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Evolution of the Secondary Symbiont "*Candidatus* Serratia symbiotica" in Aphid Species of the Subfamily Lachninae▽†

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***Buchnera aphidicola* BCc, the primary endosymbiont of the aphid *Cinara cedri* (subfamily Lachninae), is losing its symbiotic capacity and might be replaced by the coresident "*Candidatus* Serratia symbiotica." Phylogenetic and morphological analyses within the subfamily Lachninae indicate two different "*Ca.* Serratia symbiotica" lineages and support the longtime coevolution of both symbionts in *C. cedri*.**

Aphids are plant sap-feeding insects that harbor the gammaproteobacterium *Buchnera aphidicola* as their primary endosymbiont (3). *B. aphidicola* genome sequencing confirmed its role in supplying the nutrients lacking in the aphid diet (25). Several aphids harbor additional bacteria known as secondary symbionts (S-symbionts). S-symbionts are considered facultative, as despite conferring positive effects to their hosts (11, 17, 22, 24, 29), they are not essential to host survival and reproduction (1, 16). Although normally transmitted vertically through host generations, their distribution patterns suggest that sporadic horizontal transmission can occur (21).

B. aphidicola BCc, the primary endosymbiont of the cedar aphid *Cinara cedri*, possesses the smallest known *B. aphidicola* genome, with only 422 kb (7). Unlike the other sequenced *B. aphidicola* strains (25, 28, 30), this strain cannot synthesize tryptophan and riboflavin, which must come from another source (19). One particular feature of *C. cedri* is the abundant and nonfacultative presence of the S-symbiont "*Candidatus* Serratia symbiotica" (8). Moreover, "*Ca.* Serratia symbiotica" and *B. aphidicola* BCc are similar in size and shape and housed in separate, specific bacteriocytes. These observations and the rapid evolution of most of the retained genes led us to postulate that *B. aphidicola* BCc is losing its symbiotic capacity and might be replaced by "*Ca.* Serratia symbiotica" (19). Its complete genome sequence (in progress) will tell us whether this bacterium can perform all the metabolic functions necessary for host fitness or, alternatively, if some pathways have been lost. Then, the metabolic complementation of both bacteria would be the expected evolutionary outcome. In the sharpshooter *Homalodisca coagulata*, the endosymbionts "*Candidatus* Baumannia cicadellinicola" and "*Candidatus* Sulcia muelleri" have complementary biosynthetic abilities, needed to provide their host with nutrients lacking in the xylem sap (13). Molecular phylogenetic studies showed that both symbionts represent ancient associations (15), being "coprimary" symbionts (27).

Most studies on S-symbiont presence in aphids have been conducted on members of the subfamily Aphidinae, mainly on *Acyrthosiphon pisum*. Regarding the subfamily Lachninae, to which *C. cedri* belongs, the few studies carried out report the presence of gammaproteobacterial S-symbionts (4, 6, 21).

The aim of the present work was to determine whether "*Ca.* Serratia symbiotica" is consistently present in the subfamily Lachninae, thus indicating a long-term association with *B. aphidicola*, or whether its presence in the aphid *C. cedri* represents a singular case.

We have obtained sequences from 14 species of the subfamily Lachninae, either by sequencing the genes from aphids collected from natural populations between 2004 and 2006 or by retrieving them from databases (Table 1). Total DNA was extracted from aphids as described previously (10), and 16S rRNA genes were amplified (for primers and PCR conditions, see the supplemental material). The resulting PCR fragments were cloned into a pGEM-T vector (Promega) and diagnostic digestions made to assess whether the 16S rRNA gene belonged to *B. aphidicola* or a S-symbiont (23). Sequencing was performed with an ABI Prism BigDye Terminator v3.0 kit (Applied Biosystems), and the resulting sequences were analyzed with the Staden software package (26). BlastN searches confirmed the nature of the sequences. In addition to *B. aphidicola*, all aphid species harbored one S-symbiont, except *Stomaphis cupressi*. In most cases, the S-symbiont was "*Ca.* Serratia symbiotica" (Table 1).

We carried out 16S rRNA gene phylogenetic analyses with the sequences from *B. aphidicola* (Fig. 1A) and "*Ca.* Serratia symbiotica," obtained in this work, and from different aphid subfamilies (in addition to Lachninae) of the family Aphididae, previously studied (21) (Fig. 1B) (see methods in the supplemental material). As expected, *B. aphidicola* from members of the subfamily Lachninae formed a well-solved cluster with respect to the two members of the family Aphididae used as outgroups (Fig. 1A). However, at the tribe level, although the

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TABLE 1. Taxonomic status, S-symbionts, locations, and accession numbers of the aphid species included in the present study

Subfamily and tribe	Host species	S-symbiont	Location	Accession no.		
				16S rRNA gene from S-symbiont	16S rRNA gene from *B. aphidicola*	*atpD* from "*Ca.* Serratia symbiotica"
Lachninae						
Eulachnini	*Cinara* (*Cinara*) *schimitscheki*	"*Ca.* Serratia symbiotica"	Spain	EU348318	EU334766	
	Cinara (*Cinara*) *pinea*	"*Ca.* Serratia symbiotica"	Spain	EU348316	EU334770	EU348331
	Cinara (*Cinara*) *gudaris*	"*Ca.* Serratia symbiotica"	Spain	EU348317	EU334771	
	Cinara (*Cinara*) *maghrebica*	"*Ca.* Serratia symbiotica"	Spain	EU348319	EU334772	EU348330
	Cinara (*Cinara*) *cedri*[b]	"*Ca.* Serratia symbiotica"	Spain	AY620432[a]	AY620431[a]	EU360774
	Cinara (*Cinara*) *cedri*[b]	"*Ca.* Serratia symbiotica"	Spain	EU348324	EU334777	EU348327
	Cinara (*Cinara*) *pinimaritimae*	"*Candidatus* Hamiltonella defensa"	Spain	EU348313	EU334774	
	Cinara (*Cinara*) *pilicornis*	"*Ca.* Serratia symbiotica"	Spain	EU348320	EU334776	EU348332
	Cinara (*Cupressobium*) *tujafilina*	"*Ca.* Serratia symbiotica"	Spain	EU348323	EU334773	EU348333
	Cinara (*Cupressobium*) *juniperi*	unidentified	Spain	EU348311	EU334768	
	Cinara (*Cupressobium*) *cupressi*[b]	"*Ca.* Serratia symbiotica"	Spain	EU348321	EU334775	EU348328
	Cinara (*Cupressobium*) *cupressi*[b]	"*Ca.* Serratia symbiotica"	Spain	EU348322	EU334769	EU348329
Lachnini	*Stomaphis cupressi*	"*Candidatus* Arsenophonus triatominarum"	Spain	EU348325	EU334767	
		unidentified	Spain	EU348326	EU334767	
	Maculolachnus submacula	unidentified	Spain	EU348312	AJ296755[a]	
	Lachnus roboris	"*Ca.* Serratia symbiotica"	Spain	EU348314	AJ296756[a]	EU348334
	Tuberolachnus salignus	"*Ca.* Serratia symbiotica"	Spain	EU348315	AJ296754[a]	EU348335
	Pterochloroides persicae	"*Ca.* Serratia symbiotica"	Spain	AY136155[a]		
Aphidinae						
Macrosiphini	*Macrosiphoniella helichrysi*	"*Ca.* Serratia symbiotica"	Spain	AY136151[a]		
	Acyrthosiphon pisum	"*Ca.* Serratia symbiotica"	Japan	AB033777[a]		
	Acyrthosiphon pisum	"*Ca.* Serratia symbiotica"	Japan	AB033778[a]		
	Acyrthosiphon pisum	"*Ca.* Serratia symbiotica"	Japan	AB033779[a]		
	Acyrthosiphon pisum	"*Ca.* Serratia symbiotica"	USA	AF293617[a]		
	Acyrthosiphon pisum	"*Ca.* Serratia symbiotica"	USA	AY136139[a]		
	Acyrthosiphon pisum	"*Ca.* Serratia symbiotica"	USA	AY136140[a]		
	Acyrthosiphon pisum	"*Ca.* Serratia symbiotica"	USA	AY296732[a]		
	Uroleucon caligatum	"*Ca.* Serratia symbiotica"	USA	AF293624[a]		
Aphidini	*Aphis craccivora*	"*Ca.* Serratia symbiotica"	Spain	AY136137[a]		
Chaitophorinae						
Chaitophorini	*Periphyllus bulgaricus*	"*Ca.* Serratia symbiotica"	Spain	AY136157[a]		
Eriosomatinae						
Fordini	*Smynthurodes betae*	"*Ca.* Serratia symbiotica"	Israel	AY136159[a]		

[a] From the databases.
[b] Different localities.

two Eulachinini clades *Cinara* (*Cinara*) and *Cinara* (*Cupressobium*) are monophyletic groups, they did not cluster in a monophyletic tribe separated from the Lachnini, thus confirming uncertainties about the taxonomic status of these two tribes (12, 18).

Regarding the "*Ca.* Serratia symbiotica" phylogeny, the most interesting result is the existence of two major clusters (Fig. 1B). Cluster A encompasses representatives of the aphid subfamilies Aphidinae, Chaitophorinae, Eriosomatinae, and some Eulachnini members of the subfamily Lachninae from the clade *Cinara* (*Cupressobium*). Cluster B comprises solely members of the subfamily Lachninae, belonging to the clade *Cinara* (*Cinara*) from Eulachnini, plus species *Lachnus roboris* and *Tuberolachnus salignus* from tribe Lachnini. These clusters do not match those based on either the *B. aphidicola* 16S rRNA gene phylogeny (Fig. 1A) or the aphid phylogeny obtained with morphological traits (9).

To give support to the hypothesis of two "*Ca.* Serratia symbiotica" clusters, additional phylogenetic analyses were made with the protein-coding gene *atpD* in selected species from the subfamily Lachninae (for additional information, see the supplemental material). This gene was chosen because the ATPase operon has been lost in *B. aphidicola* BCc (19), and previous attempts to amplify a gene fragment in *B. aphidicola* from members of the subfamily Lachninae did not give positive results (2), indicating the possible loss of all the genes of this operon in *B. aphidicola* before the Lachninae split. The topology obtained (Fig. 1C) is similar to that with the ribosomal gene. Differences in branch length between the two clusters are more evident in this case, with longer branches in cluster B than in A. This feature indicates that this gene is evolving faster in "*Ca.* Serratia symbiotica" species in cluster B. Similar results for topology and branch length were obtained when 16S rRNA gene and *atpD* sequences were concatenated (data not shown).

In summary, we report the existence of at least two "*Ca.*

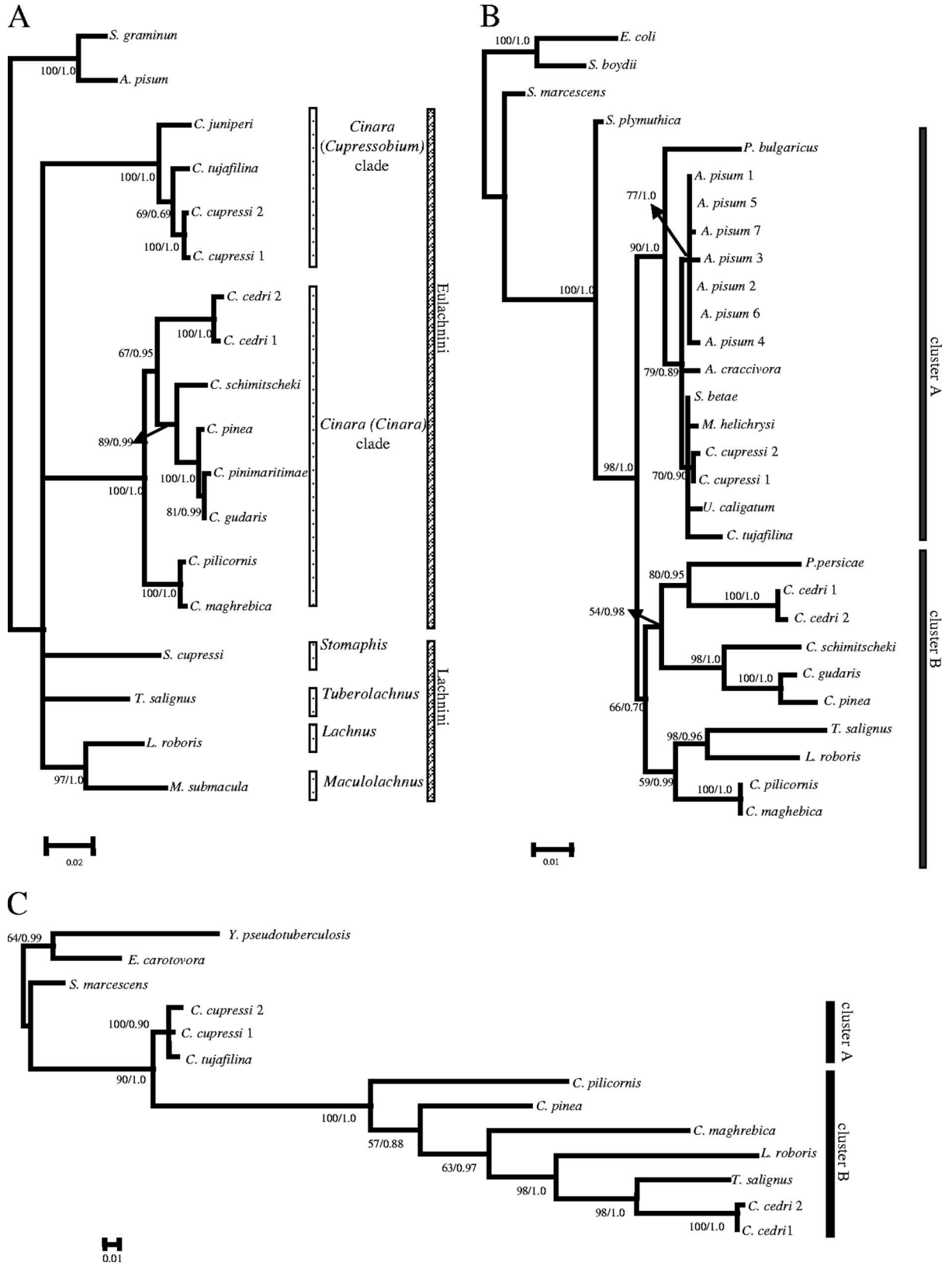

FIG. 1. Maximum likelihood phylogeny. (A) *B. aphidicola* 16S rRNA gene. The outgroups are *B. aphidicola* from *Acyrthosiphon pisum* and from *Schizaphis graminum* (GenBank accession numbers M27039 and NC_004061, respectively). (B) "*Ca.* Serratia symbiotica" 16S rRNA gene. Free-living bacteria are *Serratia plymuthica* (AY394724), *Serratia marcescens* (AF124038), and the outgroups *Escherichia coli* (AB045731) and *Serratia boydii* (AY696681). (C) Gene *atpD* from some selected "*Ca.* Serratia symbiotica" spp. Free-living bacteria are *S. marcescens* (ABI21950) and the outgroups *Erwinia carotovora* (IP_052595) and *Yersinia pseudotuberculosis* (BX936398). Numbers in nodes indicate support values in the form of proportions of bootstrap pseudoreplicates and Bayesian a posteriori probabilities for the corresponding inner branch. Branches with support values higher than 55% are collapsed. See Table 1 for species information.

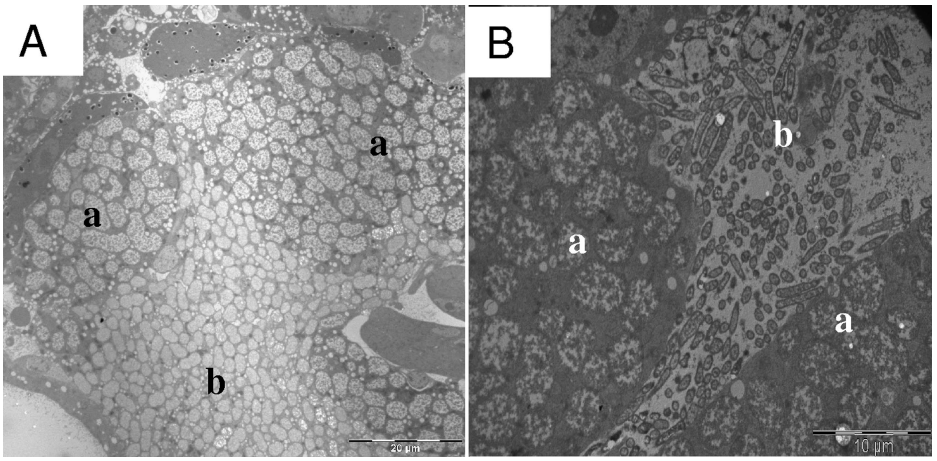

FIG. 2. Electron microscopy of *C. cedri* (A) and *C. tujafilina* (B). Primary and secondary bacteriocytes harbor *B. aphidicola* (a) and "*Ca.* Serratia symbiotica" (b).

Serratia symbiotica" clades in the subfamily Lachninae, which is compatible with the two aphid subgenera *Cinara (Cupresobium*) and *Cinara (Cinara*), proposed by entomologists according to morphological and other biological features (20). Moreover, while clade A encompasses "*Ca.* Serratia symbiotica" from aphids belonging to different subfamilies of the family Aphididae, clade B comprises only species from the subfamily Lachninae.

To further ascertain the presence of two different "*Ca.* Serratia symbiotica" clades, we performed electron microscopy studies of primary (*B. aphidicola*) and secondary ("*Ca.* Serratia symbiotica") symbionts in two selected aphids as representatives of each clade: *C. cedri* (Eulachnini from clade B) and *Cinara tujafilina* (Eulachnini from clade A) (for details, see methods in the supplemental material). The most remarkable result concerns the differences in morphology of "*Ca.* Serratia symbiotica" (Fig. 2). *C. cedri* exhibited an unusually large cell size and spherical shape, which is characteristic of primary symbionts (1), and were detected only in their specific bacteriocytes. In *C. tujafilina*, by contrast, "*Ca.* Serratia symbiotica" displayed more typical cell size and shape (bacilliform) and was located in the sheath cells, in secondary bacteriocytes, and extracellularly, as previously reported for *A. pisum* (5, 14).

All these results suggest a long-term relationship between "*Ca.* Serratia symbiotica" and aphids of the subgenus *Cinara* (*Cinara*) of the subfamily Lachninae (and probably also of the tribe Lachnini). The hypothetical evolutionary scenario could be that infection by an ancestor of "*Ca.* Serratia symbiotica" took place before the tribes Lachnini and Eulachnini split. Afterwards, clades *Cinara* (*Cinara*) and *Cinara* (*Cupressobium*) diverged and evolved. Horizontal transfer events between members of *Cinara* (*Cupressobium*) and members of the other aphid subfamilies would explain why they cluster together with a very low level of divergence. However, in members of clade *Cinara* (*Cinara*), to which *C. cedri* belongs, "*Ca.* Serratia symbiotica" would have established a deep association due to the loss of some essential functions of *B. aphidicola*, which were taken over by "*Ca.* Serratia symbiotica" as previously proposed (19). The differences in branch length obtained indicate that in this lineage, the species has undergone accelerated evolution. More difficult to explain is the clustering of "*Ca.* Serratia symbiotica" of two members of the tribe Lachnini with members of clade B, and thus, further studies are needed with more representatives of the tribe Lachnini.

In conclusion, we postulate the presence of two types of "*Ca.* Serratia symbiotica" in aphids, one an S-symbiont but the other a primary-like endosymbiont.

Nucleotide sequence accession numbers. The DNA sequences determined in this study were deposited in the EMBL/GenBank nucleotide sequence databases with the accession numbers shown in Table 1.

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