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Evolution and Diversity of Facultative Symbionts from the Aphid Subfamily Lachninae^{∇†}

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Many aphids harbor a variety of endosymbiotic bacteria. The functions of these symbionts can range from an obligate nutritional role to a facultative role in protecting their hosts against environmental stresses. One such symbiont is “*Candidatus Serratia symbiotica*,” which is involved in defense against heat and potentially also in aphid nutrition. Lachnid aphids have been the focus of several recent studies investigating the transition of this symbiont from a facultative symbiont to an obligate symbiont. In a phylogenetic analysis of *Serratia* symbionts from 51 lachnid hosts, we found that diversity in symbiont morphology, distribution, and function is due to multiple independent origins of symbiosis from ancestors belonging to *Serratia* and possibly also to evolution within distinct symbiont clades. Our results do not support cocladogenesis of “*Ca. Serratia symbiotica*” with *Cinara* subgenus *Cinara* species and weigh against an obligate nutritional role. Finally, we show that species belonging to the subfamily Lachninae have a high incidence of facultative symbiont infection.

Many insect species harbor heritable endosymbiotic bacteria. Among the best studied of these species are aphids. Almost all aphids are infected with the obligate nutritional symbiont *Buchnera aphidicola*, which is generally required for the survival of aphids and provides essential amino acids that are rare in their phloem sap diet (32). Many aphids also possess additional symbionts that may be facultative from the host's perspective and that coexist with *Buchnera* (20).

Three lineages of facultative symbionts that are prevalent in aphids belong to the *Enterobacteriaceae*. Two of these lineages (“*Candidatus Hamiltonella defensa*” and “*Candidatus Regiella insecticola*”) form well-defined clades distinct from free-living bacterial species (4, 20) and confer clear advantages to their hosts by protecting them against natural enemies. “*Ca. Hamiltonella defensa*” prevents wasp parasitism by arresting development of wasp larvae in pea aphids, and “*Ca. Regiella insecticola*” provides resistance against the fungal pathogen *Pandora neoaphidis* (24, 31). The third lineage, “*Candidatus Serratia symbiotica*,” is closely related to free-living members of the genus *Serratia*. This symbiont is distributed sporadically among aphid species and has been proposed to have a variety of effects on hosts. In pea aphids (*Acyrtosiphon pisum*; Macrosiphini), “*Ca. Serratia symbiotica*” ameliorates the deleterious fitness effects of heat shock by protecting symbiont-harboring bacteriocyte cells (2, 19, 29). Additionally, a strain of “*Ca. Serratia symbiotica*” provided some resistance to parasitoid wasp attack (24). “*Ca. Serratia symbiotica*” has been proposed to play a role in nutrition by producing amino acids for its aphid host and by decreasing its host's reliance on *Buchnera*

(10, 15, 16, 26). In contrast to most *Buchnera* strains, *Buchnera* strains from *Cinara cedri* (Lachnini) have lost the genes for biosynthesis of the essential amino acid tryptophan, while “*Ca. Serratia symbiotica*” in the same host possesses at least part of the pathway, suggesting that it has a mutualistic role in the nutrition of aphids (26).

In *A. pisum*, “*Ca. Serratia symbiotica*” cells are rod-shaped bacteria that are present in the sheath cells, hemolymph, and bacteriocytes of some individuals. In contrast, in *C. cedri* “*Ca. Serratia symbiotica*” occurs in all individuals, and its cells are large, round, and pleomorphic, similar to the cells of many obligate bacterial aphid endosymbionts, including *Buchnera* (10, 26). Furthermore, “*Ca. Serratia symbiotica*” has consistently been present in other *Cinara* species sampled (28). Both the rod-shaped and pleomorphic forms are assigned to “*Ca. Serratia symbiotica*” based on phylogenetic analyses of several gene sequences, but they fall into two distinct sister clades of symbiont lineages that seem to coincide with bacterial morphology (17, 20).

This diversity in “*Ca. Serratia symbiotica*” morphology, distribution, and functions may represent evolution of different features within lineages of a single symbiont clade. If “*Ca. Serratia symbiotica*” is an obligate nutritional symbiont in *Cinara* hosts, it is expected that *Cinara*-associated symbionts would form a clade in which the intraclade relationships mirror those of the hosts (cocladogenesis), as observed for *Buchnera* and other obligate nutritional symbionts of insects (13, 21, 38). Indeed, Lamelas et al. postulated that, based on their similar phylogenies, *Serratia* symbionts from aphids belonging to the subgenus *Cinara* have had a long-term relationship with their hosts (17).

In addition to the three most common facultative symbiont types found in aphids described above, several other symbiont lineages with unknown functions have been identified by amplification of bacterial 16S rRNA gene sequences from various aphid species (10, 28, 39). Here we examine the diversity of

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Serratia and other facultative symbionts in aphids belonging to the subfamily Lachninae. We investigated the distribution of symbionts in aphid species and geographic locations and looked for coevolutionary patterns that may correspond to the functions of facultative symbionts within their hosts.

MATERIALS AND METHODS

Extraction, PCR analysis, cloning, and sequencing of DNA from aphid samples. A panel of aphids belonging to the aphid subfamily Lachninae (with a focus on species of the genus *Cinara*) were screened for bacterial endosymbionts (Table 1; see Table S1 in the supplemental material). In most cases, screening was based on a single individual per aphid species or population, unless indicated otherwise (Table 1).

DNA extraction was performed with single aphids using a DNeasy kit (Qiagen). PCR was used to amplify 16S rRNA using primers 10F (5'-AGTTGATCATGGCTCAGATTG-3'), 35R (5'-CCTTCATCGCCTCTGACTGC-3'), and 1507R (5'-TACCTTGTACGACTTCACCCAG-3'). Each reaction mixture (50 μ l) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl₂, 1 mM deoxynucleoside triphosphates (Eppendorf), 250 pmol of each primer, and 2 U *Taq* polymerase (Eppendorf). PCR amplification was carried out with a Mastercycler Gradient (Eppendorf) as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 3 min and then a final incubation at 72°C for 10 min. Amplified products were examined using standard 1% agarose gel electrophoresis and staining with ethidium bromide. Single, discrete bands were cleaned using Agencourt AMPure PCR purification (Agencourt, Beverly, MA).

In order to separate *Buchnera* and facultative symbiont 16S rRNA gene sequences, cloning was performed using a pGEM-T Easy kit (Promega), and pGEM-T Easy with its insert was transformed into *Escherichia coli* competent cells (Promega). The insert size for eight colonies was checked using colony PCR performed with primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAACAGCTATGAC-3'), followed by digestion with SalI (Roche) as described by Sandström et al. to differentiate between *Buchnera* and facultative symbiont clones (30). The amplicon was sequenced from both ends using the M13F and M13R primers at the Genomic Analysis and Technology Core, University of Arizona. Base calls were verified and contigs were assembled using the Sequencher 4.7 DNA software (Gene Codes Corporation).

Symbiont classification and phylogenetic analyses. Symbiont types were classified based on best BLAST hits and were verified by placement in a strongly supported clade on phylogenetic trees with other bacterial taxa that are members of the lineages to which they belong. Sequences were aligned using Clustal X and were checked by hand using MacClade 4.06 (18). The best maximum likelihood (ML) trees were obtained using a heuristic search with the GTR+I+ γ model and bootstrapped with 100 replicates using RAxML (34). A Bayesian analysis was completed using MrBayes 3.1.2 (27) with the settings nst = 6 and rates = invgamma and two independent runs with four chains for 1,250,000 generations, with sampling every 1,000 generations. The first 250 samples were discarded, and posterior probabilities were obtained using the remaining 1,000 samples.

A Shimodaira-Hasegawa (S-H) test was used in PAUP* (37) to compare the best ML tree to a tree in which subgenus *Cinara* aphids possessing “*Ca. Serratia symbiotica*” cluster B are monophyletic to the exclusion of aphid species possessing other symbiont lineages (except *Cinara pinimaritimae*, which belongs to a clade with “*Ca. Serratia symbiotica*” cluster B-containing aphid species with a high level of support). Using the parameters estimated for the best ML tree, the S-H test was performed with full optimization and 1,000 bootstrap replicates.

Branch lengths were estimated using the ML parameters employed for phylogenetic reconstruction of the ML tree.

Nucleotide sequence accession numbers. The GenBank accession numbers of the 16S rRNA gene sequences determined in this study are FJ655482 to FJ655545.

RESULTS

Phylogenetic reconstruction of symbiotic *Serratia* lineages in aphids. Partial 16S rRNA gene sequences were obtained for a range of *Serratia* symbionts and other bacterial facultative symbionts, as well as *Buchnera* (GenBank accession numbers FJ655482 to FJ655545 [see Table S1 in the supplemental material]). ML and Bayesian phylogenetic analyses of the 16S

rRNA gene of these taxa resulted in a gene tree showing the evolutionary relationships among symbionts and free-living species in the genus *Serratia* (Fig. 1).

Most aphid symbionts belonging to the genus *Serratia* fall into two sister clades corresponding to clusters A and B, as described by Lamelas et al. (17). These symbionts include almost all of the *Serratia* symbionts from the aphid family Aphididae, including the Lachninae.

Cluster A contains symbionts from members of both the Aphididae and the Lachninae, including *Cinara*. The majority of the symbionts belonging to cluster A show little diversity; the greatest pairwise distance is 0.064 substitution per site. Cluster B contains symbionts solely from the Lachninae. These symbionts show greater diversity than those in cluster A (the greatest pairwise distance is 0.096 substitution per site), and lineages from closely related aphid hosts are generally more closely related to each other than to other symbiont lineages in the clade (Fig. 1). “*Ca. Serratia symbiotica*” lineages from *C. cedri* and *Pterochloroides persicae* cluster with high levels of support.

A third group of *Serratia* strains from aphids does not branch with clusters A and B. The 16S rRNA gene tree indicates that these strains are more closely related to *Serratia marcescens* than to other symbiotic clades.

Phylogenetic reconstruction of *Buchnera* lineages. 16S rRNA gene sequences were obtained from all bacterial strains from Lachninae aphids, including *Buchnera*. Phylogenetic reconstruction of the *Buchnera* sequences supports clustering of lineages into tribes and shows general congruence with expected relationships of the aphid hosts (Fig. 2) (6, 23), with the exception of the Cinarini. The Cinarini are split into two clades corresponding to the subgenus *Cupressobium* and the subgenus *Cinara*. The latter clade is grouped with *Stomaphis* aphids with high levels of support, excluding subgenus *Cupressobium* aphids. The evolutionary relationships of aphids in the subgenus *Cinara* clade are relatively unresolved, but the best ML tree and Bayesian posterior probabilities (Fig. 2) do not support monophyletic grouping of these aphids. However, constraining aphids infected with “*Ca. Serratia symbiotica*” cluster B into a monophyletic clade with respect to aphids with other symbiont types results in a significantly worse tree ($P = 0.01$, S-H one-tailed test).

Prevalence of facultative symbionts and their distribution among aphid species. In aphids, facultative symbionts are generally defined as intracellular, vertically transmitted bacterial associates that are facultative from the perspective of the host. Bacteria identified in this study that are closely related to other insect-associated bacteria or are known to reside inside insect cells were assumed to be facultative symbionts, although this assumption remains to be verified experimentally. Almost all aphids screened had a facultative symbiont infection, but infection by more than one facultative symbiont in the same aphid was rare (Fig. 2 and Table 2). Based on data from a survey of aphid species belonging to the entire aphid family (Aphididae), the level of infection with “*Ca. Serratia symbiotica*” or any facultative symbiont is 13.9% or 45.8%, respectively (28). In contrast, for species of the subfamily Lachninae, the frequencies of infection with “*Ca. Serratia symbiotica*” and with any facultative symbionts were higher, 43.9% and 78.0%, respectively ($P = 0.0006$ and $P = 0.0014$, Fisher's exact test). In

TABLE 1. Facultative symbionts of aphids

Aphid host subfamily	Aphid host				Facultative symbiont(s) ^a	Reference
	Tribe	Species, group, or genus	Collection locality	Geographic distribution		
Aphidinae	Aphidini	<i>Aphis craccivora</i>	Japan	Cosmopolitan	" <i>Ca. Serratia symbiotica</i> " cluster A	
	Macrosiphini	<i>Acyrtosiphon pisum</i>	United States	Almost cosmopolitan	" <i>Ca. Serratia symbiotica</i> " cluster A	
		<i>Macrosiphoniella helichrysi</i>	Spain	Palearctic	" <i>Ca. Serratia symbiotica</i> " cluster A	28
		<i>Uroleucon caligatum</i>	United States	North America	" <i>Ca. Serratia symbiotica</i> " cluster A	30
Chaitophorinae		<i>Periphyllus bulgaricus</i>	Spain	NA ^b	" <i>Ca. Serratia symbiotica</i> " cluster A, " <i>Ca. Hamiltonella defensa</i> "	28
Lachninae	Cinarini	<i>Cinara brauni</i>	Poland	Europe	" <i>Ca. Serratia symbiotica</i> " cluster B	
		<i>Cinara cedri</i>	Chile	Palearctic, Argentina	—	
		<i>Cinara cedri</i>	Spain	Palearctic, Argentina	" <i>Ca. Serratia symbiotica</i> " cluster B	17
		<i>Cinara (Cupressobium) cupressi</i>	United States	Cosmopolitan, except Australasia	—	
		<i>Cinara (Cupressobium) cupressi</i>	United States	Cosmopolitan, except Australasia	" <i>Ca. Serratia symbiotica</i> " cluster A	17
		<i>Cinara edulis</i>	United States	Southwestern North America	" <i>Ca. Serratia symbiotica</i> " cluster B	
		<i>Cinara etsuhoe</i>	Japan	Japan	" <i>Ca. Serratia symbiotica</i> " cluster B	
		<i>Cinara glabra</i>	United States	United States	—	
		<i>Cinara gudarisi</i>	Spain	NA	" <i>Ca. Serratia symbiotica</i> " cluster B	17
		<i>Cinara (Cupressobium) juniperi</i>	Spain	Europe, Middle East, Australia, New Zealand, North America	" <i>Ca. Serratia symbiotica</i> " cluster A	17
		<i>Cinara maghrebica</i>	Spain	Mediterranean, Argentina	" <i>Ca. Serratia symbiotica</i> " cluster B	17
		<i>Cinara maritima</i>	Spain	Mediterranean, Middle East, introduced to South America	" <i>Ca. Serratia symbiotica</i> "	28
		<i>Cinara pilicornis</i>	Spain	Europe, introduced to Australia, New Zealand, and North and South America	" <i>Ca. Serratia symbiotica</i> " cluster B	17
		<i>Cinara pinea</i>	Spain	Palearctic, introduced to North America	" <i>Ca. Serratia symbiotica</i> " cluster B	17
		<i>Cinara pinea</i> group	Czech Republic	Palearctic, introduced to North America	" <i>Ca. Serratia symbiotica</i> " cluster B	
		<i>Cinara pinimaritimae</i>	Spain	NA	—	17
		<i>Cinara ponderosae</i>	United States	Western North America	" <i>Ca. Serratia symbiotica</i> " cluster A	
		<i>Cinara pseudotaxifoliae</i>	United States	Western North America	—	
		<i>Cinara schimitscheki</i>	Spain	Europe	" <i>Ca. Serratia symbiotica</i> " cluster B	17
		<i>Cinara</i> sp.	Poland	NA	" <i>Ca. Serratia symbiotica</i> " cluster B, cluster L	
<i>Cinara</i> sp.	United States	NA	" <i>Ca. Hamiltonella defensa</i> "	4		
<i>Cinara</i> sp.	United States	NA	—			
<i>Cinara terminalis</i>	United States	Western North America	" <i>Ca. Serratia symbiotica</i> " cluster A			
<i>Cinara (Cupressobium) tujafilina</i>	Spain	Cosmopolitan	" <i>Ca. Serratia symbiotica</i> " cluster A	17		
<i>Cinara whatolca</i>	United States	Southwestern North America	" <i>Ca. Serratia symbiotica</i> " cluster A			

Continued on following page

TABLE 1—Continued

Aphid host subfamily	Aphid host				Facultative symbiont(s) ^a	Reference
	Tribe	Species, group, or genus	Collection locality	Geographic distribution		
		<i>Essigella californica</i>	United States	Nearctic	?	28
		<i>Eulachnus brevipilosus</i>	United Kingdom	Europe, introduced to New Zealand and North America	" <i>Ca. Hamiltonella defensa</i> "	23
		<i>Eulachnus pallidus</i>	Spain	Palaearctic	<i>Sodalis</i>	28
		<i>Eulachnus rileyi</i>	United States	Palaearctic, introduced to North, South, and Central America and Africa	<i>Sodalis</i>	
Fordini		<i>Smynthuroides betae</i>	Israel	Cosmopolitan	" <i>Ca. Serratia symbiotica</i> " cluster A	28
Lachnini		<i>Lachnus roboris</i>	Spain	Palaearctic	" <i>Ca. Serratia symbiotica</i> " cluster B	17
		<i>Lachnus roboris</i>	Poland	Palaearctic	" <i>Ca. Serratia symbiotica</i> " cluster B	
		<i>Lachnus roboris</i>	Poland	Palaearctic	—	
		<i>Lachnus shiicola</i>	Japan	Japan	" <i>Ca. Serratia symbiotica</i> " cluster A	
		<i>Lachnus</i> sp.	United States	NA	—	28
		<i>Lachnus takahashii</i>	Japan	Japan	" <i>Ca. Serratia symbiotica</i> " cluster A	
		<i>Lachnus tropicalis</i>	Japan	East and Southeast Asia	" <i>Ca. Serratia symbiotica</i> " cluster A	
		<i>Maculolachnus submacula</i>	Spain	Palaearctic	<i>Enterobacteriaceae</i>	17
		<i>Maculolachnus submacula</i>	United Kingdom	Palaearctic	<i>Enterobacteriaceae</i>	23
		<i>Nippolachnus piri</i>	Japan	Asia	" <i>Ca. Hamiltonella defensa</i> "	23
		<i>Nippolachnus piri</i>	Japan	Asia	<i>Sodalis</i>	
		<i>Pterochloroides</i>	Spain	Palaearctic	" <i>Ca. Serratia symbiotica</i> " cluster B	
		<i>Pterochloroides persicae</i>	Spain	Palaearctic	" <i>Ca. Serratia symbiotica</i> " cluster B	28
		<i>Tuberolachnus salignus</i>	Poland	Cosmopolitan, except Australasia	—	
		<i>Tuberolachnus salignus</i>	Spain	Cosmopolitan, except Australasia	" <i>Ca. Serratia symbiotica</i> " cluster B	17
		<i>Stomaphis aphananthae</i>	Japan	Japan	<i>Serratia</i>	
		<i>Stomaphis cupressi</i>	Spain	Kenya, France, Italy	Cluster L, <i>Enterobacteriaceae</i>	17
		<i>Stomaphis fagi</i>	Japan	Japan	<i>Arsenophonus</i>	
		<i>Stomaphis longirostris</i>	Poland	Europe, Western Siberia	<i>Arsenophonus</i>	
		<i>Stomaphis longirostris</i>	Poland	Europe, Western Siberia	Cluster L	
		<i>Stomaphis pini</i>	Japan	Japan	" <i>Ca. Serratia symbiotica</i> " cluster A	
		<i>Stomaphis quercus</i>	Poland	Europe	Cluster L	
		<i>Stomaphis quercus</i>	Poland	Europe	<i>Arsenophonus</i>	
		<i>Stomaphis takahashii</i>	Japan	Japan	<i>Arsenophonus</i>	
		<i>Stomaphis yanonis</i>	Japan	Asia	<i>Serratia</i>	
		<i>Stomaphis yanonis</i>	Japan	Asia	<i>Serratia</i>	
		<i>Stomaphis</i> sp.	Japan	NA	<i>Serratia</i>	
		<i>Stomaphis</i> sp.	Japan	NA	" <i>Ca. Serratia symbiotica</i> " cluster A	
		<i>Stomaphis</i> sp.	Japan	NA	—	
Tramini		<i>Protrama ranunculi</i>	United Kingdom	Palaearctic	—	22
		<i>Trama troglodytes</i>	United Kingdom	Palaearctic	" <i>Ca. Serratia symbiotica</i> " cluster A	22

^a ?, ambiguous; —, no identifiable facultative symbiont.
^b NA, not available.

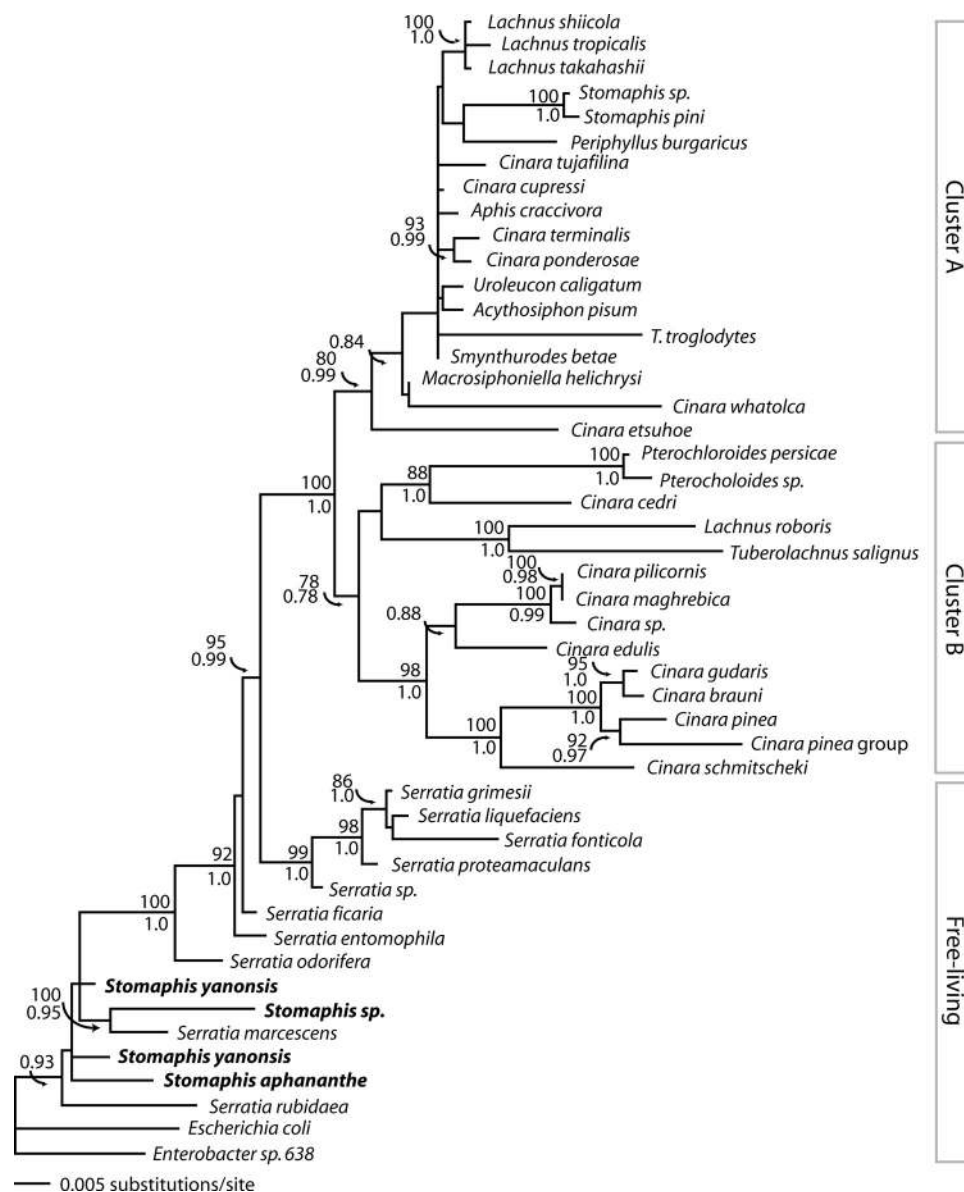


FIG. 1. ML tree for *Serratia* based on 16S rRNA genes. Symbiotic bacterial taxa are indicated by the name of the aphid species from which they were isolated and sequenced. ML bootstrap support values are indicated above nodes, and Bayesian posterior probabilities are indicated below nodes. Only support values greater than 75 or 0.75 are shown. There have been at least two independent acquisitions of aphid symbionts in the genus *Serratia*, one in the common ancestor of clusters A and B and the other in associates of the aphid genus *Stomaphis* (bold type).

Cinara species, the frequency of “*Ca. Serratia symbiotica*” infection was quite high, 64.3% ($P = 0.0002$, Fisher’s exact test). Only data from this study and the study of Russell et al. (28) were used in this analysis, as other studies may not have reported the absence of symbiont infections.

Aphids in *Cinara* subgenus *Cinara* were infected with “*Ca. Serratia symbiotica*” cluster B or cluster A, “*Ca. Hamiltonella defensa*,” *Wolbachia*, relatives of *Sodalis*, or a gammaproteobacterial lineage referred to in this study as “cluster L” (closely related to a gut-associated symbiont of honey bees [see Fig. S2 in the supplemental material]). Some species had no facultative symbionts. *Stomaphis* aphids were infected with “*Ca. Serratia symbiotica*” cluster A, *Arsenophonus*, relatives of free-living *Serratia*, and “cluster L” bacteria. The *Buchnera*

sequences from *Stomaphis* aphids infected with bacteria similar to free-living *Serratia* form a monophyletic clade (Fig. 2), while the *Serratia* symbionts themselves appear to be polyphyletic (Fig. 1). *Lachnus* aphids had “*Ca. Serratia symbiotica*” cluster A and B infections. One sample from *Nippolachnus piri* was infected with *Sodalis*, and another sample from *N. piri* was infected with “*Ca. Hamiltonella defensa*.” Finally, *Cinara* (*Cupressobium*) aphids were infected only with “*Ca. Serratia symbiotica*” cluster A.

Closer examination of symbiont sequences shown in Fig. 2 shows that there is a geographical pattern of infection with “*Ca. Serratia symbiotica*.” All aphids with “*Ca. Serratia symbiotica*” cluster B were collected in Spain, Poland, and the Czech Republic, except for *Cinara edulis* collected in the

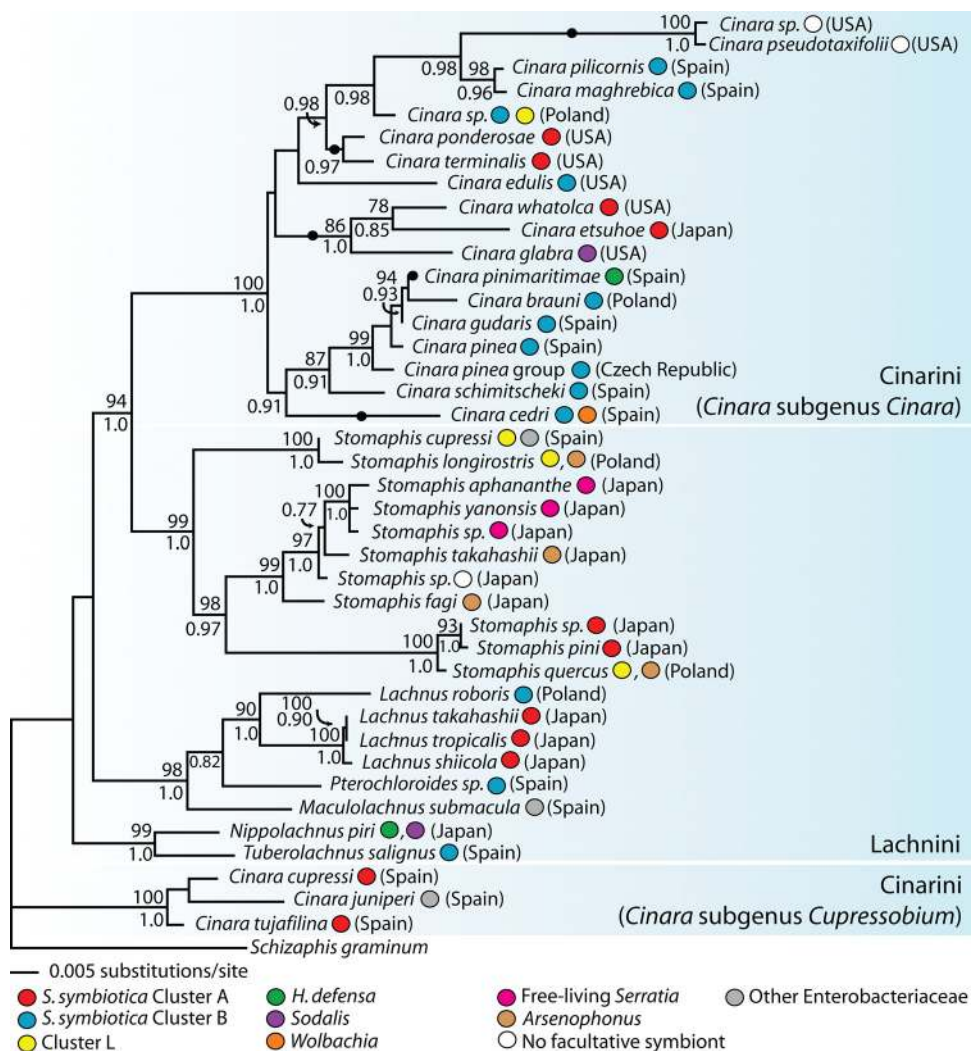


FIG. 2. ML tree for *Buchnera* associated with Lachninae, based on 16S rRNA genes. For a description of the taxa and phylogenetic support see the legend to Fig. 1. Most species of Lachninae sampled were infected with a facultative symbiont (all lineages except Other *Enterobacteriaceae*). Circles separated by a comma indicate taxa that were from different individuals in the same population or belonging to the same species. Taxa infected with “*Ca. Serratia symbiotica*” cluster B are not monophyletic in the *Cinara* subgenus *Cinara* clade. Cluster B lineages have been lost from the *Cinara* subgenus *Cinara* clade at least five times, including a loss in *C. cedri* from Chile, as indicated by filled circles on branches. There is a strong geographic pattern for infection with facultative symbionts, with “*Ca. Serratia symbiotica*” cluster A found mainly in Asia and North America and cluster B found in Europe.

United States. Excluding *C. edulis*, all *Cinara* species infected with the cluster B lineage are endemic in Europe, although several of them have been introduced into other parts of the world (Table 1) (1). All aphids infected with “*Ca. Serratia*

symbiotica” cluster A were collected from Japan or the western United States, with the exception of the subgenus *Cupressobium* aphids found in Spain. Subgenus *Cupressobium* aphids are the only cosmopolitan aphids found to be infected with “*Ca. Serratia symbiotica*” cluster A in our samples; all other Lachninae aphids infected with this symbiont lineage were collected from western North America and Asia.

TABLE 2. Frequency of symbiont infections in groups of aphids, based on data from the current study and the study of Russell et al.^a

Aphid group	No. of aphid species			% of aphid species	
	Infected with “ <i>Ca. Serratia symbiotica</i> ”	Infected with any facultative symbiont	Total screened	Infected with “ <i>Ca. Serratia symbiotica</i> ”	Infected with any facultative symbiont
Lachninae	18	32	41	43.9	78.1
<i>Cinara</i> only	9	11	14	64.3	78.6
Other Aphididae	10	33	72	13.9	45.8

^a See reference 28.

DISCUSSION

Reconstruction of aphid evolution using *Buchnera* sequences. As *Buchnera* evolves synchronously with aphid host lineages (21), the phylogenetic relationships of these lineages can be used as a proxy for the evolutionary relationships of aphids. Here, the phylogeny of *Buchnera* strains showed general congruence with the relationships of their aphid hosts

except the Cinarini, which were split into clades corresponding to *Cinara* subgenus *Cinara* and subgenus *Cupressobium*. The Cinarini were not monophyletic in the analyses of Normark (23), but subgenus *Cupressobium* taxa were not included in those analyses. The proposed close relationship of the genera *Nippolachnus* and *Tuberolachnus* (23) is supported here.

Multiple acquisitions of *Serratia* symbionts in aphids. “*Ca. Serratia symbiotica*” was first identified as an organism closely related to free-living and other insect-associated *Serratia* lineages based on sequence similarity (20, 30, 41). Its vertical transmission and scattered presence in numerous aphid species around the world suggested that this bacterium is a facultative symbiont of aphids (3, 14, 20, 28, 30, 33, 39). Early phylogenetic reconstructions of symbiotic *Serratia* lineages showed that they arose once from a common ancestor (17, 28, 30, 40, 41). We have identified additional *Serratia* types associated with *Stomaphis* aphids in Japan; these types are more closely related to free-living *S. marcescens* than to “*Ca. Serratia symbiotica*,” indicating that free-living *Serratia* strains have been acquired more than once as symbionts in aphids. Although the additional *Serratia* types associated with *Stomaphis* species are themselves polyphyletic in our tree (Fig. 1), they are located in a region of the tree with little resolution. Although vertical transmission of these bacteria in *Stomaphis* aphids has not been demonstrated yet, these facultative symbionts have been visualized in the bacteriocytes of *Stomaphis yanonsis* using immunohistochemical staining techniques (7), so they are likely symbiotic associates rather than environmental contaminants.

“*Ca. Serratia symbiotica*” encompasses lineages belonging to both clusters A and B (20). While aphid symbionts in the “*Ca. Serratia symbiotica*” clade are closely related to insect-associated bacteria, including *Serratia ficaria* in fig wasps (12) and *Serratia entomophila* in beetles (36), there is no clear evidence that “*Ca. Serratia symbiotica*” evolved directly from insect-associated bacterial species. The *Stomaphis* symbionts seem to have evolved from a close relative of *S. marcescens*, a free-living bacterial entomopathogen that infects many insect orders and is also found as an opportunistic pathogen in humans (11). The independently derived symbiotic lineages of *Serratia* may have different functions within their hosts and may reflect a propensity for these bacteria to become successful long-term associates of aphids.

Functional roles of symbiont lineages in the “*Ca. Serratia symbiotica*” clade. The potential functions of several “*Ca. Serratia symbiotica*” lineages in particular host species have been evaluated in genetic and experimental studies. Phylogenetic reconstruction of the evolution of “*Ca. Serratia symbiotica*” lineages allows us to assign potential functional roles and to determine host specificity based on relatedness and evidence of codiversification.

Given that “*Ca. Serratia symbiotica*” cluster A has been demonstrated to confer heat tolerance in *A. pisum* (19, 29), it is likely that other symbiont lineages in this clade have similar effects on their aphid hosts, although this has not been tested experimentally.

It has been proposed that symbionts of *Cinara* aphids have transitioned from facultative symbionts to obligate symbionts for host survival and reproduction (9, 17, 26). A pattern observed for all obligate symbionts studied to date is their cocladogenesis with hosts as a result of long-term vertical transmission

and rare horizontal transfer between lineages (13, 20, 21, 38). We have shown that “*Ca. Serratia symbiotica*” and other symbiont lineages from subgenus *Cinara* species do not form a clade and have not undergone the strict vertical transmission characteristic of obligate symbionts. The absence of cocladogenesis is not sufficient to deem a symbiotic lineage “not obligate”; however, the loss of symbiont lineages from taxa in a clade whose ancestor was infected with “*Ca. Serratia symbiotica*” cluster B demonstrates that these symbionts remain facultative in at least some lineages. Loss of cluster B symbionts has occurred five times in the subgenus *Cinara* according to the ML tree (Fig. 2). Two examples are *C. cedri* from Chile, which appears to lack any symbiont other than *Buchnera*, while in Europe “*Ca. Serratia symbiotica*” seems to be an obligate symbiont of this species (26), and *Cinara pseudotaxifoliae* from the United States, which is closely related to European aphids infected with “*Ca. Serratia symbiotica*” cluster B, yet also appears to lack any facultative symbiont. Although many European aphids are infected with “*Ca. Serratia symbiotica*” cluster B, close relatives or even aphids belonging to the same species in other geographic locations do not require the presence of this organism.

A more plausible scenario than ancient obligate symbiosis in lachnid aphids is that “*Ca. Serratia symbiotica*” cluster B was a relatively lachnid-specialized facultative symbiotic lineage that was horizontally transferred among clades during the diversification of the Lachninae.

Although “*Ca. Serratia symbiotica*” from *C. cedri* may be an obligate symbiont in European aphids, it seems to have been horizontally transferred from more distantly related host species in the Lachninae. Phylogenetic reconstruction using both 16S rRNA and *atpD* genes suggested that “*Ca. Serratia symbiotica*” from *C. cedri* is more closely related to symbionts from non-*Cinara* lachnids (*Pterochloroides* species for the 16S rRNA gene and *Lachnus roboris* and *Tuberolachnus salignus* for *atpD*) than to other lineages from the genus *Cinara* (17). Thus, characteristics of its genome may not be representative of characteristics of “*Ca. Serratia symbiotica*” lineages from other *Cinara* aphids.

High incidence of facultative symbionts in Lachninae aphids. Almost all aphids in the Lachninae are infected with a bacterial symbiont in addition to *Buchnera* (Table 2). Many of these symbionts are “*Ca. Serratia symbiotica*” or *Serratia* relatives, but bacteria belonging to other lineages are present in several of the lachnid species screened. The smallest known *Buchnera* genomes are found in this subfamily, and *Buchnera* has lost genes for tryptophan biosynthesis in at least one lineage (26). Due to their high incidence, we propose that facultative symbionts such as “*Ca. Serratia symbiotica*” may be beneficial in the Lachninae due to their ability to supplement nutrition and thus compensate for inadequate provisioning of nutrients by *Buchnera*. Alternatively, some lineages of facultative symbionts found in these aphids are known reproductive manipulators and could have negative effects on fitness in their hosts (8, 35).

Although facultative symbiont coinfections were rare, the presence of multiple bacterial lineages in an individual or host population could facilitate intersymbiont competition detrimental to host fitness (25). However, competition might be avoided by partitioning of bacterial cells into different host

cells, a feature commonly observed for *Buchnera* and other facultative symbionts in aphids (7, 10, 20, 30). Additionally, the presence of so many bacterial types in the host populations may provide increased opportunity for gene exchange between facultative symbionts, despite the rarity of coinfections. Examination of APSE phage sequences implied that in the past there was phage-mediated horizontal transfer of genes between “*Ca. Hamiltonella defensa*” and *Sodalis* or its relatives (5), and our results document the cooccurrence of these two symbiont lineages in the same host species (*N. piri*) for the first time, showing one potential route by which transfer could have occurred.

In this phylogenetic analysis, we learned that diversity in *Serratia* symbiont lineages is due to multiple independent origins of symbiosis from free-living *Serratia* and also to evolution within distinct symbiont clades. We show here that codiversification of “*Ca. Serratia symbiotica*” cluster B lineages with all subgenus *Cinara* aphids is unlikely, as is an obligate role. Finally, we show that there is a high incidence of facultative symbiont infection across diverse lineages in the subfamily Lachninae, which may be advantageous due to impaired *Buchnera* function.

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