTGGTCAGGCTCTGAAGCTAAC	48	530	25
*Rr*190.602n			TGCAGTTTGATAACCGACAGTCTC			

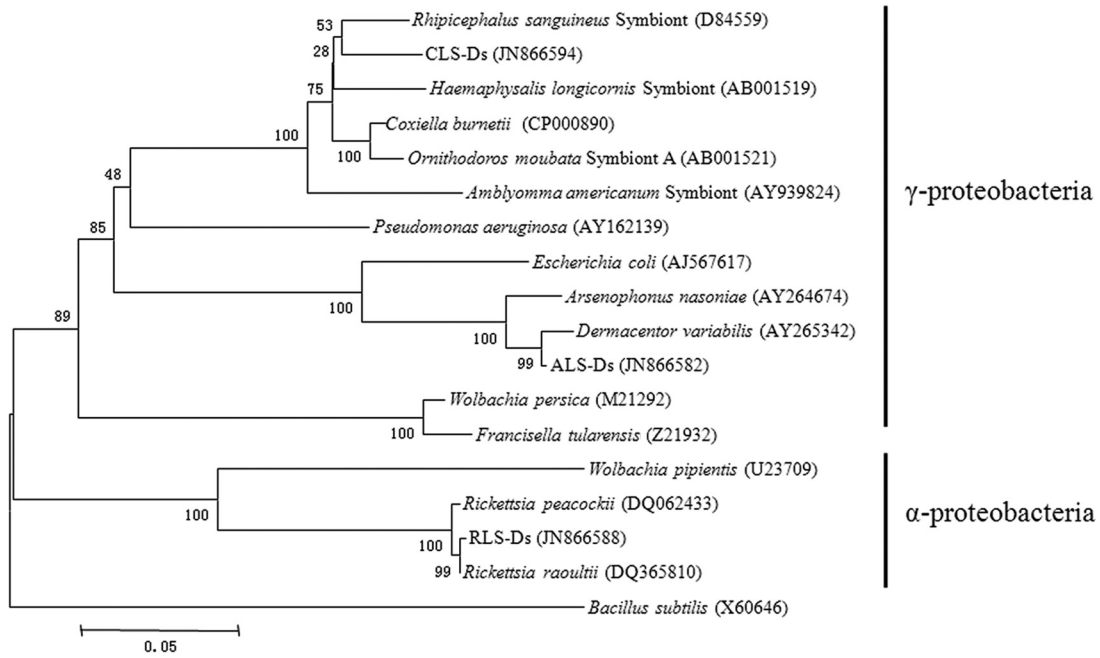

FIG 1 Phylogenetic tree of three symbionts (CLS-Ds, ALS-Ds, and RLS-Ds) of *D. silvarum* and related tick-associated symbionts based on 16S rRNA gene sequence similarity. The tree was rooted with *Bacillus subtilis* (X60646) and constructed using the neighbor-joining method, and clustering nodes were also recovered using the maximum-likelihood method. Numbers at nodes represent the levels of bootstrap support (percent) based on neighbor-joining analysis of 1,000 replicated data sets. GenBank accession numbers are given in parentheses. The bar represents 5% sequence divergence. α-proteobacteria, alphaproteobacteria; γ-proteobacteria, gammaproteobacteria.

gene library was constructed by amplifying an approximately 1,500-bp fragment of the 16S rRNA gene using eubacterial universal primers 27F and 1492R (22). The eubacterial 16S rRNA gene libraries were analyzed by restriction fragment length polymorphism (RFLP) using both HaeIII and RsaI restriction endonucleases.

To assess the prevalences, vertical transmission characteristics, and infection sites of three putative symbionts, diagnostic PCR assays were performed with three sets of primers specific for each of them (Table 1). The PCR mixtures contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates (dNTP), 2.5 U Platinum *Taq* DNA polymerase (Invitrogen), and 0.5 mM (each) primer. The PCR cycling conditions were as follows: 1 cycle of 94°C for 2 min; 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 15 s; and, finally, 72°C for 10 min. Products were cloned into the plasmid pCR2.1-TOPO (Invitrogen) and sequenced by the Sangon Biotech Company (China).

The results of RFLP analyses revealed diverse microbial associations (Fig. 1). We found that the sequences of Dc-9 and Dc-54 from the female gene library and of Dx-6 and Dx-11 from the male gene library were closely related to those of *Arsenophonus*-like symbionts (ALSs) of *D. variabilis* (GenBank accession no. AY265342) (26), with 98% to 98.15% similarity, and those symbionts were designated ALS-Ds; the sequences of Dc-8 and Dc-71 from the female gene library and of Dx-56 and Dx-68 from the male gene library shared 94.1% to 94.3% similarity with those of the *Coxiella*-like symbiont of *Haemaphysalis longicornis* (GenBank accession no. AB001519) (27), and those symbionts were designated CLS-Ds; and the sequences of Dc-3 and Dc-24 from the female gene library and Dx-7 and Dx-21 from the male gene library of symbionts designated RLS-Ds shared the highest sequence similarity with sequences of the *Rickettsia* symbiont (99.8%), which had been detected in many *Dermacentor* species (23, 28). Further studies showed that the sequences obtained had the highest (99.1% and 99.8%, respectively) similarity with those of the *gltA* gene (GenBank accession no. DQ365804) and *rompA* gene (GenBank accession no. DQ365801) of *R. raoultii*.

All three putative symbionts were detected from ticks at differ-

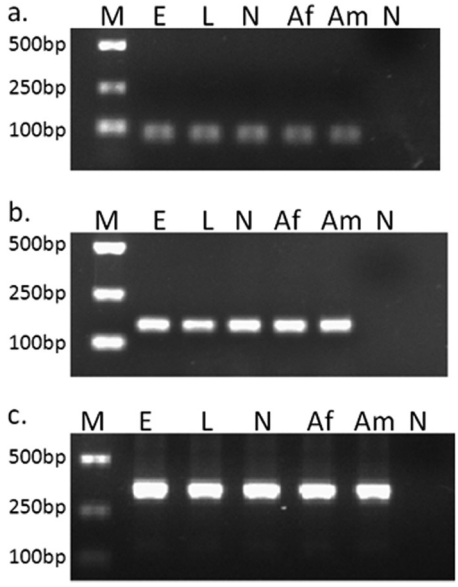

FIG 2 Detection of vertical transmission of CLS-Ds (a), ALS-Ds (b), and RLS-Ds (c) by diagnostic PCR amplification from *D. silvarum* at different developmental stages. Lanes 1 to 7: M, DNA ladder; E, eggs; L, larvae; N, nymphs; AF, adult females; AM, adult males; N, negative control.

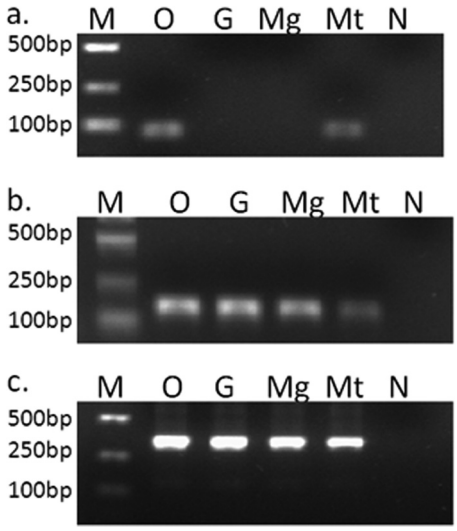

FIG 3 Detection of infection sites of CLS-Ds (a), ALS-Ds (b), and RLS-Ds (c) by diagnostic PCR amplification from different tissues of *D. silvarum*. Lanes 1 to 6: M, DNA ladder; O, ovaries; G, salivary glands; Mg, midguts; Mt, Malpighian tubules; N, negative control.

ent developmental stages, indicating that they can be transmitted vertically (Fig. 2). The infection site analyses showed that CLS-Ds infected ovaries and Malpighian tubes, while the others were found in all tissues tested (Fig. 3). The prevalence analyses revealed that CLS-Ds showed 100% infection in adults; for ALS-Ds, 42% (36/86) infection in females and 22% (10/46) infection in males; and for RLS-Ds, 86% (74/86) infection in females and 91% (42/46) infection in males. All field-collected *D. silvarum* samples harbored one symbiont, and at least 40% of the females and 22% of the males were infected by all three symbionts.

The results demonstrated that *D. silvarum* harbored diverse assemblages of putative symbionts, including *Coxiella*-like symbionts (CLS-Ds), *Arsenophonus*-like symbionts (ALS-Ds), and *Rickettsia*-like symbionts (RLS-Ds). This is the first report proving that various vertically transmitted symbionts coinhabit *D. silvarum*. To date, only a few reports have concerned the coinfection of multiple symbionts in ticks. For example, Noda et al. (27) and Reinhardt et al. (29) reported that *Ornithodoros moubata* hosted two kinds of symbionts, namely, *Rickettsia*-like and *Francisella*-like symbionts. Besides, it has been previously reported that *Amblyomma americanum* simultaneously harbored *Coxiella*-like symbionts, which are primary and are closely related to host reproduction (30, 31), and *Rickettsia*-like and *Arsenophonus*-like symbionts (21). In this study, we found an important coinfection phenomenon in *D. silvarum*, which provides a new model and clue for elucidating the issues about the interaction and interrelationship between symbionts and their hosts.

Interestingly, two of three putative categories of symbionts, CLS-Ds and ALS-Ds, in *D. silvarum* are novel. They have highest (95.6% and 96.7%) similarity with the phylogenetically most closely related species of the genera *Coxiella* and *Arsenophonus*, respectively. Phylogenetic analyses (Fig. 4 and 5) also revealed that CLS-Ds and ALS-Ds formed clear and unique clusters in their respective phylogenetic trees, and they were distinguished from those of the other species and tick-associated microorganisms in this genus.

To date, various *Coxiella*-like microorganisms have been detected in both hard and soft ticks (21, 32, 33, 34, 35, 36, 37, 38). Interestingly, *Coxiella*-like microorganisms exhibited diverse 16S rRNA genotypes from different tick species. Phylogenetic analyses (Fig. 4) revealed that *Coxiella*-like microorganisms from different tick species formed different independent branches. Moreover,

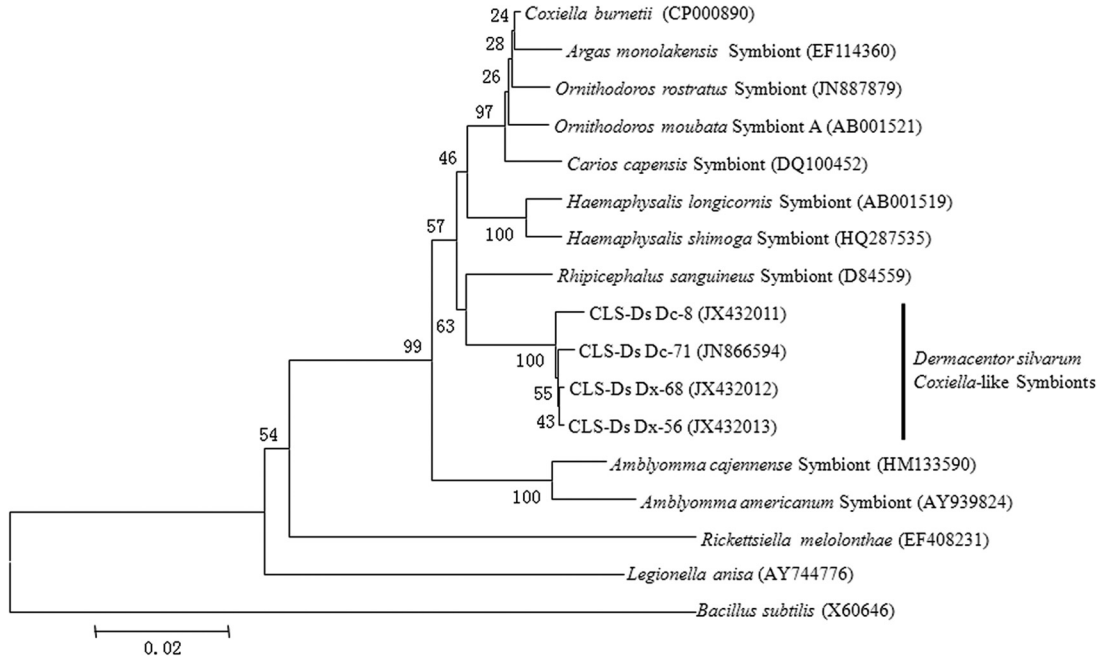

FIG 4 Phylogenetic tree of *Coxiella*-like symbionts (CLS-Ds) of *D. silvarum* and *Coxiella*-like microorganisms from other tick species based on 16S rRNA gene sequence similarity. The tree was rooted with *Bacillus subtilis* (X60646) and constructed using the neighbor-joining method, and clustering nodes were also recovered using the maximum-likelihood method. Numbers at nodes represent the levels of bootstrap support (percent) based on neighbor-joining analysis of 1,000 replicated data sets. GenBank accession numbers are given in parentheses. The bar represents 2% sequence divergence.

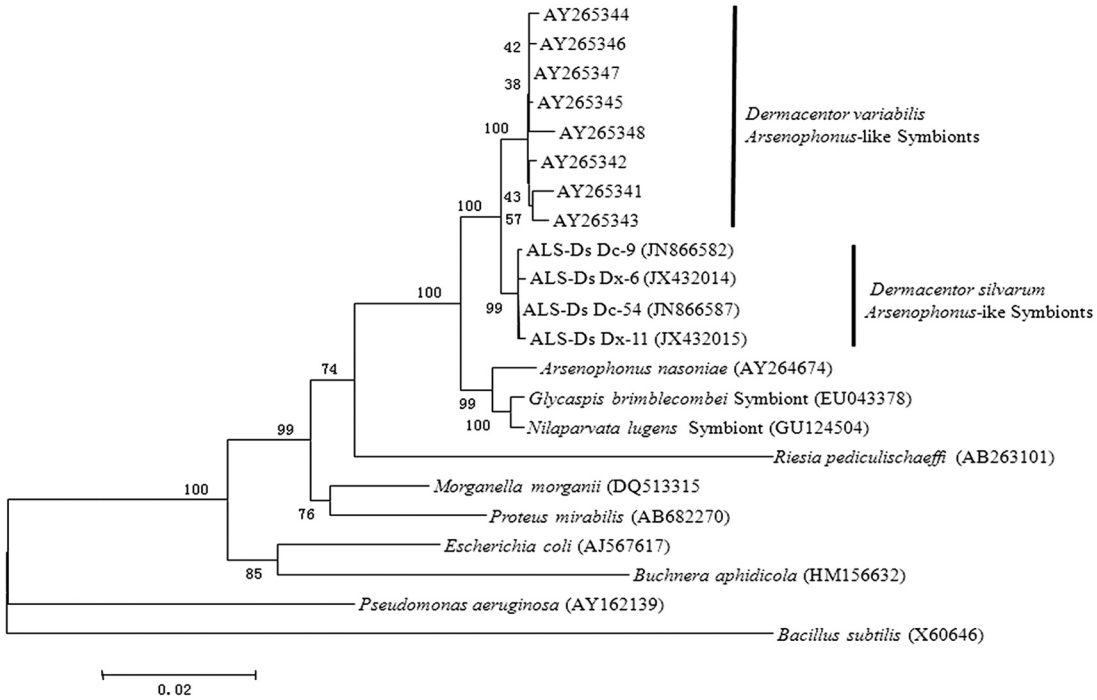

FIG 5 Phylogenetic tree of *Arsenophonus*-like symbionts (ALS-Ds) of *D. silvarum* and *Arsenophonus*-like symbionts of *D. variabilis* based on 16S rRNA gene sequence similarity. The tree was rooted with *Bacillus subtilis* (X60646) and constructed using the neighbor-joining method, and clustering nodes were also recovered using the maximum-likelihood method. Numbers at nodes represent the levels of bootstrap support (percent) based on neighbor-joining analysis of 1,000 replicated data sets. GenBank accession numbers are given in parentheses. The bar represents 2% sequence divergence.

the *Coxiella*-like microorganisms in soft and hard ticks obviously were grouped separately. Previous studies have suggested that the *Coxiella*-like symbiont in *A. americanum* is a primary symbiont because of its ubiquitous distribution (21, 30), vertical transmission (13, 21), infection of specific tissues (13), loss of fitness with antibiotic treatment (31), and reduced genome (30). In this study, we found that CLS-Ds exhibited vertical infection and infected specific tissues. Thus, we hypothesize that CLS-Ds might be a primary symbiont and essential for the survival of its tick host. It may be involved in regulation of host reproduction, because it inhabits the ovary.

Besides CLS-Ds, another novel symbiont, ALS-Ds, which belongs to the genus *Arsenophonus*, was also detected. ALSs have been found in *D. variabilis* (26), *D. andersoni* (17) and *A. americanum* (21). *Arsenophonus* is one of the four major inherited symbionts of arthropods; about 5% of the species of arthropods have been found to be infected by *Arsenophonus* (39). The type species, *A. nasoniae*, can give rise to sex ratio bias of the wasp *Nasonia vitripennis* (40). However, there is no evidence that the *Arsenophonus*-like symbionts in ticks can lead to sex ratio bias. In this study, we found that ALS-Ds exhibited wide tissue distribution and imperfect infection. Thus, it appears to be a facultative and unessential symbiont for *D. silvarum*.

The third vertical transmitted microorganism screened here was *R. raoultii*, which has been detected in many *Dermacentor* species (41, 42, 43, 44). The present study reported for the first time the vertical transmission of *R. raoultii* in *D. silvarum*, suggesting a closer relationship between *R. raoultii* and its tick host.

Nucleotide sequence accession numbers. The 16S rRNA gene GenBank accession numbers for CLS-Ds are JN866594, JX432011, JX432012, and JX432013; for ALS-Ds are JN866582, JN866587, JX432014, and JX432015; and for RLS-Ds are JN866588, JX432016, JX432017, and JX432018.

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