The pine bark adelgid *Pineus strobi* contains two novel bacteriocyte-associated gammaproteobacterial symbionts

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Abstract

Bacterial endosymbionts of the pine bark adelgid Pineus strobi (Insecta: Hemiptera: Adelgidae) were investigated using transmission electron microscopy, 16S and 23S rRNA-based phylogeny, and fluorescence in situ hybridization. Two morphologically different symbionts affiliated with the Gammaproteobacteria were present in distinct bacteriocytes. One of them (‘Candidatus Annandia pinicola’) is most closely related to an endosymbiont of Adelges tsugae suggesting that they originate from a lineage already present in ancient adelgids before the hosts diversified into the two major clades, Adelges and Pineus. The other P. strobi symbiont (‘Candidatus Hartigia pinicola’) represents a novel symbiont lineage in members of the Adelgidae. Our findings lend further support for a complex evolutionary history of the association of adelgids with a phylogenetically diverse set of bacterial symbionts.

Introduction

Ten percent of all investigated insects harbour bacterial symbionts which serve an essential function by supplying nutrients to their hosts (1–4). These obligate (primary) symbionts are vertically transmitted from mother to offspring and usually reside in specialized host cells, the bacteriocytes. This long-term association led to co-speciation of symbionts and insect hosts. One of the best-studied obligate symbionts is Buchnera aphidicola, which infected an ancestor of modern aphids (Insecta: Hemiptera: Aphididae) more than 180 million years ago (1, 5, 6) and provides its insect partner with essential amino acids lacking in the host diet (7–9). Moreover, facultative (secondary) symbionts co-residing in bacteriocytes or located in other tissues were recognized in various insects such as whiteflies, psyllids and aphids (10–13). Facultative symbionts provide protection against heat stress and natural enemies, but may also be involved in host nutrition (1, 14, 15).
Adelgids (Insecta: Hemiptera: Adelgidae), comprising the two major clades Adelges and Pineus, live exclusively on various conifers where they feed on parenchyma cell sap or phloem (16). To date, bacteriocyte-associated symbionts of only few Adelges species have been investigated on the molecular level (17–19). In contrast to their aphid sister group, these insects harbour surprisingly diverse symbionts belonging to the Gammaproteobacteria and the Betaproteobacteria. While aphids generally contain a single obligate symbiont that coevolved with its host (1), adelgids seem to have a more complex evolutionary history involving multiple symbiont acquisition and replacement events (17–19).

The pine bark adelgid Pineus strobi (Hartig 1837) feeds on the outer tissue of the phloem of pines (Pinus spp.; 16, 20). Early histological studies demonstrated coccoid and polymorphic symbiont morphotypes in single and multinucleated bacteriocytes, respectively (21, 22). Here we investigated whether P. strobi harbours symbionts that are phylogenetically related to known symbionts of other adelgids of the Adelges clade, or whether distinct symbionts were acquired during evolution.

Methods

Sampling

Exules (parthenogenetic life stages on secondary host trees among adelgids; 16) and eggs of two natural adelgid populations were sampled from Pinus spp. (Table S1, Fig. S1). Samples were stored in 96% ethanol for DNA purification, or fixed for electron microscopy and fluorescence in situ hybridization.
Ultrastructure analysis

Transmission electron microscopy (TEM) was conducted with individuals of both insect populations. Insects were prefixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C, fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 hours at room temperature. Subsequently specimens were dehydrated with 2,2-dimethoxypropane and embedded in low viscosity resin (Agar Scientific). Ultra-thin sections were stained with uranyl acetate and lead citrate and were examined with a ZEISS EM 902 electron microscope at 80 kV.

PCR, cloning and sequencing

DNA of up to 20 individuals from each insect population was extracted using the DNeasy Blood and Tissue Kit (Qiagen) or a Chelex-based method (23). Altogether eight extractions were done. For the identification of the insects, the partial cytochrome c oxidase subunit 1 (coI) and the partial nuclear elongation factor 1-alpha (ef1alpha) genes were amplified and sequenced with the primers listed in Table S2. For the identification of the symbionts, 16S and 23S rRNA gene sequences were amplified with general bacterial primers, cloned and sequenced. PCR reaction mixtures typically contained 1 Unit of Taq DNA polymerase (Fermentas); 50 pmol of each primer, 10x Taq buffer with KCl; 2mM MgCl₂; 0.2mM of each deoxynucleotide and up to 5 µl template DNA in a final volume of 50 µl. PCR conditions were as follows: 95 °C for 4 min; 35 cycles of 95 °C for 45 s, annealing for 45 s, 72 °C for 1.5-2 min; and 72 °C for 10 min. For one of the symbionts (phytotype 2), initially only a partial 16S rRNA sequence could be obtained using the 909f and Ba1492R primers together with Phu DNA polymerase (New England Biolabs) under the following conditions: 98°C for 3 min; 35 cycles of 98°C for 30 s, 52°C for 30 s, 72°C for 30 s; and 72°C for 5 min. PCR cocktail contained 25 µl Phu HF master mix, 0.5 µM of each primers, 3% DMSO and 3 µl of DNA template in a final volume of 50 µl. PCR products were
cloned with TOPO TA and TOPO XL cloning kits (Invitrogen Life Technologies) and sequenced with vector specific primers. For phylotype 2, additional symbiont-specific primers (listed in Table S2) were employed in standard PCR reactions to obtain a near full-length 16S rRNA gene sequence. Sequencing was performed using an ABI 3130 XL genetic analyzer and the BigDye Terminator kit v3.1 (ABI).

**Phylogenetic analyses**

The obtained 16S and 23S rRNA sequences were submitted to BLASTn similarity searches against the GenBank database (24). Sequence alignments were performed and manually curated by using the software package ARB (25). Only alignment positions conserved in at least 50% of all sequences were considered in phylogenetic analyses. Three approaches were used for phylogenetic reconstruction. We used maximum likelihood (ML) in PhyML v. 3.0 (26), Bayesian inference (BI) by MrBayes v. 3.2.1 (27) with the best-fit model of evolution (GTR+I+G) selected by JModelTest 2.1.3 (28). Six gamma categories were used. PhyML estimated nucleotide frequencies, the gamma shape parameter and the proportion of invariable sites; and optimized tree topology using a BIONJ tree as a starting tree. In MrBayes, two independent analyses were run from different random trees until they reached stationarity and convergence (average standard deviation in split frequencies were lower than 0.01). Trees resulted by the first 25% of generations were discarded. Additionally, we employed a non-stationary non-homogenous model implemented in nhPhyML which allow variable substitution rates and base composition among lineages (29). Bayesian and maximum likelihood trees were used as input for the analyses. Transversion/transition ratio and gamma shape parameter were estimated by nhPhyML; five equilibrium frequency categories were employed.
The obtained col and ef1alpha sequences were concatenated and analysed with a reference dataset using ARB (25), PhyML (26), and MEGA (30) as described (17; Fig. S2).

Fluorescence in situ hybridization

To confirm the identification of the two symbionts and to correlate observed phylotypes with morphotypes fluorescence in situ hybridization (FISH) was performed on eggs and exules of both populations as described earlier (18). Briefly, samples were fixed in 4% paraformaldehyde for 4 h at 4°C. Symbiont-specific probes were designed with the program ARB and were used together with a general bacterial probe. Specificity of probes was tested with increasing formamide concentrations in the hybridization buffer. Probe sequences and optimal formamide concentrations are listed in Table S2. Samples were hybridized for at least 1.5 hours and were analysed on the same day with a laser scanning confocal microscope (Zeiss LSM 510 Meta and Leica TCS Sp8).

Results

Ultrastructure of bacteriocyte-associated symbionts in Pineus strobi

Two natural adelgid populations were sampled from pine trees (Table S1). Phylogenetic analyses of concatenated col and ef1alpha sequences indicated that the adelgids from both populations were affiliated to P. strobi of the family Adelgidae (Fig. S2). TEM analysis revealed bacteriocytes located in proximity to the gut in the insect abdomen (Fig. 1A). Two morphologically different symbiont types, coccoid and polymorphic were located in distinct densely packed bacteriocytes. Morphologically similar symbionts have recently been described for Adelges nordmannianae/piceae (18). The polymorphic and the coccoid morphotype had a length of 1.8 - 5.2 µm and 0.9 - 3.6 µm, respectively. Coccoid bacteria showed an electron-
translucent granular cytoplasm, while the cytoplasm of the polymorphic symbionts was more homogenous and electron-dense (Fig. 1). Both bacterial morphotypes were surrounded by a Gram-negative type cell wall and by a host-derived membrane, the so-called symbiosome membrane (Fig. 1C, D, and E). Similarly to other intracellular symbionts of insects (31), cell-wall structure of the polymorphic symbiont seemed to be reduced, as it was enclosed by three layers corresponding to the inner and outer membranes and the symbiosome membrane, respectively (Fig. 1E). In case of the coccoid symbiont, a fourth layer was apparent between the inner membrane and the outer membrane, possibly representing peptidoglycan (Fig. 1D). In addition, membrane vesicles were present between the outer membrane of the coccoid symbionts and the symbiosome membrane (Fig. 1C, D). Such vesicles are known from diverse Gram-negative bacteria including pathogens and are released from the outer membrane of the bacteria. They play an important role in growth, reproduction, bacterial stress response, and may act as vehicles for bacterial toxins, for cell-cell communication, nutrient acquisition, and inhibition of phagosome-lysosome fusion and immune recognition (32–34).

Two distinct gammaproteobacterial symbionts

Two 16S rRNA gene sequence types were recovered, and both were almost identical between the two insect populations (99.3-100% sequence similarity). Sequence type 1 showed highest similarity to various Providencia rettgeri strains (95.5-95.7%), isolated from corals (35) or pathogenic to silkworms (36) among others. Phylotype 2 was most similar to ‘Candidatus Annandia adelgestsuga’ (94.9%), a bacteriocyte-associated symbiont of the hemlock woolly adelgid A. tsugae (19). Both sequence types showed only low sequence similarity (89.2-90.7% for type 1, and 83.4-84.9% for type 2) to bacteriocyte-associated gammaproteobacterial
symbionts of other adelgids including *A. nordmannianae/piceae*, *A. abietis/viridis*, *A. laricis/tardus*, and *A. cooleyi/coweni*.

In general, different tree calculation methods resulted in partly inconsistent tree topologies within as well as between the 16S rRNA and 23S rRNA-based analyses (Fig. 2), which is in agreement with previous reports on the phylogeny of free-living and symbiotic bacteria among the *Gammaproteobacteria* (37–40). However, 16S rRNA analyses clearly demonstrated the affiliation of sequence type 1 to *Providencia rettgeri;Providencia vermica* isolated from an entomopathogenic nematode (41), *Moellerella wisconsensis* found in human stool specimens and *Arsenophonus nasoniae* (90.5-90.7% sequence similarity), a facultative symbiont in parasitoid wasps and whiteflies (42) (Fig. 2A). No close relationship was found to symbionts of other adelgids or any other sternorrhynchan insects.

Phylotype 2 and ‘*Candidatus Annandia adelgestsuga*’ grouped together with strong support in each analysis and appeared as a long branch nested in a clade containing gut symbionts of plataspid stinkbugs (*Candidatus Ishikawaella capsulata*); (43) and acanthosomatid stinkbugs (*Candidatus Rosenkranzia clausaccus*); (44), *Buchnera aphidicola*, and ‘*Candidatus Purcelliella pentastirinorum*’, a bacteriome-associated symbiont in cixiid planthoppers (45). Within this clade their relationship to other insect symbionts could not be resolved with confidence. They represented a sister clade of ‘*Candidatus Purcelliella pentastirinorum*’, but their relationship was not well established (BI=88%, ML=55%). While phylogenetic analysis of ‘*Candidatus Annandia adelgestsuga*’ by von Dohlen et al. (19) suggested *B. aphidicola* as closest relative, its position was, similar to our results, only weakly supported. In agreement with the 16S rRNA data, 23S
rRNA-based phylogenies of phylotype 1 and 2 suggested an affiliation to *Providencia* sp., and 'Candidatus Purcelliella pentastirinorum', respectively (Fig. 2B).

**In situ localization of 'Candidatus Hartigia pinicola' and 'Candidatus Annandia pinicola'**

The two bacterial morphotypes observed by transmission electron microscopy were readily detected by FISH with 16S rRNA and 23S rRNA-targeted oligonucleotide probes specific for each of the two phylotypes (Fig. 3). The obtained 16S and 23S rRNA sequences could thus be assigned to the coccoid (phylotype 1) and the polymorphic symbionts (phylotype 2), respectively. All bacteria within the bacteriocytes were stained with either of the symbiont-specific probes, demonstrating the absence of additional bacteria. Both symbionts were detected in the exulis life stage (Fig. 3A and B) as well as in eggs (Fig. 3C), suggesting their vertical transmission from mother to offspring as known for other adelgid symbionts.

The low degree of phylogenetic relationship to other bacteria requires classification of the coccoid *Pineus strobi* symbiont in a novel genus within the *Gammaproteobacteria*. Given its similarity to 'Candidatus Annandia adelgestsuga' (19), the polymorphic symbiont likely represents a novel species in the candidate genus *Annandia* within the *Gammaproteobacteria*. We thus propose two novel tentative names according to Murray and Stackebrandt (46).

‘Candidatus Hartigia pinicola’

‘Hartigia’, in honour of the entomologist Theodor Hartig, who first described *Pineus strobi* in 1837; ‘pin-icola’, friend or lover of pine. This bacterial endosymbiont of *Pineus strobi* is coccoid with a cell size between 0.9 and 3.6 µm, has a Gram-negative type cell wall and is surrounded by a symbiosome membrane within bacteriocytes. ‘Candidatus Hartigia pinicola’ (also referred to as
phytotype 1 in this study) represents a novel genus within the class *Gammaproteobacteria*
(phylum *Proteobacteria*). Basis of assignment: 16S rRNA and 23S rRNA genes
(GenBank/EMBL/DDBJ accession numbers KC764415, KC764416, KC764419).

‘*Candidatus Annandia pinicola*’

‘Annandia’, referring to the close phylogenetic relationship shared with ‘*Candidatus Annandia adelgestsuga*’ (19); ‘pin-icola’, friend or lover of pine. This symbiont of *Pineus strobi* is polymorphic with a cell size between 1.8 and 5.2 µm, has a Gram-negative type cell wall and is surrounded by a symbiosome membrane within bacteriocytes. ‘*Candidatus Annandia pinicola*’
(also referred to as phylotype 2 in this study) represents a novel species of the candidate genus *Annandia* (19) within the class *Gammaproteobacteria* (phylum *Proteobacteria*). Basis of assignment: 16S rRNA and 23S rRNA genes (GenBank/EMBL/DDBJ accession numbers KC764417, KC764418, KC764420).

**Discussion**

‘*Candidatus Hartigia pinicola*’ (phytotype 1) and ‘*Candidatus Annandia pinicola*’ (phytotype 2) represent the first symbionts identified in a member of the *Pineus* clade of the Adelgidae. Both symbionts are affiliated to the *Gammaproteobacteria*, are localized in bacteriocytes and are vertically transmitted from mother to offspring.

Interestingly, ‘*Candidatus Annandia pinicola*’ formed a well-supported monophyletic group with the *Adelges tsugae* symbiont ‘*Candidatus Annandia adelgestsuga*’ (19)(Fig. 2). Thus these two symbionts likely originate from an ancient symbiont already present in the ancestral adelgids before they diversified into the two major lineages, *Adelges* and *Pineus*, ~88 million years ago.
A long-term association between adelgids and this symbiont lineage is also supported by long branches of *Annandia* symbionts in phylogenetic analyses, their universal occurrence in *A. tsugae* (19) and *P. strobi* populations and congruent phylogeny observed between ‘*Candidatus* Annandia adelgestsuga’ and its host populations earlier (19). However the relationship of ‘*Candidatus* Annandia pinicola’ and ‘*Candidatus* Annandia adelgestsuga’ to other insect symbionts remains uncertain. One of their closest relatives is *B. aphidicola*, which might indicate their origin from a symbiont harbored by the ancestor of adelgids and their aphid sister group, but this hypothesis is not well supported by the current data and needs further investigations including the analyses of additional genes and adelgid species.

‘*Candidatus* Hartigia pinicola’, the second symbiont of *Pineus strobi* represents a novel symbiont lineage among adelgids, which is not closely related either to known symbionts of adelgids or any other sternorrhynchan insects such as aphids, psyllids, scale insects or whiteflies. Whether this symbiont is obligatory for the host insect or occurs occasionally as a facultative symbiont is unknown. Nevertheless, given its sequence similarity (>95% at the 16S rRNA level) to free-living bacteria and its well-preserved cell wall it might represent a more recent association with *P. strobi*, which fits well to previous observations suggesting a complex evolutionary history of adelgids and their symbionts (17–19).

Each adelgid species investigated so far contained two bacteriome inhabiting symbionts (17–19), similar to the situation seen among many members of the suborder Auchenorrhyncha (Insecta: Hemiptera). Planthoppers, leafhoppers, treehoppers, cicadas and spittlebugs share an ancient symbiont ‘*Candidatus* Sulcia muelleri’ (*Bacteroidetes*) (48) which typically co-occurs with different symbiont lineages in major insect groups, for instance with ‘*Candidatus* Baumannia
cicadellinicola’ in sharpshooters (49) or ‘Candidatus Zinderia insecticola’ in spittlebugs (50) respectively. These joint symbionts are co-obligatory and coevolved with their respective hosts.

The role of adelgid symbionts in host ecology is still unknown. However, taking all available data together (17–19), a picture is beginning to emerge in which, compared to other plant-sap sucking insects, the diversity of bacteriocyte-associated symbionts of adelgids is much larger (Fig. 4). So far, seven symbiont lineages were identified among six adelgid species (17–19), which suggest multiple symbiont acquisition and symbiont replacement events during the evolution of adelgids (Fig. 4). One clear example of symbiont replacement has been demonstrated among three species complexes previously (17). *A. cooley/coweni, A. laricis/tardus* and *A. abietis/viridis* underwent co-speciation with a betaproteobacterial symbiont lineage (‘Candidatus Vallotia’). However, they recruited additional symbionts from two different gammaproteobacterial lineages, suggesting the replacement of ‘Candidatus Gillettellia cooleya’ still found in *A. cooley/coweni* by ‘Candidatus Proftia tarda’ and ‘Candidatus Proftia virida’ in *A. laricis/tardus* and *A. abietis/viridis*, respectively (Fig. 4).

Symbiont replacement has also been found in other insect groups, e.g. in weevils of the family Dryophthoridae (51), and spittlebugs in the tribe Philaenini (52). Similarly, acquisition of a novel symbiont has been reported for aphids, where a former facultative symbiont partly took over the nutritional function of the long-term associated *B. aphidicola* (53, 54). The acquisition of novel symbionts by adelgids during their evolution might have helped to assure survival and to invade new niches e.g. by expanding the host range or by allowing the use of different food sources (phloem and parenchyma cell sap). Additional analysis of further *Pineus* species with respect to their bacterial symbionts as well as genome sequence analyses of known symbionts will provide...
further insights into the evolution of this symbiosis and the role of the bacterial symbionts in these associations.

Acknowledgements

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Figure legends

Figure 1. Ultrastructure of bacteriocyte-associated symbionts of Pineus strobi. (A, B) Ultra-thin sections of the adelgid abdomen showing two symbiont morphotypes located in distinct bacteriocytes. (C, D) The coccoid (phytotype 1) and (E) the polymorphic (phytotype 2) symbionts show a typical Gram-negative type cell wall and are surrounded by a symbiosome membrane. (C) Coccoid symbiont dividing by binary fission. (D) Vesicles are present between bacteria and symbiosome membrane. The cell wall of this symbiont includes three layers likely corresponding to outer membrane, peptidoglycan, and inner membrane. (E) The polymorphic symbiont is enclosed by three membrane layers corresponding to the inner and outer membranes and the symbiosome membrane. Bars in A and B represent 2 µm; bar in C represents 1 µm; bar in D represents 200 nm, bar in E represents 500 nm. g = gut, b = bacteriocytes, ms = membrane stacks, s = symbiont, cm = cell membrane, sm = symbiosome membrane, cw = bacterial cell wall, v = vesicles.

Figure 2. Phylogenetic relationships of the bacteriocyte-associated symbionts of Pineus strobi with the Gammaproteobacteria. (A) A 16S rRNA-based and (B) a 23S rRNA-based Bayesian tree are shown. Symbionts of adelgids are indicated by grey boxes. Nodes with <50% Bayesian posterior probability were collapsed. Bayesian support values and >50% maximum likelihood bootstrap values (1000 replicates) are indicated on internal nodes. Nodes labelled with asterisks were also supported by a non-homogenous and non-stationary model implemented in nhPhyML using the Bayesian and maximum likelihood trees as starting tree. Bars represent the number of changes per site. GenBank/EMBL/DDBJ accession numbers are given in squared
brackets. Selected members of the *Alphaproteobacteria* were used as outgroup [NC_002678, NC_011988, NC_002978, NC_006142, NC_009883], which is indicated by the arrow.

Figure 3. In situ identification of ‘*Candidatus Hartigia pinicola*’ and ‘*Candidatus Annandia pinicola*’ in different life stages (exulis, egg) of *Pineus strobi*. Bacterial symbionts were labelled by using symbiont-specific 16S and 23S rRNA-targeted oligonucleotide probes together with a general bacterial probe (Table S2). Probes specific for phylotype 1 and phylotype 2 were labelled with Cy5 (blue) and Cy3 (red), respectively. The general bacterial probe (EUB338-I) was double-labelled with FLUOS (green). All probes were used simultaneously. The combined signal from the general and the symbiont-specific probe appear blue-green for ‘*Candidatus Hartigia pinicola*’ (coccoid symbiont, phylotype 1) and yellow to orange for ‘*Candidatus Annandia pinicola*’ (polymorphic symbiont, phylotype 2). (A) Bacteriocytes of *P. strobi* at the exulis life stage visualized by 16S rRNA-targeted symbiont-specific probes HarPi-265, and AnnPi-327. (B) Bacteriocytes in the exulis life stages visualized by 23S rRNA-targeted symbiont-specific probes HarPi-378, and AnnPi-1439. (C) Symbionts inside a *P. strobi* egg (probes HarPi-265 and AnnPi-1439). Bars represent 10 µm.

Figure 4. Diversity and multiple acquisition and replacement events of bacteriocyte-associated symbionts in the insect family Adelgidae. A schematic representation of host and symbiont phylogeny is shown. Host phylogeny and divergence time points indicated in dark grey are based on a concatenated data set of *mtDNA* and *ef1alpha* and were taken from Havill *et al.* (47). Colored lines represent known symbionts of adelgids and aphids. Symbiont phylogeny is based on 16S and 23S rRNA analyses and was partly taken from Toenshoff *et al.* (17, 18) and von Dohlen *et al.* (19). Capital letters indicate the estimated divergence time points of the
Adelgidae (in millions of years ± standard deviation; D = 88 ± 14.09, F = 65.05 ± 12.03, G = 60 ± 11.84, I = 55 ± 11.67). (Adapted from reference 47 with permission from Elsevier.)