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Diversity of proteobacterial endosymbionts in hemlock woolly adelgid (*Adelges tsugae*) (Hemiptera: Adelgidae) from its native and introduced range

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Summary

Knowledge of intraspecific variation in symbioses may aid in understanding the ecology of widespread insects in different parts of their range. We investigated bacterial symbionts of *Adelges tsugae*, a pest of hemlocks in eastern North America introduced from Asia. Amplification, cloning, and sequencing of bacterial 16S rDNA, *in situ* hybridizations, and electron microscopy revealed that *A. tsugae* harbours up to five bacterial phylotypes, according to population. Three *Gammaproteobacteria* species are maternally transmitted. The first, designated '*Ca*. Pseudomonas adelgestsugas' resides in the haemocoel, and was detected in all populations except Taiwan. The second phylotype, '*Ca*. Serratia symbiotica', resides in bacteriocytes of populations on *Tsuga sieboldii* in Japan and in E. North America. The third phylotype, designated '*Ca*. Annandia adelgestsuga', clustered within a lineage of several insect endosymbionts that included *Buchnera aphidicola*. It was detected in bacteriocytes in all populations, and in salivary glands of first instars. Two *Betaproteobacteria* phylotypes were detected in some Japanese *T. sieboldii* and eastern North America populations, and were observed only in salivary glands with no evidence of maternal transmission. Our results support the ideas that symbiont gain and loss has been volatile in adelgids, and that symbionts may help to trace the source of invasive species.

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Introduction

Over two decades of studies have documented the remarkable diversity of heritable bacterial symbionts in insects, including members of the plant-sap-feeding Sternorrhyncha (Hemiptera) (Baumann, 2005; 2006). Mutualistic bacterial residents of insects fall broadly into two categories: obligate mutualists required by hosts for normal development (primary symbionts), and facultative mutualists not required for host reproduction (secondary symbionts) (Moran *et al*., 2008). In most Sternorrhyncha, obligate symbionts are confined to specialized host cells (bacteriocytes) typically composing a large organ (bacteriome) (Buchner, 1965; Baumann, 2005). Dietary studies, gene sequencing, and genomics have described the indispensible role of these mutualists in supplementing nitrogen-poor diets of phloem- and xylem-feeding insect hosts, with genomes reduced to functions essential for the partnership (Douglas *et al*., 2001; Wernegreen, 2002; Moran *et al*., 2008), and genomic complementarity in protein synthesis and DNA replication between partners (McCutcheon and Moran, 2010; Wilson *et al*., 2010; Hansen and Moran, 2011; McCutcheon and von Dohlen, 2011). Phylogenetic reconstructions show repeated patterns of ancient infection and co-diversification between sternorrhynchan hosts and obligate symbiont lineages (Baumann, 2005; Moran *et al*., 2005; Gruwell *et al*., 2007). In contrast, facultative mutualists of Sternorrhyncha invade several cell types, including bacteriocytes, and play roles in host fitness not related to nutrition (Moran *et al*., 2008; Oliver *et al*., 2010). Their presence in hosts is variable and can be shared by unrelated host lineages (Oliver *et al*., 2010).

Knowledge of the identity, distribution and functions of heritable symbionts is clearly central to building a holistic comprehension of host–insect ecology and evolution. Such knowledge may be especially important for understanding and combating the virulence of pest species. While primary bacteriome symbionts typically are universal within host lineages and their genomes relatively

stable (Tamas *et al*., 2002), certain mutations with large effects on host fitness have been measured on an ecological timescale (Dunbar *et al*., 2007). Some obligate symbionts are directly implicated in the pest status of their host (Hosokawa *et al*., 2007). Facultative symbionts, in particular, are demonstrated to affect host ecology in ways that may increase the probability of successful establishment and spread of introduced pests in novel environments (Oliver *et al*., 2010). In aphids, different strains of facultative associates confer varying levels of tolerance to high temperatures (Russell and Moran, 2006), resistance to parasitoids (Oliver *et al*., 2005), and resistance to fungal pathogens (Scarborough *et al*., 2005). Similar interactions may operate in other Sternorrhyncha (e.g. Hansen *et al*., 2007). Facultative symbionts may broaden the host-plant range of pest species (Tsuchida *et al*., 2004; 2011), alter defensive behaviour (Dion *et al*., 2011), and possibly facilitate virus transmission by insect vectors (van den Heuvel *et al*., 1994; Morin *et al*., 1999). Other direct impacts of facultative symbionts on fitness may increase the invasive potential of pests (Himler *et al*., 2011). Clearly, information on the identity and distribution of symbionts in insect pest species could be critical for the success of control programmes. Moreover, information on symbiont composition and genetic diversity from populations in native and introduced ranges could help to trace the source of pest introductions (e.g. Stenger *et al*., 2010), from which potential biological control agents could be discovered.

Adelges tsugae Annand (Sternorrhyncha: Aphidoidea: Adelgidae), the hemlock woolly adelgid, is a devastating insect pest of hemlocks (*Tsuga canadensis* and *T. caroliniana*) in eastern North America. Originally introduced from Japan, *A. tsugae* was first discovered in the eastern USA in the 1950s; since the 1980s it has been considered a severe threat to the health and sustainability of eastern hemlock forests (Souto *et al*., 1996; McClure and Cheah, 1999; Spaulding and Rieske, 2010). Molecular phylogenetic studies using host genes have implicated a particular population in southern Honshu, Japan as the source of the *A. tsugae* introduction to eastern North America (Havill *et al*., 2006). Much of the research, development and management efforts to stem the spread of *A. tsugae* in North America and mitigate damage to hemlocks have focused on physical, chemical and biological control (e.g. Orwig, 2008; Costa, 2010; Grant *et al*., 2010; Ross *et al*., 2010; Joseph *et al*., 2011), and breeding for resistance (Olsen and Bentz, 2010); however, additional research on *A. tsugae* biology is needed to develop novel approaches for management.

Early light microscopy studies established that Adelgidae harbour symbiotic bacteria within an abdominal bacteriome (Profft, 1937; Buchner, 1965; Steffan, 1968). Until recently, however, no adelgid bacterial associates had been studied with modern methods. Shields and Hirth (2005) conducted preliminary investigations of *A. tsugae* endosymbiont ultrastructure, noting four bacterial forms located variously in bacteriocytes, oocytes/eggs, and the haemocoel. Recently, Toenshoff and colleagues (2012a,b) investigated bacterial endosymbionts of four *Adelges* species complexes with microscopy and molecular methods. All *Adelges* harboured two species of intracellular, maternally inherited bacteria, localized to the bacteriomes; residents were either a *Betaproteobacteria* and a *Gammaproteobacteria*, or two species of *Gammaproteobacteria*. Phylogenies of *Gammaproteobacteria* were mostly unresolved, so that relationships of several endosymbionts to other bacteria were uncertain. *Betaproteobacteria* endosymbionts were related to *Burkholderia* species, most closely to symbionts of *Rhizopus* fungi. Toenshoff and colleagues (2012a,b) interpreted their results as suggesting a pattern of multiple symbiont acquisitions and replacements during adelgid evolution.

Investigations of additional adelgid taxa are needed to understand the full evolutionary history of endosymbiosis in this lineage. For pest species, symbiont information could help to confirm the source of invasive populations and contribute to developing potential biological control strategies (Shields and Hirth, 2005). The goals of this study were to identify, localize and characterize the diversity of bacterial associates of *A. tsugae* using molecular methods and microscopy. Multiple populations from the natural and introduced ranges of *A. tsugae* were sampled to document presence and diversity of symbionts among geographic locations, and to provide further evidence for southern Japan as the source of the eastern North America infestation. The possible roles of the symbionts in the mutualistic relationship with their host insects, and their contributions to a further understanding of endosymbiosis in Adelgidae are also discussed.

Results

Molecular identification, phylogenetics, and population distribution of endosymbionts

Bacterial 16S rRNA genes amplified from *A. tsugae* samples yielded five distinct types of sequences. All types belonged to members of *Gammaproteobacteria* and *Betaproteobacteria*, according to GenBank BLAST and RDPII Classifier searches. Sequences of the first type, designated gamma-A symbiont (GS-A) were most similar (~ 95% pairwise identity) to *Pseudomonas* spp. (e.g. *P. aeruginosa*). Sequences of the second type, designated gamma-B (GS-B) were most similar (99%) to a secondary symbiont of aphids (Aphididae), '*Candidatus* Serratia symbiotica', specifically, strains from *Cinara cupressi* and other members of '*Ca*. S. symbiotica' 'cluster A' (Lamelas *et al*., 2008; Burke *et al*., 2009). The third type, gamma-C

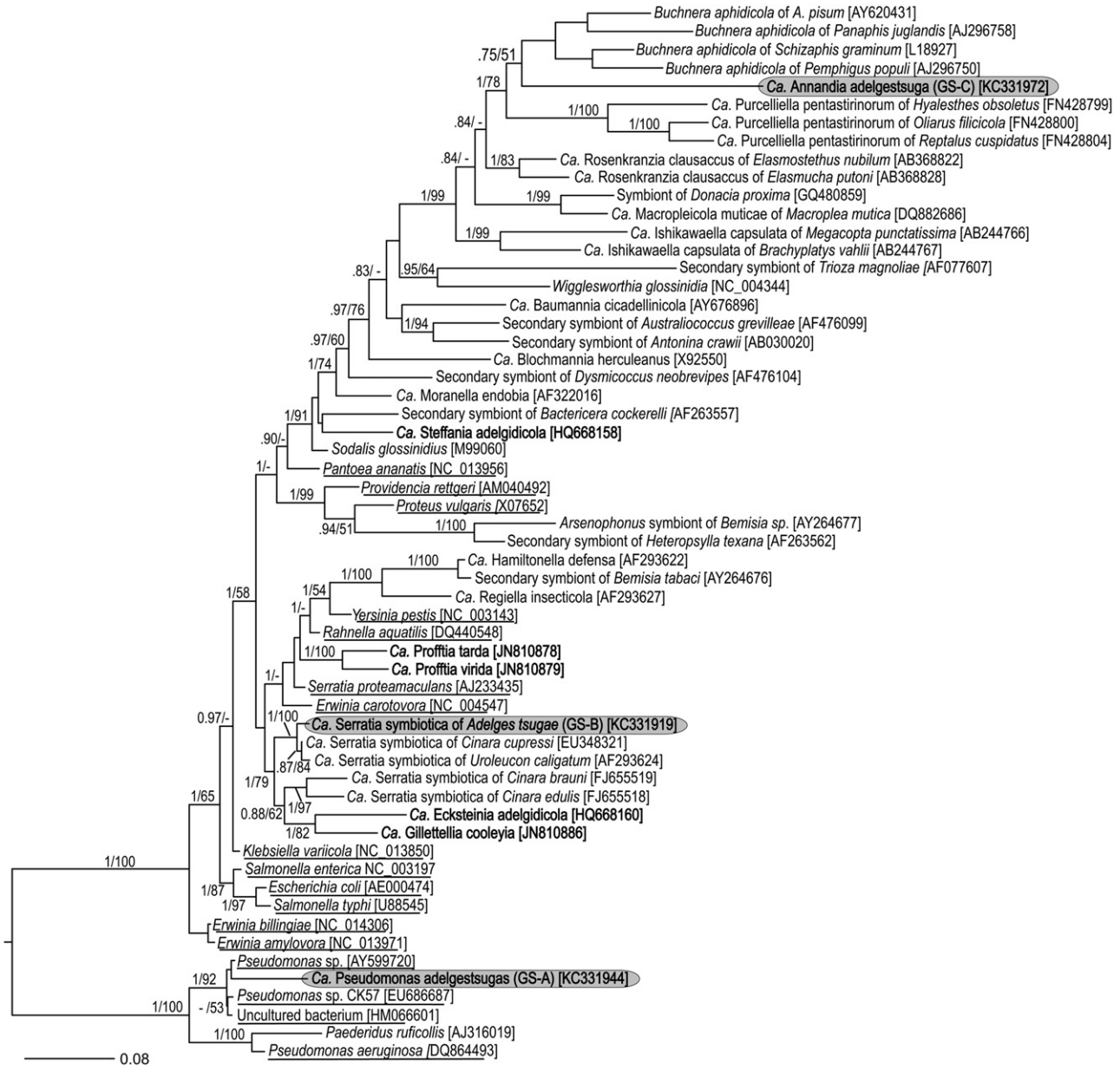

Fig. 1. Phylogeny of selected *Gammaproteobacteria*, showing relationships of *A. tsugae* endosymbionts to other insect endosymbionts and free-living bacteria. The 50% consensus topology from the Bayesian analysis is shown. *A. tsugae* symbionts are highlighted in grey; other adelgid endosymbionts are in bold; free-living bacteria are underlined. Numbers associated with branches indicate Bayesian posterior probabilities/maximum-likelihood bootstrap percentages.

(GS-C), was most similar (88–90%) to the aphid primary endosymbiont, *Buchnera aphidicola*, and endosymbionts of several heteropterans and beetles. Two additional types were members of the *Betaproteobacteria*: one type, BS-1 was similar (99%) to free-living *Burkholderia* spp. and a *Burkholderia* symbiont of *Riptortus* true bugs (Hemiptera: Alydidae), and the other type, BS-2 was most similar (98%) to free-living *Janthinobacterium* spp., Oxalobacteraceae, and *Massilia* spp. in the Burkholderiales.

Phylogenetic analysis of the *Gammaproteobacteria* data set, including insect endosymbionts and free-living bacteria, placed the *A. tsugae* endosymbionts with confidence in three unrelated lineages (Fig. 1). The GS-A symbiont was placed in a well-supported cluster with several free-living *Pseudomonas* spp. [BI (Bayesian Inference) posterior probability (PP) = 1.0, maximum-likelihood bootstrap support (maximum-likelihood, ML) = 92%]. The GS-B symbiont was placed with high support (PP = 1.0,

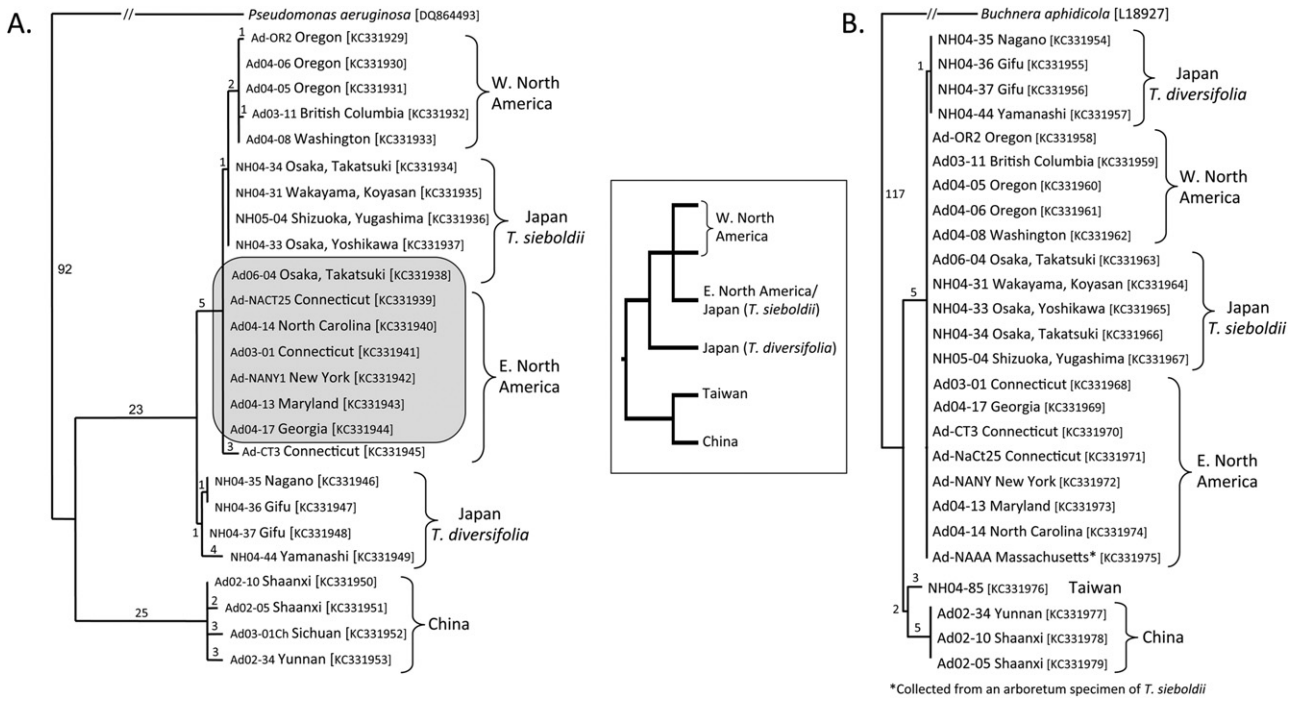

Fig. 2. Maximum-parsimony phylograms of *Gammaproteobacteria* symbionts GS-A and GS-C.
A. GS-A ('*Ca*. Pseudomonas adelgestsugas') sequences from all populations except Taiwan, which yielded no amplifications of that phylotype. Identical eastern North American and one Osaka *T. sieboldii* samples are highlighted in grey.
B. GS-C ('*Ca*. Annandia adelgestsuga') sequences amplified from all populations. Numbers on branches indicate the inferred substitutions along the branch. GenBank accessions are indicated in brackets. Inset: Relationships of *A. tsugae* populations inferred from mitochondrial DNA, simplified from Havill and colleagues (2006).

ML = 100%) with '*Ca*. S. symbiotica' 'cluster A' sequences (Lamelas *et al*., 2008; Burke *et al*., 2009). This cluster formed the sister to a group including 'cluster B' '*Ca*. S. symbiotica' (Lamelas *et al*., 2008; Burke *et al*., 2009) plus two previously discovered *Adelges* endosymbionts, '*Ca.* Ecksteinia adelgidicola' and '*Ca*. Gillettellia cooleyi'; the latter two species were well-supported sister taxa. The GS-C symbiont was placed with high support (PP = 1.0, ML = 78%) in a clade containing *Buchnera aphidicola* representatives and symbionts of Pentastirini planthoppers. Within this clade, GS-C was sister to *Buchnera aphidicola*; however, BI support (PP = 0.75) and ML support (51%) for this relationship were low. The exact positions of the other previously recognized *Adelges* endosymbionts were not certain, although each was placed with confidence within a larger lineage. '*Ca*. Steffania adelgidicola' was firmly placed within a large lineage of insect symbionts, closest to a secondary symbiont from potato psyllid (*Bactericera cockerelli*), *Sodalis glossinidius* from tsetse fly, and '*Ca*. Moranella endobia' from mealybug. '*Ca*. Profftia virida' and '*Ca*. P. tarda' were well-supported sister taxa, and placed with high support within a lineage including several free-living bacteria plus secondary symbionts of aphids and whiteflies.

Phylogenetic analyses of the individual *Gammaproteobacteria* symbiont data sets from *A. tsugae* revealed population-level associations and genetic structure in some, but not others. The GS-A symbiont was present in all populations and most samples, except for Taiwan (Fig. 2A). Genetic variation among populations was low (~ 99.7% pairwise identity, except for the divergence of Chinese samples from all others at ~ 97% similarity). Despite the low variation, symbiont sequences were generally clustered with respect to population and host–insect relationships (Fig. 2 inset). W. North American samples from *T. heterophylla* were nested within an unresolved cluster of Japanese samples from *T. sieboldii*; these in turn were nested within an unresolved cluster of E. North American samples, with the exception that one *T. sieboldii* sample was identical to all but one E. North American sample. Sister to the preceding samples was a clade of Japanese samples from *T. diversifolia*. All North American and Japanese samples clustered in a basal split with the Chinese samples. The GS-B symbiont was detected only in *T. sieboldii* and E. North American populations (Fig. S1). Sequence variation was low (99.9% identity) and phylograms revealed no phylogenetic structure by population; however, some *T. sieboldii* and E. North

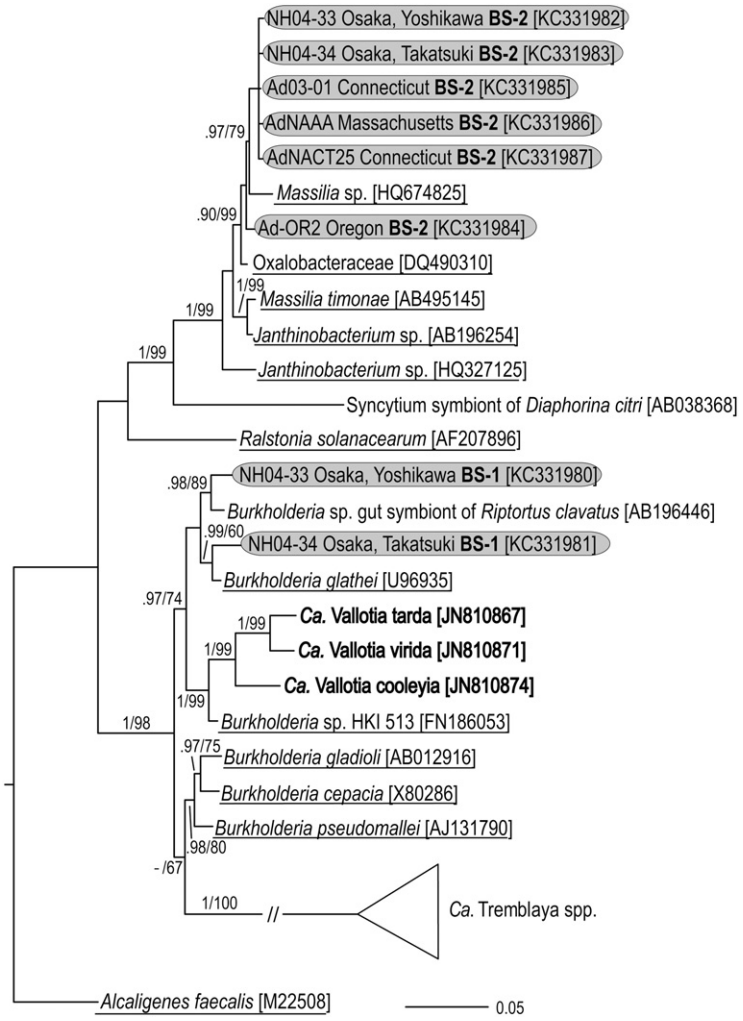

Fig. 3. Phylogeny of *Betaproteobacteria* showing relationships of BS-1 and BS-2 sequences from *A. tsugae* to selected insect endosymbionts and free-living bacteria. Topology shown is the 50% consensus of post-burn-in trees from Bayesian analysis. BS-1 and BS-2 were amplified only from Japanese *T. sieboldii*, and North American populations; other populations yielded no *Betaproteobacteria*. Sequences amplified from *A. tsugae* are highlighted in grey; other adelgid endosymbionts are in bold; free-living bacteria are underlined. Numbers associated with branches indicate Bayesian posterior probabilities/maximum-likelihood bootstrap percentages.

American sequences were identical. The GS-C symbiont was found in all populations sampled, including Taiwan (Fig. 2B). GS-C also exhibited low genetic variation: all sequences from W. North America, E. North America, and *T. sieboldii* were identical. Samples from *T. diversifolia* had one nucleotide difference from the former. Chinese and Taiwanese samples formed a basal split with the former samples.

Phylogenetic analyses of the *Betaproteobacteria* data set placed the two sequence types from *A. tsugae* into two lineages (Fig. 3). The BS-1 sequences clustered within a lineage of *Burkholderia* spp. that included a symbiont from the heteropteran bug, *Riptortus*. BS-1 was detected in only two samples, both from Osaka; these sequences formed independent lineages within a *Burkholderia* subclade (Fig. 3). The BS-2 sequences were amplified only from one W. North American sample, three E. North American samples, and two *T. sieboldii* samples, and exhibited low genetic variation (99% pairwise identity). BS-2 sequences were placed in a lineage including *Janthinobacterium* spp. and *Massilia* spp.; their combined lineage was sister to a psyllid (*Diaphorina citri*) symbiont. Japanese and E. North American samples formed a well-supported monophyletic group sister to *Massilia* sp.; the W. North American sample was placed outside that lineage. Previously discovered '*Ca*. Vallotia spp.' symbionts of other *Adelges* spp. (Toenshoff *et al*., 2012a) composed a well-supported lineage within *Burkholderia*, but were not closest relatives of sequences from *A. tsugae*.

FISH detection and localization of endosymbionts

Fluorescent *in situ* hybridization (FISH) experiments with the general eubacterial probe, 1507r, produced signal in the haemocoel, bacteriocytes, and salivary glands of first-instar stages, and in haemocoel and bacteriomes of later stages (Fig. S1). In some samples, the signal within bacteriomes was restricted to central regions or cells, but in the sample from W. North America, entire bacteriocytes were probed (Fig. S1). This indicates that 1507r did not universally probe all bacteria in *A. tsugae* (specifically, the major bacteriome resident, GS-C; below).

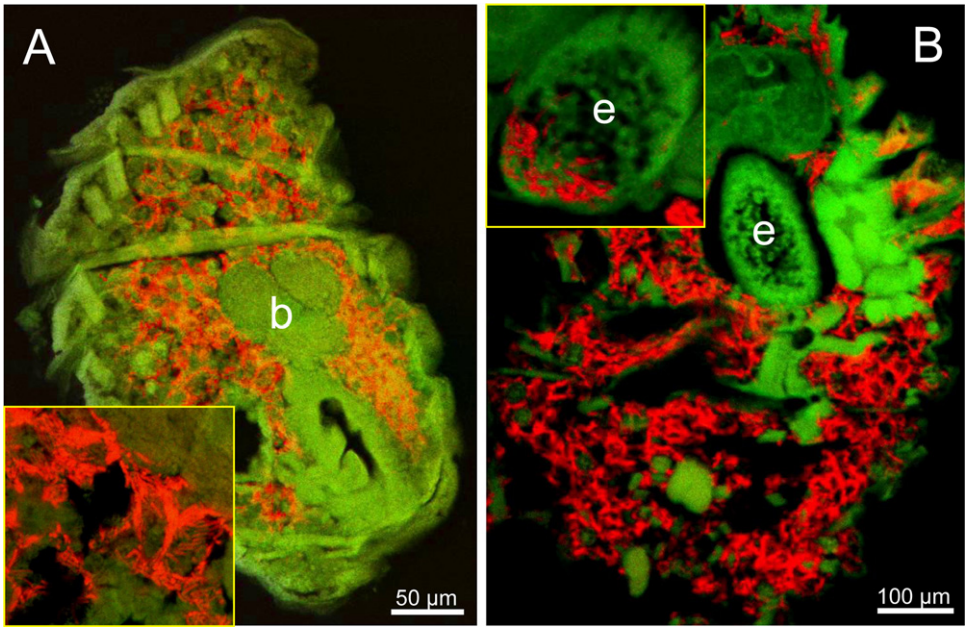

Fig. 4. Laser-scanning confocal images of *A. tsugae* after FISH with the GS-A-specific probe GANA 1118r (labelled with A-568), indicating GS-A ('Ca. Pseudomonas adelgestsugas'; in red) are found only extracellularly in the haemocoel.
A. Eastern North America sample from *T. canadensis* (third instar); inset: high magnification of bacteria.
B. Japanese sample from *T. sieboldii* adult; inset: egg infected with bacteria indicating transovariole transmission.
Green = autofluorescence from insect tissue. b, bacteriome; e, egg.

FISH with GS-A-specific probes yielded signal in the haemolymph, only (Figs 4 and S3). Positive probing was observed in all populations tested. Rod-shaped bacteria were detected in eggs, both *in vivo* and post-oviposition, indicating vertical transmission of this symbiont. Populations of the GS-A symbiont reached high densities within older insect stages, almost completely filling the body cavity in these stages.

Hybridizations with the GS-B probes indicated that this symbiont resided within the bacteriome, but appeared to be restricted to central cells or cell regions of the bacteriome (Fig. 5). The GS-B symbiont was not detected within eggs *in vivo*, but was observed within bacteriocytes clustered at one pole of the egg, strongly suggesting vertical transmission (Fig. 5A, inset). Consistent with the PCR results, GS-B probing was never observed in samples other than *T. sieboldii* and E. North American populations.

Hybridizations performed with the GS-C-specific probes yielded positive probing in all populations tested (Figs 6 and S4). Experiments with first-instar stages detected GS-C in the bacteriomes or salivary glands, or in both of these organs early in development (Fig. 6A and B; Fig. S4A–C and F). In later developmental stages (second instar and older), GS-C appeared to be absent from salivary glands (Fig. 6C and D; Fig. S4D and E). Probing within gravid females revealed signal from GS-C bacteria clustered at one pole of the egg (Fig. 6C), and experiments on freshly laid eggs revealed clusters of bacteria within the egg (Fig. S4A), confirming vertical transmission of GS-C. Older instars with fully developed bacteriomes revealed unprobed areas in the central regions, presumably where GS-B resides (Fig. S4E). Samples from W. North America, however, where GS-B was not detected, showed all bacteriocytes completely filled with GS-C (Figs 6D and S4F).

Double hybridizations with both GS-B- and GS-C-specific probes showed more clearly the pattern of GS-B endosymbionts situated in central regions or cells of the bacteriome, surrounded by regions or cells completely filled with GS-C symbionts (Fig. 7).

FISH experiments with BS-1- and BS-2-specific probes produced signal in the salivary glands, only (Fig. 8). Numerous attempts to detect these bacteria within other regions of the insect, including the bacteriome, failed to yield signal. In addition, positive probing occurred in the first-instar stage only. Numerous attempts to demonstrate vertical transmission of these bacteria by probing within eggs also failed to detect positive signal.

Transmission electron microscopy

We observed three distinct morphological forms of bacterial endosymbionts from immature and adult developmental stages and generations of *A. tsugae* (Fig. 9), which corresponded in form and location within bacteri-

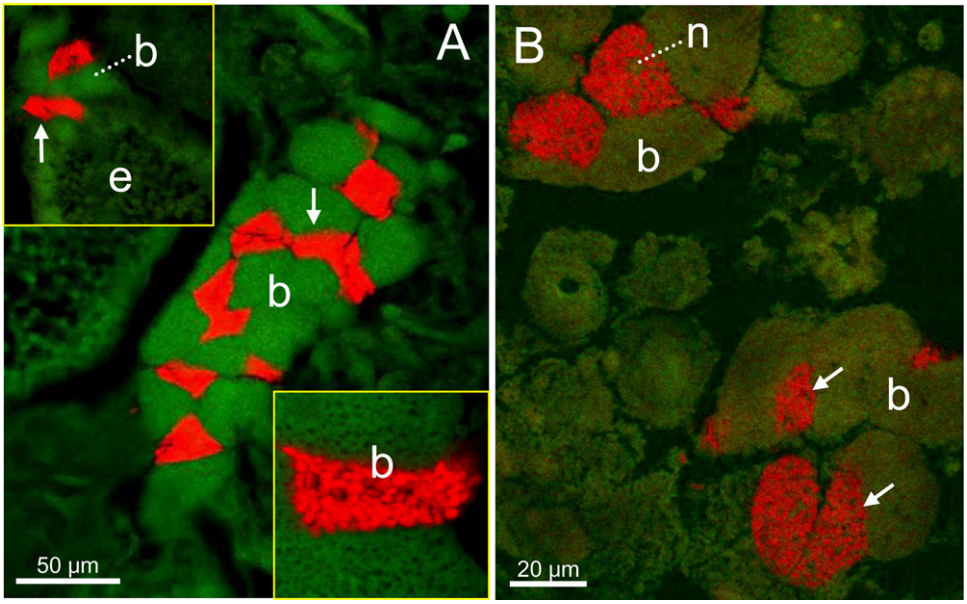

Fig. 5. Laser-scanning confocal images of *A. tsugae* after FISH with the GS-B-specific probe GaB1119r (labelled with A-568) indicating GS-B ('*Ca.* Serratia symbiotica'; in red) are located within bacteriocytes, typically in central cells or regions.
A. Japanese sample from *T. sieboldii* fourth-instar/adult; inset (upper left): ('*Ca.* S. symbiotica' in bacteriocytes (arrow) clustered at one pole of an egg, suggesting the initiation of transovariole transmission; inset (lower right): high magnification of '*Ca.* S. symbiotica' in the bacteriome.
B. Eastern North America sample from *T. canadensis* (third instar) showing '*Ca.* S. symbiotica' occupying whole and partial bacteriocytes (arrows).
Green = autofluorescence from insect tissue. b, bacteriocyte/bacteriome; e, egg; n, bacteriocyte nucleus.

ome or haemocoel to the three gammaproteobacterial species identified above. This work confirmed earlier, preliminary observations (Shields and Hirth, 2005). The morphological form corresponding to GS-C resided within bacteriocytes, mostly those in peripheral regions of the bacteriome; they were 2.1–2.9 µm in diameter and densely packed within bacteriocytes (Fig. 9). Symbionts had a typical Gram-negative cell wall consisting of an inner cytoplasmic membrane (12–20 nm thickness) and an outer membrane (19–27 nm); each was surrounded by a third symbiosome membrane (13–19 nm), which appeared to be tightly bound to the cell wall. GS-C was found in all developmental stages and oocytes, except ovipositing and post-oviposition adults, which lacked intact bacteriocytes. Some bacteriocytes contained only GS-C; others also contained GS-B. GS-B were observed in some bacteriocytes, alone or in conjunction with GS-C (Fig. 9). Where the two bacteria coexisted in bacteriocytes, they were segregated in different areas of the cell, but no membrane boundaries appeared to separate them. GS-B were coccoid, 2.3–3.1 µm in diameter, and many exhibited a central area of dense cytoplasm (Fig. 9). GS-B were also surrounded by a cell wall and symbiosome membrane similar to those enclosing GS-C; they were less densely packed in bacteriocytes than GS-C. GS-B were often seen in bacteriocytes with disrupted cell membranes; they were also found in oocytes, eggs, and all other developmental stages of E. North American *A. tsugae*, except ovipositing and post-oviposition adults; they were never observed in W. North American samples. GS-A were found exclusively in the haemocoel of *A. tsugae*. They were bacilliform, varying from 8.7 to 16.6 µm in length and 1.5–2.3 µm in diameter. Each bacterium was enclosed by two membranes of the cell wall (both 12–21 nm in width). GS-A was found in all developmental stages and oocytes.

Proposed names for Adelges tsugae *endosymbionts*

Based on its low genetic distance to other endosymbionts and the biological attributes described here, for GS-C we propose a new '*Candidatus*' name (Murray and Stackebrandt, 1995), '*Ca.* Annandia adelgestsuga', in honour of P. N. Annand, who described *A. tsugae* and other adelgids in the early 20th century (Annand, 1924; 1928). Because the GS-A symbiont was clearly nested within a lineage of *Pseudomonas*, we propose the name '*Ca.* Pseudomonas adelgestsugas'. As GS-B was placed confidently within the '*Ca.* Serratia symbiotica' clade, we refer to this endosymbiont as '*Ca.* Serratia symbiotica' from *A. tsugae*.

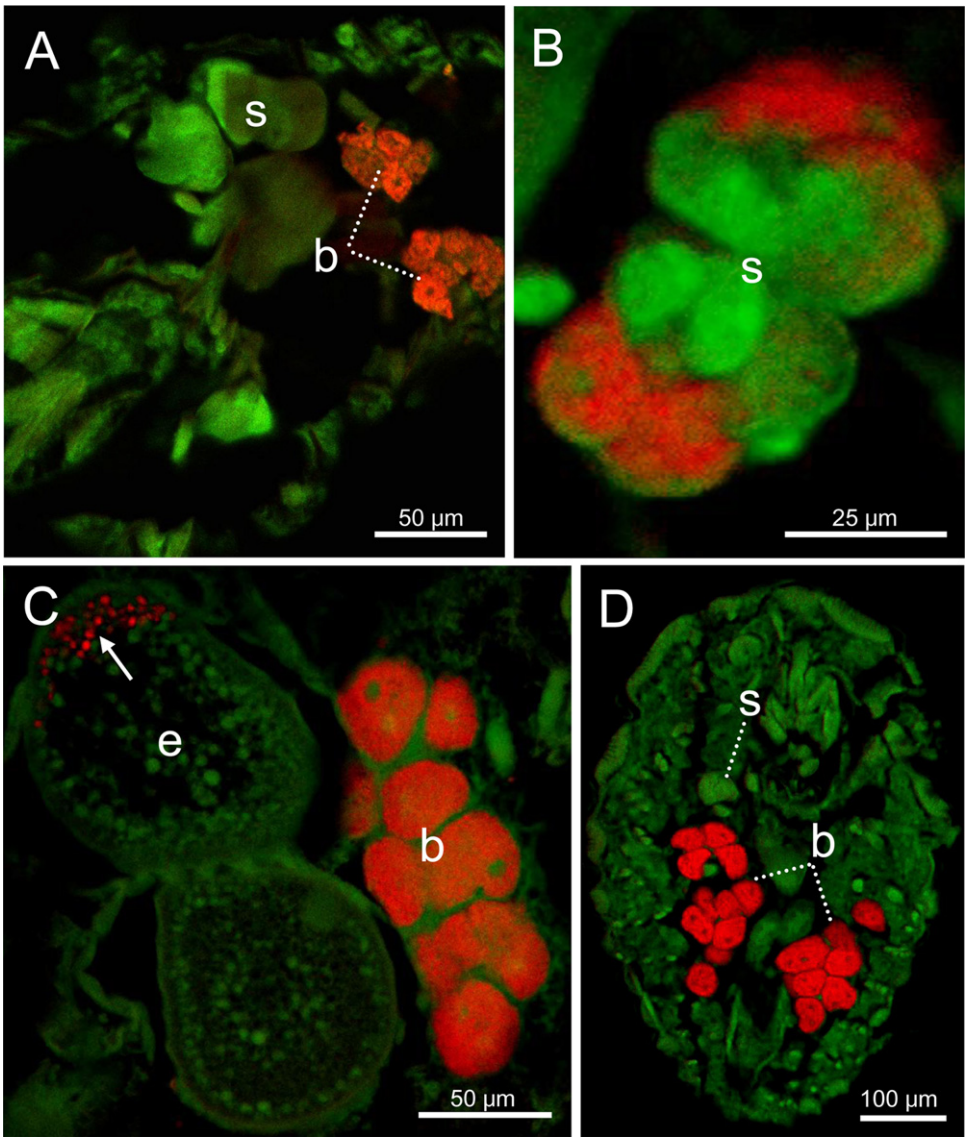

Fig. 6. Laser-scanning confocal images of *A. tsugae* after FISH with the GS-C-specific probe GamC 440r (labelled with A-568), indicating GS-C ('*Ca.* Annandia adelgestsuga'; in red) are harboured in bacteriocytes, are transmitted to eggs, and invade salivary glands in early instars.
A–C. Japanese samples from *T. sieboldii*.
A. First-instar stage showing '*Ca.* A. adelgestsuga' within bacteriocytes of the bipartite bacteriomes; probing not evident in the salivary gland.
B. First-instar stage showing '*Ca.* A. adelgestsuga' in the salivary gland.
C. Adult stage with bacteriocytes packed with '*Ca.* A. adelgestsuga'; some bacteria have infected one pole of an egg (arrow).
D. Western North American sample, third-instar stage showing '*Ca.* A. adelgestsuga' occupying all regions of all bacteriocytes; no probing is evident in salivary glands.
Green = autofluorescence from insect tissue. b, bacteriocyte/bacteriome; e, egg; s, salivary gland.

Discussion

Physical locations and phylogenetic affinities of A. tsugae Gammaproteobacteria *endosymbionts*

Evidence from 16S gene sequences, *in situ* localizations, and transmission electron microscopy documents that Asian and North American populations of *A. tsugae* harbour up to three species of symbiotic bacteria belonging to the *Gammaproteobacteria*. All are transmitted from mother to offspring through eggs. One symbiont, '*Ca.* Annandia adelgestsuga', is universal in all populations sampled, while another, '*Ca.* Pseudomonas adelgestsugas', is present in all but the Taiwan population. However, we were able to analyse only one Taiwan sample; therefore, this finding must be tested with additional samples. The third symbiont, '*Ca.* S. symbiotica', is present only in E. North American and Japanese *T. seiboldii* populations. The universal '*Ca.* Annandia

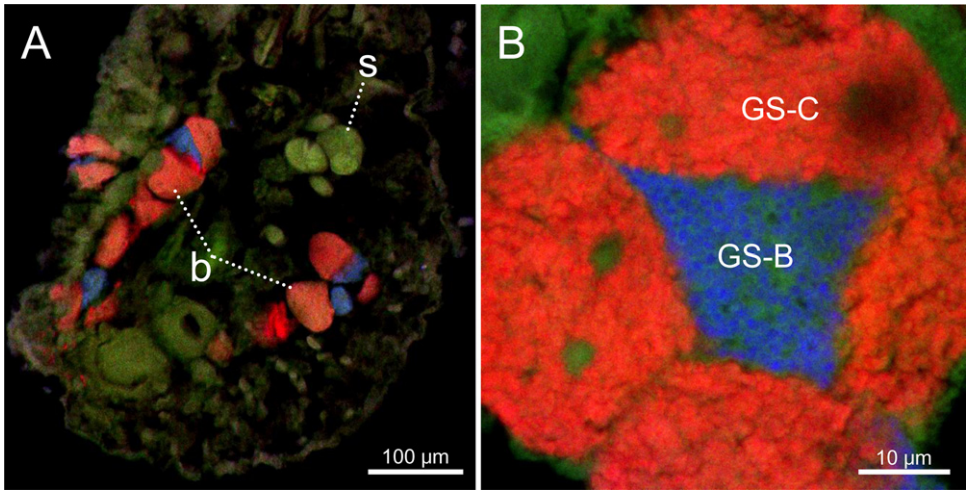

Fig. 7. Laser-scanning confocal images of *A. tsugae* after dual FISH with GS-B-specific probe GamB 1119r (labelled with A-647, in blue) and GS-C specific probe GamC 440r (labelled with A-568, in red) on samples from eastern North America.
A. Third-instar stage showing bipartite bacteriome with probed bacteria; the salivary gland is unprobed. '*Ca*. Annandia adelgestsuga' is more numerous and in more peripheral bacteriocyte regions/cells; GS-B ('*Ca*. S. symbiotica') less numerous and in central regions of the bacteriocytes.
B. High magnification of the bacteriome, illustrating central location of '*Ca*. S. symbiotica' with respect to '*Ca*. A. adelgestsuga' within bacteriocytes and bacteriome.
Green = autofluorescence from insect tissue. b, bacteriocyte/bacteriome; s, salivary gland.

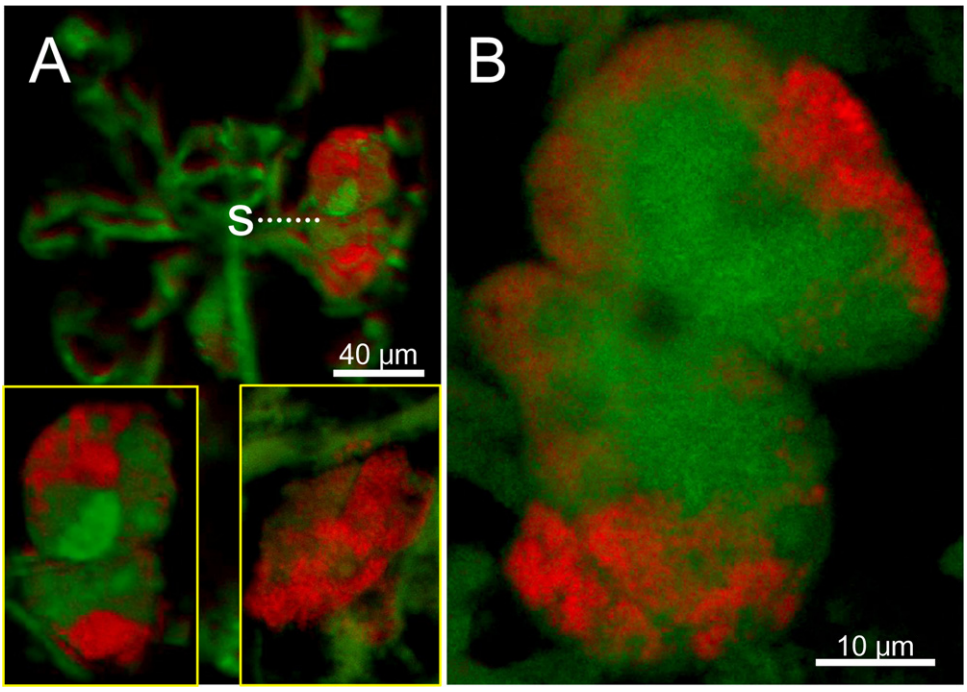

Fig. 8. Laser-scanning confocal images of *A. tsugae* samples from *T. sieboldii* (Osaka) after FISH with *Betaproteobacteria*-specific probes (labelled with A-568).
A. First-instar stage probed with BS-1 mix (BetaA 454r, BetaA 1003r), indicating BS-1 in the salivary gland; insets: higher magnifications of salivary glands.
B. First-instar stage probed with BS-2 mix (BetaB 454r, BetaB 1003r), indicating BS-2 in the salivary gland.
Green = autofluorescence from insect tissue. s, salivary gland.

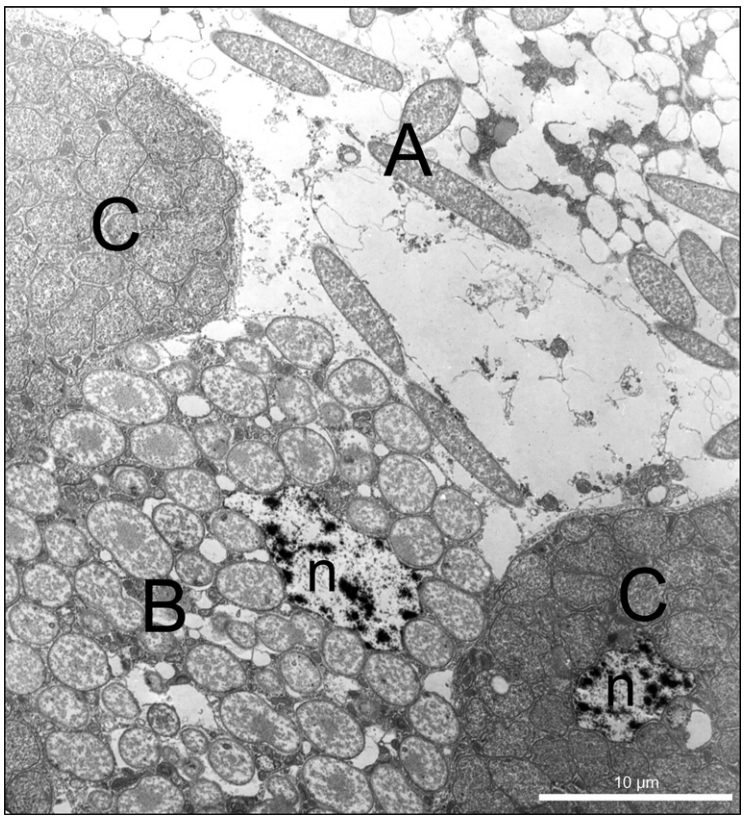

Fig. 9. Transmission electron micrograph of eastern North America *A. tsugae* bacteriocytes and adjacent haemocoel, showing ultrastructure and locations of three forms of bacteria: bacilliform GS-A ('*Ca.* Pseudomonas adelgestsugas') cells (A) living extracellularly in the haemolymph; loosely packed, light-staining coccoid GS-B (*Ca.* Serratia symbiotica') cells (B) contained within central bacteriocytes; tightly packed, dark-staining GS-C (*Ca.* Annandia adelgestsuga') cells (C) housed intracellularly in peripheral bacteriocytes. n, nucleus.

adelgestsuga' is housed intracellularly within bacteriocytes of the bacteriome, and in salivary glands of the *A. tsugae* first developmental stage. The bacilliform '*Ca.* P. adelgestsugas' is free-living in the haemocoel and never contained in host insect cells. '*Ca.* S. symbiotica' is located within bacteriocytes but in comparatively low numbers; when present, it occupies central bacteriocytes, sometimes sharing bacteriocytes with '*Ca.* A. adelgestsuga' but never intermingled. This curious central concentration of '*Ca.* S. symbiotica' in the bacteriomes is also observed in *Cinara cedri* aphids (Gomez-Valero *et al.*, 2004), in which '*Ca.* S. symbiotica' has become co-obligate with *Buchnera* (Lamelas *et al.*, 2011). Notably, this arrangement is also observed in '*Ca.* Ecksteinia adelgidicola' of *A. piceae*, in which interior cells occupied by this symbiont are multinucleated (Toenshoff *et al.*, 2012b). The significance of this arrangement is as yet unclear.

Phylogenetics of gammaproteobacterial symbionts revealed relatively close relationships of *A. tsugae* endosymbionts to other insect endosymbionts; however, none of the *A. tsugae* symbionts was most closely related to those previously reported from other *Adelges* species. Based on its genetic similarity to '*Ca.* Serratia symbiotica' facultative mutualists of aphids, '*Ca.* S. symbiotica' of *A. tsugae* was possibly acquired in the Japanese *T. sieboldii* population through horizontal transfer from aphids, and subsequently introduced with hosts into eastern North America.

'*Ca.* P. adelgestsugas' belongs to a lineage of mostly free-living *Pseudomonas* spp.; this genus is apparently without precedence as symbionts of Sternorrhyncha, although *Paederus* beetles harbour a *Pseudomonas* symbiont (Kellner, 2002). The only other hints of *Pseudomonas* occurrence in Sternorrhyncha are unpublished sequences in GenBank that were reported as isolated from bacteria within *Sitobion miscanthi* aphids in China (GenBank Accessions HM156660, HM156661).

The sister relationship of '*Ca.* Annandia adelgestsuga' to *Buchnera aphidicola*, the primary endosymbiont of aphids, is notable, as Adelgidae and Aphididae are two of three major lineages within Aphidoidea (the third aphidoid lineage, Phylloxeridae, does not contain bacteriome-associated primary endosymbionts (Vorwerk *et al.*, 2007; Medina *et al.*, 2011). It is tempting to speculate that the association of this bacterial lineage with aphidoid insects might date to the common ancestor of Aphididae and Adelgidae, inferred from fossils to be ~150–170 Ma (Heie, 1987; Heie and Pike, 1996; Grimaldi and Engel, 2005). However, we abstain from this interpretation for now, because although '*Ca.* A. adelgestsuga' and *Buchnera* are sister taxa (Fig. 1), their relationship is not statistically supported by BI and only weakly supported by ML.

If the *Buchnera* and '*Ca.* A. adelgestsuga' symbionts had in fact co-evolved from a most recent common ancestor, then we would expect this to be reflected in strong support for their relationship, as has been found in other studies of insect–bacterial co-evolution (Munson *et al*., 1991; Clark *et al*., 2000; Sauer *et al*., 2000; Thao *et al*., 2000; Spaulding and von Dohlen, 2001).

Gammaproteobacteria *endosymbiont population structure, obligate versus facultative status, and origins of the eastern North American* A. tsugae *infestation*

Population-level relationships of *Gammaproteobacteria* symbionts of *A. tsugae* supported the conclusions based on host insect genes with regard to cryptic species within what is currently called *A. tsugae*, and with respect to the Asian source of the *A. tsugae* invasion in E. North America (Fig. 2) (Havill *et al*., 2006; Foottit *et al*., 2009). As found for *A. tsugae* genes, '*Ca.* A. adelgestsuga' sequences from China and Taiwan and '*Ca.* P. adelgestsugas' from China were distinctly divergent from Japanese and North American populations; in '*Ca.* P. adelgestsugas', genetic differences were on the order of those observed between distinct bacterial species. For both '*Ca.* P. adelgestsugas' and '*Ca.* A. adelgestsuga' sequences, Japanese populations from *T. seiboldii* were identical or one substitution different from E. North American samples (W. North American sequences, however, were also identical to J-Ts and E. North America). In the more variable '*Ca.* P. adelgestsugas' sequences, the Ad06-04 sample from Takatsuki, Osaka, on the island of Honshu, was identical to E. North American samples and possibly pinpoints the source of the E. North American introduction to that population. Japanese samples of '*Ca.* P. adelgestsugas' and '*Ca.* A. adelgestsuga' from *T. diversifolia* were always divergent from E. North American samples. In further support of an E. North American introduction from Honshu, sequences of '*Ca.* S. symbiotica' were recovered only from E. North American and Honshu *T. sieboldii* samples.

Long-term, obligate, and vertically transmitted symbionts typically show a pattern of co-speciation with their insect hosts since the original infection (Moran *et al*., 2005). The congruent pattern of relationships in '*Ca.* P. adelgestsugas' and '*Ca.* A. adelgestsuga' symbionts with those of their host insects is thus consistent with a history of host–symbiont co-diversification at the population and/or species level within the *A. tsugae* species complex, and supports a status as obligate symbionts (with the caveat that '*Ca.* P. adelgestsugas' has yet to be found in the Taiwan species). It was surprising, however, that '*Ca.* A. adelgestsuga' exhibited less genetic variation than '*Ca.* P. adelgestsugas', because the former is sequestered intracellularly; such an intimate physical association implies a longer history of association. Long-term endosymbionts typically show accelerated substitution rates and A : T nucleotide compositional bias (Moran, 1996; Lambert and Moran, 1998; Spaulding and von Dohlen, 2001). '*Ca.* A. adelgestsuga' does exhibit biased base composition (55.5% A/T) compared with '*Ca.* P. adelgestsugas' (48.2% A/T). In addition, the '*Ca.* A. adelgestsuga' branch in the *Gammaproteobacteria* phylogeny (Fig. 1) is comparatively long, even longer than the accelerated *Buchnera* lineage. More surprising is that '*Ca.* P. adelgestsugas' shows strict co-evolution with its hosts, because it occurs extracellularly in large numbers in the haemocoel and thus seems more prone to horizontal transfer, e.g. through parasitoids (as may happen with facultative symbionts in aphids (Gehrer and Vorburger, 2012). However, Adelgidae apparently lack parasitoid attackers (Havill and Foottit, 2007), so this might explain why periodic horizontal transmission apparently has not occurred. Population structure, rather than physical location may in fact have more to do with traits typically evolved in maternally transmitted endosymbionts. Genomic changes and rapid evolution seem to be consequences of a long history of association, in which obligate symbiosis and lack of recombination among variants is ensured by faithful maternal transmission (Moran, 2007). The symbiont of plataspid stink bugs (Hemiptera), for example, exhibits accelerated substitution rates and reduced genome size even though it is housed extracellularly in the gut and is transmitted externally when progeny ingest the egg casings (Hosokawa *et al*., 2006).

The sporadic occurrence of '*Ca.* Serratia symbiotica' from *A. tsugae* in only a few Japanese populations on *T. sieboldii* and in eastern North America demonstrates that this symbiont is facultative in *A. tsugae*. This is consistent with intraspecific, population-level patterns of cluster A '*Ca.* S. symbiotica' and other facultative, mutualistic endosymbionts from several aphid species, in which presence and infection rates may vary widely across populations (Tsuchida *et al*., 2006). In contrast, other representatives of the '*Ca.* S. symbiotica' lineage in adelgids, '*Ca.* Ecksteinia' (in *A. piceae*) and '*Ca.* Gillettellia' (in *A. cooleyi*) were consistently present in host insects within and among populations (Toenshoff *et al*., 2012a,b). While the populations sampled were few, this suggests these symbionts may hold co-obligate status, as does the related cluster B member, '*Ca.* S. symbiotica' from *Cinara cedri*.

Hypothesized functions of endosymbionts and possible contributions to virulence of A. tsugae

The roles that gammaproteobacterial symbionts fulfil in their coexistence with *A. tsugae*, and what connection these functions might have to its virulence in E. North

America are so far unknown. Yet, some speculation is possible based on the relationships of these bacteria to endosymbionts of other insects and their documented contributions to hosts. Researchers have remarked on the magnitude of *A. tsugae*'s effects on hemlocks and its lethality compared with a co-occurring introduced pest, elongate hemlock scale (*Fiorinia externa*) (Radville *et al*., 2011; Gómez *et al*., 2012) with informal speculations that endosymbionts might play a part in these effects. The relative toxicity of *A. tsugae* to hemlock has been attributed to salivary components (Radville *et al*., 2011), which we hypothesize could be regurgitated bacteria (or their products) from the salivary glands. This hypothesis makes sense given that the lethality of *A. tsugae* appears to be related to induction of a hypersensitive response and systemic induction of H_2O_2, which is a well-known response of plants to pathogens such as bacteria (Dixon *et al*., 1994).

The striking presence of '*Ca.* P. adelgestsugas' cells packed in high numbers in the haemocoel (Fig. 4) certainly suggests an important function in the bacterial–insect partnership. *Pseudomonas* spp. are unprecedented as symbionts of Sternorrhyncha. However, a *Pseudomonas* sp. symbiont produces a powerful defensive toxin in *Paederus* beetles (Piel *et al*., 2004). This suggests the idea that '*Ca.* P. adelgestsugas' might play a defensive role for *A. tsugae*, with enhanced effectiveness in a novel geographic region (E. North America), where predators are naïve and maladapted to a new toxin or other defence. Another hypothesis is that '*Ca.* P. adelgestsugas' might amplify *A. tsugae*'s feeding damage. Young and colleagues (1995) suggested that detrimental effects of *A. tsugae* on hemlocks might be due to factors other than direct feeding, for example, through effects of some toxin. Feeding by *A. tsugae* causes dramatic increases and compositional changes of amino acids in the tree (Gómez *et al*., 2012) and the formation of false rings that inhibit water transport (Gonda-King *et al*., 2012). Possibly, '*Ca.* P. adelgestsugas' produces a substance related to these impacts.

We speculate that '*Ca.* S. symbiotica' could play a role in adelgids similar to that in aphids, perhaps contributing to the successful invasion and spread of *A. tsugae* on *T. canadensis* in E. North America. In aphids, this symbiont is involved in mitigating effects of high temperatures, in endoparasitoid defence, and in expanding host plant range (Montllor *et al*., 2002; Russell and Moran, 2006; Oliver *et al*., 2010). The southern Honshu source population may be especially adapted to high temperatures because of the presence of '*Ca.* S. symbiotica', making the pest populations in E. North America especially virulent in the south. In addition, invasion of E. North America involved a host-plant shift (from *Tsuga seiboldii* to *T. canadensis* and *T. caroliniana*), and perhaps '*Ca.* S. symbiotica' assisted in the transition to these new host plants through other unknown mechanisms.

We hypothesize that the major bacteriome resident, '*Ca.* A. adelgestsuga', may fulfil a role in *A. tsugae* related to nutrition, as does *Buchnera aphidicola* and other obligate symbionts of Sternorrhyncha. As the primary endosymbiont of aphids, *Buchnera* supplements the incomplete diet of its host by overexpressing genes coding for amino acids that are scarce or absent in its phloem-sap diet (Moran and Degnan, 2006). In contrast to aphids, *A. tsugae* feeds on parenchyma cells of the xylem rays (Young *et al*., 1995). These cells might constitute a more complete diet for *A. tsugae* than sugar-rich but nitrogen-poor phloem. What essential nutrients might be missing from this diet and whether '*Ca.* A. adelgestsuga' assists in supplying them remains to be studied.

Perhaps the most surprising result of this study was the presence of '*Ca.* A. adelgestsuga' in salivary glands of early developmental stages (Figs 6 and S4). By the second instar, '*Ca.* A. adelgestsuga' disappears from the salivary gland, and presumably is either eliminated there or migrates to the bacteriome. What function '*Ca.* A. adelgestsuga' may perform in the salivary glands is unknown; however, their location in the settling instar hints that these bacteria might aid in establishment at a feeding site.

To our knowledge, the documentation of endosymbiotic, and presumably mutualistic, bacteria residing both in the bacteriome and in the salivary glands (transiently) is unprecedented in Sternorrhyncha. Given our results, however, investigations for endosymbionts in salivary glands of other sternorrhynchans should be pursued. The claims of Haracsi (1938) hint that the situation in *A. tsugae* might be found in other Aphidoidea [although Buchner (1965) dismissed his findings]: in *Pemphigus* aphids, Haracsi observed that the salivary gland and bacteriome differentiate during embryogenesis from the same syncitial tissue, and for a significant part of embryonic development exist in close proximity to each other. He suggested that microbes in the salivary gland are the same as symbionts of the bacteriome. Haracsi thought that the role of salivary gland symbionts might be to furnish their insect host's saliva with enzymes such as cellulase and hemicellulase to aid in digestion of plant tissue as well as in gall formation.

Other types of bacteria in various insect hosts have been documented in salivary glands and other tissues; these appear to be mostly cases of plant pathogenic bacteria within insect vectors, or bacteria manipulating reproduction. Examples include *Spiroplasma* and *Phytoplasma* plant pathogens (Fletcher *et al*., 1998; Kwon *et al*., 1999; Ammar and Hogenhout, 2005; Marzorati *et al*., 2006; Hogenhout *et al*., 2008), *Wolbachia* and *Cardinium* (Bacteroidetes) reproductive manipulators (Cheng

and Aksoy, 1999; Mitsuhashi *et al*., 2002; Chen *et al*., 2005; Marzorati *et al*., 2006), and Rickettsiae-like organisms of unknown function (Thomopoulos *et al*., 1991). In tsetse flies, the secondary endosymbiont, *Sodalis*, but not the primary endosymbiont, *Wigglesworthia*, is found in salivary glands as well as other tissues (Cheng and Aksoy, 1999; Aksoy and Rio, 2005). The *Sodalis* genome indicates functional adaptations intermediate between a free-living and mutualistic lifestyle (Toh *et al*., 2006); however, its role in salivary glands is unknown.

The *Betaproteobacteria* BS-1 and BS-2 variants are more puzzling residents of *A. tsugae*. BS-1 was more closely related than BS-2 to the *Betaproteobacteria* symbionts of other Adelgidae ('*Ca*. Vallotia spp.'), but was not a member of their exclusive lineage and thus does not share a recent common ancestor (Fig. 3). Both BS-1 and BS-2 were found only in salivary glands, but were apparently restricted to immature developmental stages (Fig. 8). Samples from which these bacterial sequences were PCR-amplified included adult and egg stages, but these were also mixed with early instars. Despite repeated FISH experiments on all stages, positive signal was found only in first instars, and never in bacteriomes or eggs. Although unlikely, it is possible that these bacteria are transmitted to eggs *in vivo*, but in such low numbers that they were not detected. Given the apparent lack of cross-generational transmission, it is surprising that sequences of BS-2 from *T. seiboldii* on southern Honshu were identical or nearly so to E. North American and one W. North American sample. We can hypothesize two possible scenarios to explain these findings: (i) BS-2 is an environmental *Betaproteobacteria* sp. associated with *Tsuga* in both Japan and North America, and is acquired by *A. tsugae* as it feeds, or (ii) BS-2 was introduced to E. North America from Japanese populations and has spread (possibly through feeding) on *Tsuga* in North America along with *A. tsugae*.

The apparently limited incidence of BS-1 (found only in two Osaka samples) suggests that this bacterium is perhaps rarely acquired from the environment. The ecological role of *Burkholderia glathei*, closest to the BS-1 NH04-34 sample, is unknown. However, the BS-1 NH04-33 sample was closely related to a *Burkholderia* midgut symbiont of the broad-headed bug [*Riptortus pedestris* (= *R. clavatus*)] (Kikuchi *et al*., 2005). These bugs acquire *Burkholderia* symbionts from the environment in each generation, and symbionts significantly increase several measures of fitness in their hosts (Kikuchi *et al*., 2007). Numerous heteropteran species in five families of two sister superfamilies harbour a diversity of *Burkholderia* midgut symbionts, which are interrelated with environmental and gall-forming strains (Kikuchi *et al*., 2011). Notably, soil-derived pesticide-degrading strains of *Burkholderia* can establish symbioses with *R. pedestris* experimentally (and naturally with a chinch bug, *Cavelerius saccharivorus*) and confer pesticide resistance to their hosts (Kikuchi *et al*., 2012).

Evolutionary patterns of bacterial endosymbiosis in Adelgidae

Prior to this study, Toenshoff and colleagues (2012a,b) found that each of four *Adelges* species complexes harboured two types of maternally transmitted bacterial endosymbionts. Three species (*A. abeitis/viridis, A. laricis/tardus, A. cooleyi/coweni*) each contained a *Betaproteobacteria* and a *Gammaproteobacteria* interspersed in the same bacteriocytes. *A. nordmannianae/piceae* harboured two *Gammaproteobacteria*, each located in separate bacteriocytes. Relationships of *Betaproteobacteria* symbionts ('*Ca.* Vallotia spp.') mirrored those of their hosts (Havill *et al*., 2006). *Gammaproteobacteria* of *A. abeitis/viridis* and *A. laricis/tardus* ('*Ca.* Profftia' spp.) were sister taxa, consistent with their hosts' relationships in Adelgidae phylogeny. '*Ca.* Gillettellia cooleyi' and '*Ca.* Ecksteinia adelgidicola' from *A. cooleyi/coweni* and *A. nordmannianae/piceae* were also sister taxa. The position of the second symbiont in *A. nordmannianae/piceae* ('*Ca.* Steffania adelgidicola') was unresolved in their *Gammaproteobacteria* phylogeny, which lacked resolution in deeper relationships.

The pattern of endosymbiosis in *A. tsugae* found here is most similar to *A. nordmannianae/piceae*: '*Ca.* Vallotia sp.' is absent, and a member of the '*Ca*. S. symbiotica' lineage is a secondary endosymbiont. Unlike the previously explored adelgids, however, *A. tsugae* harbours up to three vertically transmitted symbionts, and two belong to novel lineages. In addition, '*Ca*. S. symbiotica' of *A. tsugae* does not share a most recent common ancestor with '*Ca.* Gillettellia cooleyi' and '*Ca.* Ecksteinia adelgidicola' within the '*Ca*. S. symbiotica' lineage, and thus represents an independent acquisition of this symbiont. Our results therefore further support the conclusion that endosymbiont evolution in adelgids has been a complex history of acquisition and replacement of (presumed) obligate and facultative symbionts. Toenshoff and colleagues (2012a) present one parsimonious evolutionary hypothesis of symbiont infection and replacement, wherein the ancestor of adelgids was infected by an ancestral '*Ca.* Vallotia sp.' and a common ancestor of '*Ca.* Gillettellia'/'*Ca.* Ecksteinia'; in certain lineages, '*Ca.* Vallotia' was replaced by '*Ca*. Steffania' and *S. symbiotica* by '*Ca*. Profftia'. Under this hypothesis, our results suggest that the *A. tsugae* lineage would have undergone a replacement of '*Ca.* Vallotia' by '*Ca*. Annandia', a loss of '*Ca.* Gillettellia'/'*Ca.* Ecksteinia' and gain of another member of the '*Ca*. S. symbiotica' lineage in Japanese populations, as well as an acquisition of '*Ca*. P. adelgestsugas'. As

Toenshoff and colleagues (2012a) note, several alternative scenarios are also plausible. A complete history of bacterial symbiosis in adelgids will be best understood with information on symbiont composition in additional species, as well as genomic information to illuminate the different ages of bacterial–host associations (Toenshoff *et al*., 2012b).

Experimental procedures

Taxon sampling, gene amplification, cloning, and sequencing

Adelges tsugae were sampled from populations in eastern North America, western North America, Japan, Taiwan, and China (Table S1). Whole-genomic DNA was extracted from whole insects using a protein salting-out protocol (Sunnucks and Hales, 1996). Bacterial small-subunit ribosomal 16S rRNA genes were amplified from whole-genomic preparations by polymerase chain reaction (PCR) with the general eubacterial primers, 10f or 27f (both forward primers) and 1495R (reverse), or in some cases 340f or 341f for the forward primer (Table S2); multiple forward primers were used to increase the probability of amplifying all possible bacteria. PCRs were prepared in 20 μl vols and contained 1 mM dNTPs, 20 mM 10× buffer (Roche Diagnostics GmbH, Mannheim, Germany), 1 mM of each primer, 1 unit of *Taq* DNA polymerase (Roche or GenScript Corp., Piscataway), 1 μl of GeneProtein32 (Ambion, Austin), and 20 ng of whole-genomic DNA. Reaction cycles for the PCRs amplified with the general eubacterial forward and reverse primers were 94°C for 3′, then 35× (94°C 1′, 55°C 1′, 72°C 1′) with a final extension at 72°C for 10′. A 1500 base pair (bp) band containing the nearly full-length sequence of the 16S rRNA gene was the target product.

PCR products were cloned using the Topo TA Cloning Kit (Invitrogen, Carlsbad) according to manufacturer's instructions. A PCR analysis was conducted to check for correct-length inserts (instructions provided with kit) on 10–50 colonies using the vector primers M13 forward and M13 reverse. The resulting PCR products of correct lengths were purified using an isopropanol-ammonium acetate purification method prior to sequencing.

16S rRNA products obtained either through general eubacterial PCRs and cloning, or through specific PCRs (see below), were first sequenced with the forward amplification primer, or M13 forward for cloned products, using the ABI Prism Terminator Cycle sequencing v2.0 Ready Reaction kit, or Dynamic ET Terminator Cycle sequencing kit (Amersham Biosciences, Piscataway) and products were visualized on an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City) or an ABI Prism 3100. Sequences were aligned with Sequencher 4.1 (Gene Codes Corp., Ann Arbor). Ten to 50 colonies per cloning reaction were assayed. Unique sequences were identified from alignments of all test sequences, and single clones or specific PCR products representing unique molecules were sequenced fully. Primers for sequencing were the same as those used for PCR. Additional internal primers, 341f, 766f, 570r and 810r were used to obtain complete sequences in both directions (Table S2). Sequence fragments of endosymbiont rRNA genes were joined into full-length regions (contigs) with Sequencher. All unique contigs were entered into nucleotide BLAST searches in GenBank and to the Classifier analysis tool at the Ribosomal Database Project II (RDPII) website to obtain a general classification of bacterial endosymbiont types. Final sequences were deposited in the GenBank public database.

Once putative endosymbionts were identified as being present in certain populations, new samples from these populations were screened to ascertain the presence of these bacteria using highly specific bacteria primers (specific PCRs), thus circumventing the cloning step. Sequences and orientation of the specific primers used for this screening method are listed in Table S2. For *Betaproteobacteria* endosymbionts, highly specific primers could not be developed. However, general *Betaproteobacteria*-specific primers were designed and used to preferentially enrich the PCR for betaproteobacterial 16S rRNA gene copies; PCR products were then cloned. Annealing temperatures for PCRs with specific primers were varied according to the melting temperature of the primers.

During initial cloning and sequencing work using primers 340f and 1495r, a three-way chimeric sequence was encountered, which contained a ~ 450 bp central segment with a unique bacterial 16S rRNA sequence. Forward and reverse primers specific to this ~ 450 bp region were designed: GamC 476f and GamC 847r (Table S2) and were used in specific PCRs to ascertain the presence of this putative endosymbiont (later designated 'GS-C') in representative individuals from all populations. Partial 16S rRNA from this endosymbiont was amplified in two segments: the first was amplified with general eubacterial forward primer 340f in combination with specific reverse primer GamC847r, and the second was amplified using specific forward primer Gam C476f and the general eubacterial reverse primer 1495r. PCR products were then purified and sequenced directly. Interestingly, the general eubacterial primers, 8f, 10f and 27f, which are routinely used to amplify 16S rRNA genes from all lineages of Eubacteria, failed to amplify the 5′ end of this endosymbiont sequence.

Phylogenetic analysis

After sequence comparisons in GenBank and RDPII, we determined that *A. tsugae* endosymbiont sequences belonged to the *Gammaproteobacteria* and *Betaproteobacteria* lineages. Sequences with closest matches and other representative sequences from these lineages were downloaded from GenBank to prepare alignments for phylogenetic analysis. Several data sets were prepared: the first included single exemplars from three *Gammaproteobacteria* isolated from *A. tsugae*, other gammaproteobacterial insect endosymbionts and free-living bacteria. The second included all sequences of *Betaproteobacteria* isolated from *A. tsugae* and selected betaproteobacterial insect endosymbionts and free-living bacteria. Each data set of unaligned sequences was submitted to the CLUSTALW Multiple Sequence Alignment server at GenomeNet (Kyoto University Bioinformatics Center; http://www.genome.jp/tools/clustalw/). Short regions of ambiguous alignment were removed by eye. The resulting alignments were analysed to determine the relationships of

A. tsugae endosymbionts to known bacteria and other insect endosymbionts. Three additional alignments were prepared consisting of all variants of each gammaproteobacterial endosymbiont from all sampled populations, and one or more out-groups. These alignments were used to examine population-level structure in sequence variation. Because sequence variation was low, these simple alignments were compiled using Sequencher.

Phylogenetic analyses were conducted under maximum parsimony (MP) for single-endosymbiont alignments, and under BI and ML for the *Gammaproteobacteria* and *Betaproteobacteria* multi-species alignments. MP analyses were performed with Paup* 4.0 (Swofford, 2002), with all sites weighted equally during 500 random-addition heuristic searches. BI was conducted with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using the best-fit model of evolution (GTR + gamma) as determined with MrModeltest 2.2 (Nylander, 2004). Two separate analyses were run with four chains (three heated and one cold) for 10 000 000 generations, sampled every 10 000 generations. Convergence, stationarity, and appropriate burn-in for each analysis were assessed with Tracer v1.5 (Rambaut and Drummond, 2009), and posterior probabilities for each node were calculated from the collection of post-burn-in trees with MrBayes. ML bootstrap analyses were performed with GARLI v2.0 (Zwickl, 2006) at the GARLI Web Service, http://www.molecularevolution.org) using the GTR + gamma model, and with RAxML 7.0.4 at the CIPRES Science Gateway Black Box interface (http://phylobench.vital-it.ch/raxml-bb/) using the GTRCAT model to perform bootstrapping, followed by an ML search using the GTRGAMMA model (Stamatakis, 2006; Stamatakis *et al*., 2008).

In situ *hybridization and confocal microscopy*

FISH were carried out on E. North American, W. North American, and JP samples. Experiments were performed with oligonucleotide probes designed to be specific to the different putative endosymbiont sequences amplified from *A. tsugae* samples. Specific probes were designed from sequence alignments compiled from 16S rRNA sequences derived from all representative populations of *A. tsugae* as well as other related *Gammaproteobacteria* and *Betaproteobacteria* 16S rRNA sequences from GenBank, including free-living bacteria and other endosymbionts. Probes were designed from regions of the 16S rRNA molecule where a secondary structure model of *Escherichia coli* 16S rRNA (Fuchs *et al*., 1998) indicated that binding should be successful, and where sequences differed from other bacteria from the same sample. Probes were named according to their equivalent positions in the *E. coli* gene (Table S2).

FISH was conducted on insects of various developmental stages preserved in acetone and stored at –80°C, or in 95–100% ethanol and stored at –20°C. Fixation and sectioning methods were adapted from Ausubel and colleagues (1996), Chapter 14.1–14.2, and Fukatsu and colleagues (1998). Whole insects were fixed in a 4% formaldehyde solution, infused in a 0.5 M sucrose solution overnight, then embedded in Tissue Freezing Medium (Fisher Scientific, Denver), and frozen at –22°C. Blocks were cut into 12–14 μm thick serial cryo-sections using a Riechert-Jung Cryocut 1800, in transverse, frontal, or sagittal orientations. Sections were transferred to Fisherbrand Superfrost Plus slides (Fisher Scientific) and allowed to air-dry for 45 s to 2 min.

Hybridization methods were adapted from Fukatsu and colleagues (1998), Komminoth (1996) and Zarda and Schleifer (1996). After air-drying, the slides were incubated sequentially in Coplin jars containing 4% formaldehyde in 1× PBS (5 min), 3× PBS (3–5 min) and 1× PBS (3–5 min). For minute insect sections, the 3× and 1× PBS steps were occasionally omitted to improve section retention, and excess 4% formaldehyde solution was simply wiped off the slide. In such cases, slides were subjected to two washes with probe-free 1× hybridization solution (HS) prior to application of the HS as described below. Slides were processed for FISH within 2 h of sectioning. Specific probes, as well as the general eubacterial primer 1507r, were labelled with Chromatide Alexa-568-5-dUTP (A-568) (Molecular Probes, Eugene) according to the manufacturer's instructions. We also purchased a manufacturer-labelled Alexa647 GamC_440 (A-647) probe directly from Molecular Probes for experiments that involved double probing with the Gamma-C endosymbiont (see *Results*). To hybridize probes to bacterial rRNA, room-temperature sections were bathed in 20 μl of HS without probe, aspirated, then covered and sealed with PC20 CoverWell incubation chambers (Grace Bio-labs, Bend, OR) containing 20 μl of 1× HS with labelled probe. To ensure specificity, formamide concentration in HS was adjusted for T_m of the probes. Probes were used singly, as well as in mixtures to increase the signal in hybridizations. HS with single probes or mixtures of probes were held at 50 pmol ml^{-1} total probe concentration. The hybridization was conducted at 42°C for 16 h, after which slides were washed first in 2× SSC (sodium chloride-sodium citrate buffer) and then in 1× phosphate-buffered saline (PBS), then mounted with a coverslip in 1× PBS. Specificity of hybridization was confirmed with negative controls: no-probe, RNase digestion, and competitive suppression with excess unlabelled probe (Fukatsu *et al*., 1998; von Dohlen *et al*., 2001). A positive control was conducted with 1507r labelled with Alexa_568 dUTP. Visualization of the hybridized probes was achieved on a Bio-Rad MRC1024 laser-scanning confocal microscope using lasers with wavelengths of 488 nm, 568 nm, and 647 nm.

Transmission electron microscopy

Fresh, whole insects from populations on *T. canadensis* from Connecticut, USA and *T. heterophylla* from Oregon, USA, were prefixed in 3% glutaraldehyde, 4% paraformaldehyde and 2% acrolein in 0.1 M sodium cacodylate buffer overnight, then postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h. After ethanol dehydration, samples were stained with uranyl acetate, dehydrated, and embedded in Spurr's resin. Ultrathin sections from the abdominal region were prepared with an ultramicrotome, mounted on collodion-coated copper meshes, stained with uranyl acetate and lead, and examined at 60 kV with a Zeiss 902 transmission electron microscope.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Collection and voucher information for samples used in this study. All host genera are *Tsuga*.
Table S2. Primers and probes used in this study.
Fig. S1. Maximum-parsimony phylogram of *Gammaproteobacteria* GS-B ('*Ca*. Serratia symbiotica') sequences amplified from Japanese *T. sieboldii* populations from the island of Honshu and from eastern North American populations; all other samples yielded no amplifications of that phylotype. Numbers above branches indicate inferred substitutions along the branch. GenBank accessions are indicated in brackets.
Fig. S2. Laser-scanning confocal images from FISH with the general eubacterial probe 1507r (labelled with A-568).
A. Japanese sample from *T. sieboldii* first-instar stage, showing probed bacteria (in red) in both the body cavity and salivary glands. Inset: high magnification of a probed salivary gland.
B. Same sample as in A, but different insect; no differentiated bacteriome is visible.
C. Japanese sample from *T. sieboldii*, fourth-instar or adult stage, showing probed bacteria in the haemocoel and the central region of a bacteriome; insets: high magnification of bacteriome-dwelling bacteria and haemocoel bacteria.
D. Western North America sample from *T. heterophylla*, third-instar stage cross-section, showing probed rod-shaped bacteria (in red) in the haemocoel and completely filling several bacteriocytes.
Green = autofluorescence from insect tissue. b, bacteriome, s, salivary gland, h, haemocoel, n, bacteriocyte nucleus.
Fig. S3. Laser-scanning confocal images of *A. tsugae* after FISH with the GS-A-specific probe GANA 1118r (labelled with A-568), indicating GS-A (*Pseudomonas adelgestsugas*; in red) are found only extracellularly in the haemocoel.
A. Japanese sample from *T. sieboldii* (first instar) showing rod-shaped bacteria in the posterior body cavity.
B. Western North America sample from *T. heterophylla* (second instar).
C. Japanese sample from *T. sieboldii* egg showing bacteria free in the posterior cavity and clustered around developing bacteriome (arrows).
D. Japanese sample from *T. diversifolia* egg, showing bacteria in the posterior region. Green = autofluorescence from insect tissue. b, bacteriome; h, haemocoel.
Fig. S4. Laser-scanning confocal images of *A. tsugae* after FISH with the GS-C-specific probe GamC440r (labelled with A-568), indicating GS-C ('*Ca*. Annandia adelgestsuga'; in red) are harboured in bacteriocytes, are transmitted to eggs, and invade salivary glands in early instars.
A–C, samples from Japan.
A. Sample from *T. sieboldii*, egg stage, showing '*Ca*. A. adelgestsuga' clustered in two regions of one pole; not all

bacteria appear to be contained within discrete bacteriocytes at this stage (arrow).
B. Sample from *T. diversifolia*, first-instar stage showing '*Ca*. A. adelgestsuga' in both bacteriome and salivary gland.
C. Sample from *T. diversifolia*, first-instar stage, showing '*Ca*. A. adelgestsuga' in bacteriocytes.
D–F, samples from North America.
D. Sample from eastern North America, second-instar stage, showing '*Ca*. A. adelgestsuga' in most areas of the bacteriome; some areas are unprobed (arrow).
E. Sample from eastern North America, third-instar stage showing '*Ca*. A. adelgestsuga' in most areas of the bacteriome; other areas are unprobed (arrows).
F. Sample from western North America, first-instar stage, showing '*Ca*. A. adelgestsuga' in both bacteriome and salivary gland (arrow).
Green = autofluorescence from insect tissue. b, bacteriocyte/bacteriome, s, salivary gland.