universität wien

DISSERTATION

Titel der Dissertation

Diversity and ecology of bacteriocyte-associated symbionts in adelgids (Hemiptera: Adelgidae)

Verfasserin

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angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer. nat.)

Wien, 2012

Studienkennzahl lt. Studienblatt:	A 091 444
Dissertationgebiet lt. Studienblatt:	Ökologie
Betreuerin / Betreuer:	Univ.-Prof. Dr. Matthias Horn

“An enemy today may be a friend tomorrow.”

Hajime Ishikawa

"We are symbionts on a symbiotic planet, and if we care to,
we can find symbiosis everywhere."

Lynn Margulis

“Those who have much are often greedy, those who have little always share.”

Oscar Wilde

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Chapter I

Introduction & Outline

Symbiosis – an alliance with major evolutionary impact

A wide array of interactions among multicellular organisms and microorganisms occurs in nature. Some of these relationships are characterized by a physical association over a longer period of time during their lives. In 1879, Heinrich Anton de Bary, a German botanist, coined the term 'symbiosis' (from Greek *sým* "with" and *bíōsis* "living"). He defined it as two or more species living together in close association with each other, typically with one on or within the body of the other (De Bary, 1879). The definition of the term symbiosis is still controversially discussed. In this thesis I will follow de Bary's definition subdividing the umbrella term symbiosis in parasitic (benefit for only one of the involved partner on cost of the other), commensal (benefit for one partner, but neutral to the other) and mutualistic (benefit for both involved partners) relationships. However, with regard to the fitness effects on the host, symbiotic relationships can often be everything from beneficial to harmful and thus the spectra of associations often converge.

In the last century, the research in microbiology focused mainly on pathogenic bacteria, which are often parasitic causing various diseases in eukaryotic hosts. For example, *Chlamydiae*, phylogenetically well-separated bacteria, live exclusively in eukaryotic cells since several hundreds of millions of years (Wagner and Horn, 2006; Horn *et al.*, 2004). They are known as important animal and human pathogens (www.chlamydiae.com) as well as energy parasites using utilized ATP generated by the host via ATP/ADP transport proteins (Schmitz-Esser *et al.*, 2004; Tjaden *et al.*, 1999; Winkler and Neuhaus, 1999). *Chlamydiae* exhibit a broad host range and they are characterized by a biphasic developmental cycle (AbdelRahman and Belland, 2005; Horn, 2008). The phylogenetic diverse phylum *Chlamydiae* contains ~250 published 16S rRNA gene sequences >1000 nucleotides and comprises eight different families. It is divided into the pathogenic *Chlamydiaceae* including *Chlamydia trachomatis* (responsible for Trachoma and genital tract infections) and *Chlamydophila pneumoniae* (a causative agent of pneumonia) and the symbiotic *Chlamydia*-like bacteria (so-called environmental chlamydiae). The latter contain 7 families occurring in various hosts such as protozoa, worms, arthropods, crustaceans, fish, urine of fruit bats, mammals and as contaminant of a human cell line (Horn, 2008). It is hypothesized that the *Chlamydia*-like bacteria are new emerging pathogens e. g. due to their potential role in respiratory infections of humans (Haider *et al.*, 2008; reviewed in (Horn, 2008)), but a causal link has not yet been established (Horn, 2008). Free-living amoebae, especially acanthamoebae, are frequent hosts of chlamydiae and are important for survival and dispersal of chlamydiae in the environment. These amoebae are discussed to act as training ground for intracellular bacterial pathogens (Molmeret *et al.*, 2005) and thus may play a role in *Chlamydia*-like bacteria turning into new pathogens.

In the last years the research focus changed towards more non-parasitic/non-pathogenic investigations, realizing the importance of commensal and mutualistic symbioses on earth. One example is the so-called gut microbiota (microbiota= all microbial lineages that live in a particular environment), which is comprised of commensal and mutualistic bacteria living in the gut of mammals. They outcompete pathogenic bacteria for nutrients and space, and have great impact on the health of their hosts (Kranich *et al.*, 2011; Marchesi, 2010; Chow *et al.*, 2010). The microbiota is involved in metabolism and digestion as well as in the development of a functional immune system, and necessary for the prevention of certain diseases such as diabetes, asthma, rheumatoid arthritis and colitis (Kranich *et al.*, 2011). Numerous studies are illuminating this complex association and show an interrelation of the gut microbiota with the host phylogeny, diet and gut morphology (Ley *et al.*, 2008).

One of the most prominent symbiotic associations is the emergence of the first eukaryotic cell more than 1,450 million years ago as a consequence of the acquisition of the mitochondrial ancestor cell, a respiring alphaproteobacterium. Different models explaining this crucial evolutionary event are currently under debate, which can be separated in two main classes: (1) either a nucleus-bearing amitochondrial precursor cell acquired an alphaproteobacterium or (2) the fusion of an alphaproteobacterium with an archaeon followed by the acquisition of eukaryotic features (Lang *et al.*, 1999). Most recent theories postulate an archaeon as the origin of the eukaryotic cell, which is as well supported by comparative genome analyses. Most genes for replication, transcription and translation are related to archaeal homologues whereas genes encoding for metabolism and biosynthetic functions are related to bacterial homologues (Lang *et al.*, 1999; de Duve, 2007; Vesteg and Krajcovic, 2008). Nevertheless, it is still unknown if the homology of the eukaryotic genes to the *Archaea* is based on a direct descendent or due to a common ancestor. Thus, the origin of the eukaryotic cell still remains unclear. Later in evolution (>1,200 million years ago) the acquisition of a cyanobacterium forming the chloroplasts took place resulting in the origin of plant cells (Embley and Martin, 2006).

Mutually beneficial symbiotic interactions such as the evolution of the eukaryotic cell allow the exploitation of otherwise inaccessible niches and therefore trigger the emergence of diversity and colonization of the Earth. The identification of the symbiotic partners and their interactions with each other as investigated by genomic analyses is an essential step forward to the understanding of symbiotic systems (e. g. reviewed in (Chaston and Goodrich-Blair, 2010)). For example, the knowledge about microbial symbioses may change some day the management of certain diseases that includes both associated partners such as patient and microbiota (Nicholson *et al.*, 2005). However, invertebrates played an important role for the understanding of symbiotic interactions since they served as model systems due to their

great diversity and the tendency to interact with a relatively low number of bacterial symbionts compared to e. g. humans. For the reason of a better understanding of symbiotic relationships, this thesis focuses mainly on the investigation of a rather unexplored insect family and the identification of their essential obligate bacterial symbionts.

Insects – a multicolored majority with impact

Currently, there are approximately 920,000 unique insect species described (Figure 1; Grimaldi and Engel, 2005). Due to their associations with other organisms e.g. plants and animals, insects are the most successful group of higher organisms on this planet (Purvis and Hector, 2000; Labandeira and Eble, 2000), if diversity can be regarded as a measure of success.

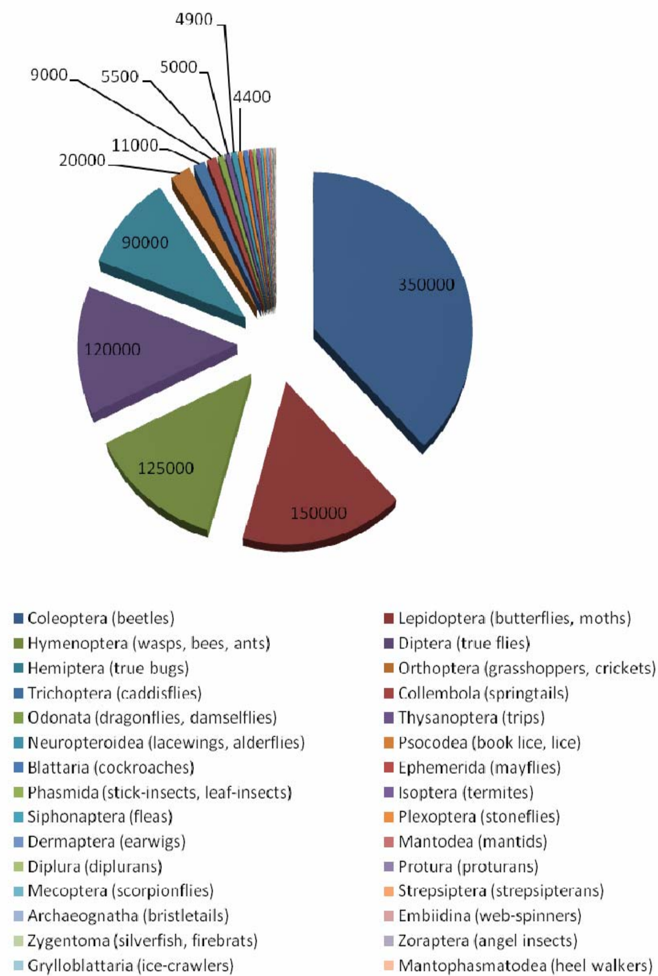

Figure 1. The pie chart depicts the currently described insect species classified in higher taxa (mainly orders). Different taxa and orders are colour-coded. Data taken from Grimaldi & Engel (2005).

Insects play an important role in nature by holding essential functions such as pollination, since 80% of all flowering plants are pollinated by insects (bees, butterflies, flies, ants). Furthermore, they take part in the decomposition, nutrient cycling and aeration of the soil (dung beetles, flies, cockroaches, termites). Various animals e.g. reptiles, birds, fish, and mammals, consume insects making them essential parts of the food chain. Additionally, insects such as ladybird beetles and parasitoid wasps can play a vital role in population control since they act as predators, parasites or parasitoids of other insects, arthropods and vertebrates. They are of economic importance by helping to produce commercial products (silk, honey) and several can be used as indicators for the health status of an ecosystem (www.savenature.org).

Furthermore, insects can also be severe pests in agriculture and forest ecosystems. They transmit pathogenic viruses and bacteria to plants and animals. For example, plant-feeding homopterans (whiteflies, leafhoppers, aphids) transmit more than 80% of insect-transmitted viruses that comprise ~400 virus species (Fereres and Moreno, 2009). Additionally, insects are of medical importance operating as vectors for several human diseases such as malaria, dengue fever, typhus and Q fever (www.savenature.org).

Identifying the players

The impact of symbiotic interactions on life on planet earth illustrates the importance of symbiosis research. In the early 20th century, symbiotic associations between bacteria and insects were histological intensively studied by light microscopy (Buchner, 1953). Insect-symbiont associations are difficult to maintain under laboratory conditions compared to culturable model organisms such as *Escherichia coli*, *Arabidopsis thaliana* and *Drosophila melanogaster* (Moran, 2006). However, more recently applied culture-independent molecular tools such as 16S rRNA gene sequencing, high-throughput genomic and transcriptomic sequencing, proteomics and metabolomics, as well as single-cell techniques such as fluorescence *in situ* hybridization, high-resolution microscopy, Raman microspectroscopy and secondary ion mass spectrometry are providing unexpected and new insights into the ecology, biology and evolution of such symbiotic consortia (Wagner and Haider, 2012; Wagner *et al.*, 2006; Walker and Parkhill, 2008; Stoecker *et al.*, 2010; Wagner, 2009; Amann *et al.*, 1993; Margulies *et al.*, 2005; Croucher *et al.*, 2009; Zhang *et al.*, 2006).

Illuminating the diversity of heritable symbionts in insects

Associations between bacteria and insects are widespread in nature. Most of the research in the symbiosis field is focusing on the transmission of pathogens, but in respect to the impact and importance of heritable (vertically and horizontally transmitted) symbionts for the insect host and therefore for the ecosystem on earth, the study of symbiont diversity, function and interaction with the host is of great importance and may represent an elegant tool for insect pest control (Bourtzis and Miller, 2006). An estimated ten percent of all insects contain 'nutritional' symbionts, which are considered as an important driving force in the evolution of their hosts. These symbionts enable the hosts to occupy or dominate ecological niches and habitats that might otherwise be unavailable and thereby influence the hosts ecology dramatically, (Dasch *et al.*, 1984; Degnan and Moran, 2008a; Buchner, 1953; Douglas, 1998). In addition, a number of heritable symbionts protect their hosts against heat stress, pathogenic fungi or parasitoid wasps (Chen *et al.*, 2000; Montllor *et al.*, 2002; Oliver *et al.*, 2003a). In contrast to those beneficial traits, symbionts can also function as reproductive manipulators that influence the insect population severely (Werren *et al.*, 2008). Bacterial symbionts of insects are often inherited by vertical transmission via the maternal or paternal germ line or by horizontal transmission spreading within or between species (Moran *et al.*, 2008). These symbionts are distributed within different phyla in the domain *Bacteria* such as *Chlamydiae*, *Bacteriodetes*, *Firmicutes* and *Proteobacteria* (*Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*) (Figure 2; Moran *et al.*, 2008).

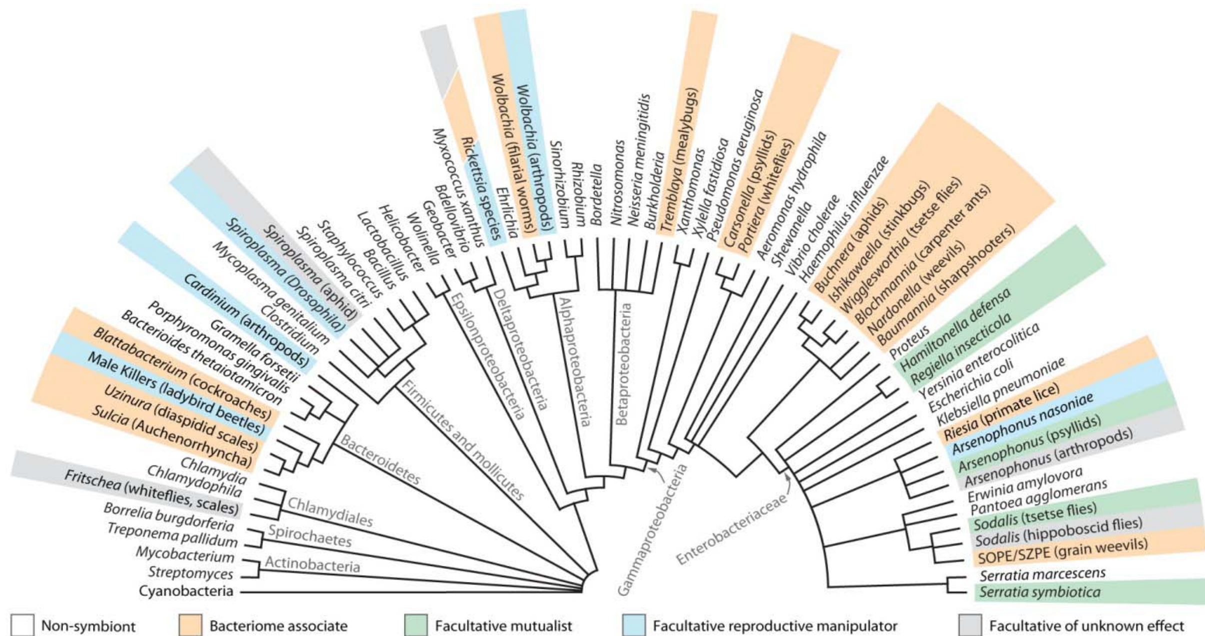

Figure 2. Diversity of heritable insect symbionts unequally distributed over the phyla within the *Bacteria*. Symbionts with diverse functions, obligate intracellular bacteria as well as symbionts with different tissue tropisms are present in this tree. The phylogeny is based on 16S rRNA gene sequences. Figure taken from Moran *et al*. (2008).

Replacement and acquisition of obligate bacteriocyte-associated symbionts

Obligate (primary) symbionts reside in specialized cells called bacteriocytes and supply the host insect with nutrients or other essential compounds, which are absent in the insects' diet (xylem, phloem, blood, omnivores, grain). In the nineteen-fifties, Paul Buchner and his co-workers pioneered the bacteriocyte/symbiont biology using light microscopy and hypothesized already their long-term association and the potential role of bacterial symbionts for their host insects (Buchner, 1953). For example, the bacteriocytes of aphids are developing in the embryogenesis of the host independently from the symbiont (Braendle *et al.*, 2003). Bacteriocytes are found embedded between midgut cells, attached to the gut and ovarioles, and in the fat body in the case of ants, aphids and cockroaches, respectively (Braendle *et al.*, 2003; Baumann, 2005). Within bacteriocytes, the symbionts are located either within a third so-called symbiosome membrane (e.g. '*Candidatus* Buchnera aphidicola') or free in the cytosol (e.g. '*Candidatus* Blochmannia spp.', *Wigglesworthia glossinida*, '*Candidatus* Baumannia cicadellicola'). In the aphid bacteriocytes, host genes for amino acid metabolism and transport were up-regulated, as well as genes responsible for bacterial defense that are potentially needed for the control of the symbiont population (Nakabachi *et al.*, 2005).

Interestingly, some insect groups harbour consistently one main obligate bacteriocyte-associated symbiont, while in a number of other insect families symbiont replacements are postulated. These are caused by a food change of a single host within this family during evolution (Lefevre *et al.*, 2004). Tsetse flies, which are vectors of African trypanosomes, feed on vertebrate blood and thus depend consistently on the obligate symbiont *Wigglesworthia glossinidia* (*Gammaproteobacteria*) since 50-100 million years. Analysis of the *W. glossinidia* genome revealed that this bacterium provides its hosts with B-complex vitamins (thiamine, pantothenic acid, pyridoxine, folic acid, and biotin) to complement the vitamin deficient vertebrate blood (Akman *et al.*, 2002; Aksoy, 1995). Similarly, cockroaches and ants, which can feed on unbalanced diets but can also be omnivorous, harbour a single obligate symbiont such as *Blattabacteria* sp. (*Bacteroidetes*) and '*Ca.* Blochmannia spp.' (*Gammaproteobacteria*), respectively (Clark *et al.*, 2001; Sauer *et al.*, 2000; Lo *et al.*, 2003). Both obligate symbionts provide certain compounds that are missed in the hosts' diet (Degnan *et al.*, 2005a; Gil *et al.*, 2003; Sabree *et al.*, 2009).

In contrast to the above-mentioned examples of consistent single symbionts, a different situation is found in weevils (Curculionoidea), which feed mostly on monocotyledonous angiosperms (leaves, stipes, roots, decaying woods) and are found in various habitats. The bacteriocyte-associated symbiont '*Candidatus* Nardonella spp.' (*Gammaproteobacteria*) was hosted by most weevils of the family Dryophtoridae for 125 million years and is thus the

ancestral obligate symbiont (Conord *et al.*, 2008; Lefevre *et al.*, 2004). About 50-100 million years ago, '*Ca.* Nardonella spp.' were replaced in grain weevils of the genus *Sitophilus* spp. by bacteriocyte-associated *Gammaproteobacteria* belonging to the S-clade (referring to *Sitophilus oryzae* primary endosymbiont (SOPE) and *Sitophilus zeamais* primary endosymbiont (SZPE)). This replacement is hypothesized as a consequence of the host weevils food change from stem-feeding to seed-feeding since the new symbionts provide amino acids (phenylalanine, proline) and vitamins (riboflavin, biotin, and pantothenic acid) to the host (Gasnier-Fauchet *et al.*, 1986; Heddi *et al.*, 1999; Heddi *et al.*, 1998; Lefevre *et al.*, 2004). These bacteriocyte-associated nutritional symbionts have a relatively long evolutionary history with their host insects owing to their vertical transmission mostly from mother to offspring and co-diversification with their respective host lineages, an assumption that is supported by congruent phylogeny between symbionts and hosts. However, the symbiont phylogeny (Figure 2) as well as genome comparison still shows the ancestry of obligate symbionts from free-living bacteria (Moran *et al.*, 2008).

Obligate symbionts of plant-sap feeding auchenorrhynchan insects (Homoptera)

Insects of the suborder Auchenorrhyncha (spittelbugs/froghoppers, cicadas, leafhoppers containing sharpshooters, treehoppers and planthoppers) are characterized by various lifestyles (roots, leaves, shoots), feeding habits (phloem, xylem, content of plant cells) and the transmission of pathogenic viruses and bacteria (Figure 3) (Gray and Banerjee, 1999). The plant-sap xylem is low in nutrients such as sugar (primarily glucose), but contains inorganic nitrogen, small amounts of non-essential amino acids (glutamate, aspartate) and organic acids (primarily malate) (Redak *et al.*, 2004). In contrast, phloem is rich in sugars and nitrogen almost exclusively in the form of free amino acid, but is lacking essential amino acids (Douglas, 2006).

As mentioned above, insects feeding on deficient diets are often depending on specialized symbionts. Histological studies of the last century demonstrated that the auchenorrhynchans harbour diverse (2 to 6) bacteriocyte-associated symbionts in their body cavity (Buchner, 1953). H. J. Müller, a student of Paul Buchner, postulated already multiple symbiont acquisitions and losses of particular symbiont lineages during diversification of Auchenorrhyncha (Müller, 1940). The oldest known bacteriocyte-associated symbiont clade is '*Candidatus* Sulcia muelleri' (*Bacteriodetes*) hosted by and co-diversied since >260 million years with most auchenorrhynchan insects since they may have been the first insects to feed on plant sap (Figure 3; Moran *et al.*, 2005a). Interestingly, some members of planthoppers and leafhoppers (Auchenorrhyncha) replaced '*Ca.* S. muelleri' completely by other symbionts

such as fungi (Moran *et al.*, 2005a). In other auchenorrhynchan subclades, additional bacteria were recruited later in evolution and turned into obligate symbionts that coexist in many cases in close proximity with '*Ca.* S. muelleri' in the bacteriocytes (Figure 3).

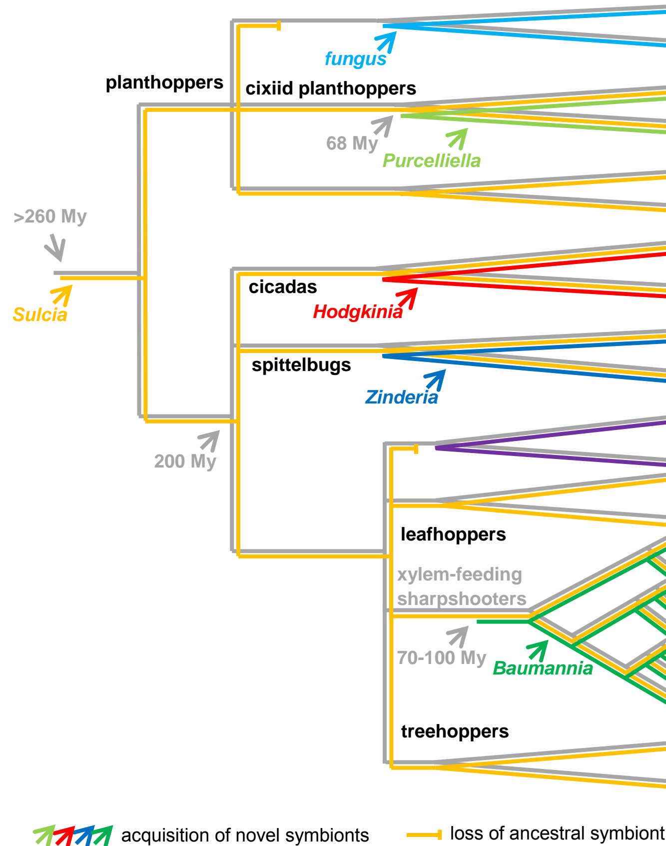

Figure 3. Schematic diagram of the acquisition of symbionts in the plant-sap feeding insect group Auchenorrhyncha (Hemiptera). Host insects and symbionts co-diversified over million of years (My) shown by the congruence of the phylogenies between symbionts (coloured) and hosts (grey). Symbiont acquisitions and replacements are indicated with differently coloured lines. Estimated time points of symbiont acquisitions based on fossil records of the hosts (modified from Moran (2007)).

In the case of xylem-feeding sharpshooters (subfamily of phloem-feeding leafhoopers), the acquisition of an additional symbiont named '*Candidatus* Baumannia cicadellinicola' (*Gammaproteobacteria*) enabled the host insect to adapt to xylem sap 70-100 million years ago based on fossil records of the late Cretaceous. Both obligate symbionts ('*Ca.* S. muelleri and '*Ca.* B. cicadellinicola') are maternally transmitted and thus co-diversified with their sharpshooter hosts (Figure 3; Moran *et al.*, 2003; Moran *et al.*, 2005a; Moran, 2007; Takiya *et al.*, 2006). In the case of spittelbugs (froghoppers) and the xylem-feeding cicadas, two novel symbionts, '*Candidatus* Zinderia insecticola' (*Betaproteobacteria*) and '*Candidatus* Hodgkinia cicadicola' (*Alphaproteobacteria*), were acquired after the divergence of all four host clades (cicadas, spittelbugs, leafhoppers, treehoppers) 200 million years ago during the

early Jurassic and co-reside with ‘*Ca.* S. muelleri’ (Figure 3; McCutcheon *et al.*, 2009b; Moran *et al.*, 2005a). ‘*Ca.* S. muelleri’ is able to synthesize seven (leucine, isoleucine, valine, threonine, lysine, arginine, and phenylalanine) or eight (before-mentioned amino acids plus tryptophan) essential amino acids depending on the strain, whereas the co-residing symbionts take over the production of the remaining two (methionine, histidine; ‘*Ca.* B. cicadellicola’, ‘*Ca.* H. cicadicola’) or three (methionine, histidine, and thryptophan; ‘*Candidatus* Zinderia insecticola’) essential amino acids. In addition, they produce a large number of vitamins and cofactors (‘*Ca.* B. cicadellicola’) and therefore perfectly complement the capabilities of the co-resident ‘*Ca.* S. muelleri’ (McCutcheon and Moran, 2007; McCutcheon and Moran, 2010; Wu *et al.*, 2006; McCutcheon *et al.*, 2009b). Members of phloem-feeding cixiid planthoppers acquired in addition to ‘*Ca.* S. muelleri’ ‘*Candidatus* Purcelliella pentastirinorum’ (*Gammaproteobacteria*) approximately 68 million years ago, which probably contributed to the diversification of this insect group (Figure 3). Both symbionts underwent a co-speciation with their respective insect host, an assumption that is well supported by the consistency of the tree topologies of host and both symbionts (Bressan *et al.*, 2009). In summary, auchenorrhynchan insects harbour phylogenetically diverse symbionts as postulated by Buchner and Müller and corroborated by a variety of recent phylogenetic and genomic studies. Symbiont replacement and acquisition is hypothesized to be triggered by the change of food sources from plant-sap to e.g. xylem.

Obligate symbionts of plant-sap feeding sternorrhynchan insects (Homoptera)

The sternorrhynchan insects (aphids, scale insects, whiteflies, psyllids; Figure 4) are common vectors of pathogenic viruses and bacteria. They harbor, like the auchenorrhynchan insects, various nutritional symbionts belonging to *Betaproteobacteria*, *Gammaproteobacteria*, *Bacteriodetes* and Eukaryota (Figure 5; Blackman and Eastop, 1994; Gray and Banerjee, 1999; Baumann, 2005). The members of the Sternorrhyncha feed all on plant-sap (mainly phloem) and transmit their obligate symbionts vertically from mother to offspring (Baumann, 2005).

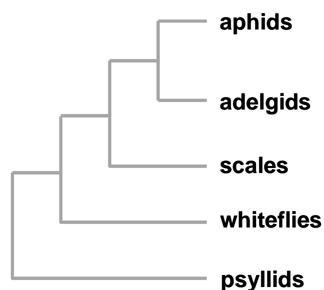

Figure 4. Schematic diagram of the phylogenetic relationship of Sternorrhynchan insects (aphids, adelgids, scale insects, whiteflies, psyllids) based on 18S rRNA gene sequences (modified from von Dohlen and Moran (1995)).

Buchnera

One of the best studied symbiont is *Buchnera aphidicola* (*Gammaproteobacteria*), which resides in most aphids and provides them with essential amino acids that are absent in the insects' diet (Figure 5; Shigenobu *et al.*, 2000; Munson *et al.*, 1991a; Munson *et al.*, 1991b; Douglas, 2006). The symbionts are essential for their host, an assumption that is corroborated by the finding that the hosts become infertile after removal of the symbiotic bacteria via antibiotic treatment. *B. aphidicola* and the aphid hosts co-diversify since ~180 million years as reflected by the congruency of the symbiont/host phylogeny and further supported by the vertically transmission from mother to offspring (Figure 5; Martinez-Torres *et al.*, 2001; Miura *et al.*, 2003; Baumann, 2005). In addition to *B. aphidicola*, aphids can be hosts for different facultative symbionts located in various tissues implementing diverse protective roles (Moran *et al.*, 2005b). Secondary symbionts such as '*Candidatus* Hamiltonella defensa' (*Gammaprotebacteria*) and '*Candidatus* Regiella insecticola' (*Gammaprotebacteria*) drastically influence the hosts' biology by providing protection against parasitoid wasps and resistance against fungal infection (von Burg et al., 2008; Ferrari et al., 2004; Oliver et al., 2003a; Degnan et al., 2009b). Interestingly, the nutritional role of *B. aphidicola* can be compensated in the cedar aphid *Cinara cedri* by the recently identified symbiont '*Candidatus* Serratia symbiotica' (*Gammaprotebacteria*). This symbiont encodes genes for tryptophan biosynthesis, which are absent in the respective co-residing *B. aphidicola* strain BCc (Gosalbes *et al.*, 2008; Burke and Moran, 2011; Burke *et al.*, 2009). Although other '*Ca.* S. symbiotica' strains found in various aphid species function as facultative symbiont providing tolerance against heat stress and resistance against parasitoid wasps, in the case of the cedar aphid, the host insect together with *B. aphidicola* and '*Ca.* S. symbiotica' represent a symbiotic consortium in which all partners benefit from each other, and thus the status of a facultative symbiont for 'Ca. S. symbiotica' has to be discussed

(Gosalbes *et al.*, 2008; Chen *et al.*, 2000; Montllor *et al.*, 2002; Oliver *et al.*, 2003a; Lamelas *et al.*, 2008; Burke *et al.*, 2009). The occurrence of '*Ca.* S. symbiotica' in the cedar aphid provides either evidence for a future replacement of *B. aphidicola* or an example for the establishment of a long-term well-balanced symbionts/host consortium (Gosalbes *et al.*, 2008). The loss and replacement of *B. aphidicola* by an extracellular fungus (Ascomycotina; Figure 5) was observed for some Cerataphidini aphids (Fukatsu and Ishikawa, 1996) and illustrates the possibility of future *B. aphidicola* replacements in other hosts.

Obligate symbionts of psyllids, whiteflies and scale insects

Psyllids and whiteflies harbour obligate symbionts named '*Candidatus* Carsonella ruddii' and '*Candidatus* Portiera aleyrodidarum', respectively; both belong to the *Gammaproteobacteria* (Figure 5). They reside in bacteriocytes localized in the insect abdomen (Baumann, 2005) and are maternally transmitted, as reflected by congruence of the host/symbiont phylogeny. It is hypothesized, that the common ancestor of modern psyllids and whiteflies was infected by the symbionts 120 and 100-200 million years ago, respectively (Thao and Baumann, 2004; Buchner, 1953; Thao *et al.*, 2000; Thao *et al.*, 2001). The symbiotic role of '*Ca.* C. ruddii' for the host insect is not yet well understood due to the lack of most genes involved in biosynthesis of essential amino acids in its genome (Tamames *et al.*, 2007; Nakabachi *et al.*, 2006), whereas for '*Ca.* P. aleyrodidarum' a nutritional function is hypothesized (Costa *et al.*, 1997).

Scale insects (Coccoidea) contain a large number of diverse symbiont lineages belonging to *Bacteriodetes*, *Betaproteobacteria*, *Gammaproteobacteria* and *Eukarya* (fungi), as originally hypothesized by Tremblay and Buchner, who described symbionts with different morphologies hosted by this insects group (Figure 5; Tremblay, 1990; Buchner, 1953). The obligate symbiont of parenchyma cell sap-feeding armored scale insects (Diaspididae) is '*Candidatus* Uzinura diaspidicola' (*Bacteriodetes*), which is showing an almost congruent phylogeny with their respective hosts (Gruwell *et al.*, 2007). The related giant and cushion scales (Monophlebidae) harbour phylogenetically closely related obligate symbionts that are called *Uzinura*-like in this thesis (Figure 5; Matsuura *et al.*, 2009), whereas members of the insect family Putoidae host symbionts belonging to *Gammaproteobacteria*. In contrast to scale insects of the insect family Diaspididae and Monophlebidae harbouring a monophyletic symbiont clade, phloem-feeding mealybugs/pseudococcids (Pseudococcidae), mostly severe pests all over the world feeding on a great diversity of host plants, contain phylogenetically diverse symbionts belonging to *Betaproteobacteria*, *Gammaproteobacteria* and *Bacteriodetes* (Figure 5). The mealybugs (Pseudococcidae) are divided into two subfamilies

Pseudococcinae and Phenacoccinae and harbour phylogenetically closely related obligate symbionts '*Candidatus* Tremblaya princeps' and '*Candidatus* Tremblaya phenacola' (*Betaproteobacteria*), respectively. Mealybugs of a subgroup of Phenacoccinae display an exception due to the presence of phylogeneticaly different symbiont belonging to *Bacteriodetes* (Gruwell *et al.*, 2010). '*Ca.* T. princeps' (*Betaproteobacteria*), the obligate symbiont of a mealybug subgroup (Pseudococcinae), infected the ancestor host insect approximately 100-200 million years ago (Thao *et al.*, 2002; Baumann and Baumann, 2005; Munson *et al.*, 1992; Fukatsu and Nikoh, 2000). This symbiosis represents a special symbiotic scenario since the bacterial symbiont '*Ca.* T. princeps' itself contains symbionts. '*Ca.* T. princeps' acquired multiple times phylogenetically different symbionts belonging to *Enterobacteriaceae* (*Gammaproteobacteria*) such as '*Candidatus* Moranella endobia', which co-reside within '*Ca.* T. princeps' resulting in co-speciation of both with the host insect (von Dohlen *et al.*, 2001; McCutcheon and von Dohlen, 2011; Lopez-Madrigal *et al.*, 2011; Thao *et al.*, 2002; Kono *et al.*, 2008). This is the first situation demonstrating that a bacterium ('*Ca.* M. endobia') is living within another bacterium ('*Ca.* T. princeps') (von Dohlen *et al.*, 2001). '*Ca.* M. endobia' and '*Ca.* T. princeps' highly compensate each other based on genome analyses. Both symbionts provide essential nutrients to the host insect *Planococcus citri* (Lopez-Madrigal *et al.*, 2011; McCutcheon and von Dohlen, 2011). Interestingly, phylogenetically deep branching members of the symbiont clade '*Ca.* T. princeps' do not contain inner symbionts (Figure 5), which may be indicative of the establishment of the mealybug/symbionts associations on molecular level (Thao *et al.*, 2002; Hardy *et al.*, 2008).

In summary, aphids, psyllids and whiteflies harbour a single lineage of obligate bacteriocyte-associated symbionts belonging mostly to *Gammaproteobacteria* that are not directly phylogenetically related (Figure 4, 5). In contrast, the scale insects harbour distantly related obligate symbionts belonging to *Gammaproteobacteria* as well as symbionts related to *Betaproteobacteria* and to *Bacteriodetes* (Figure 4, 5). Therefore, the scale insects are an interesting insect group to investigate the establishment and evolution of bacterial symbioses, symbiont acquisition and replacement.

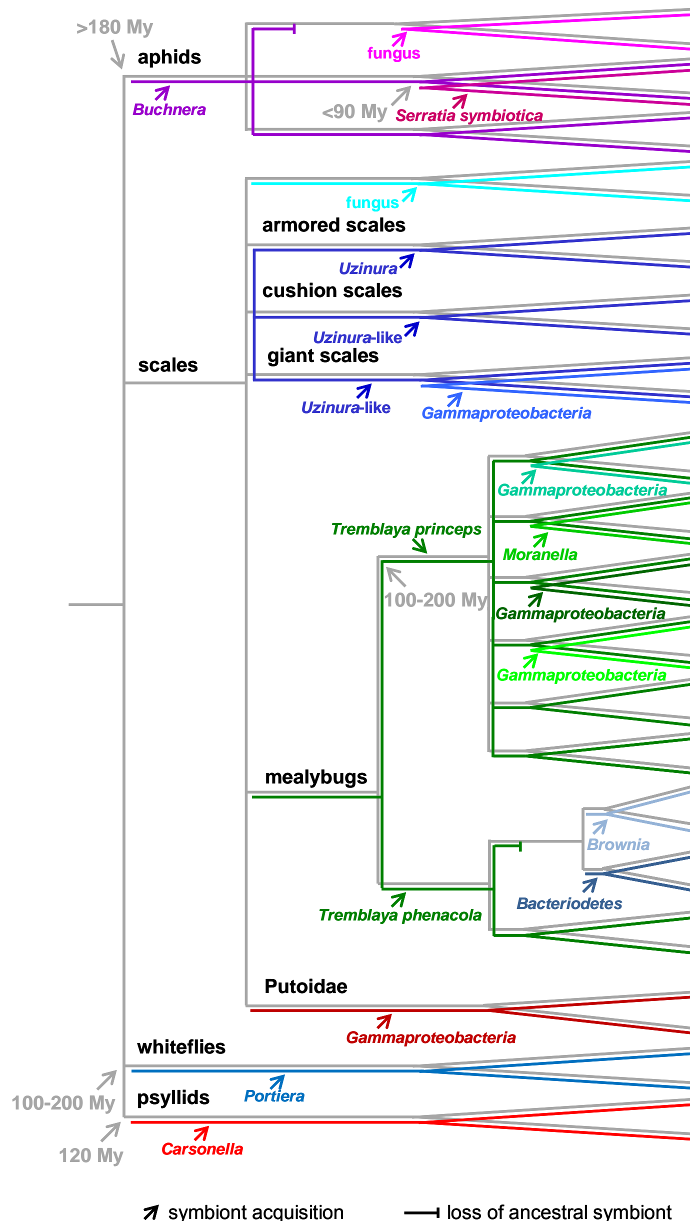

Figure 5. Schematic diagram of multiple symbiont acquisitions and replacements in the plant-sap feeding insect group Sternorrhyncha (Hemiptera). Host insects and symbionts co-diversified over million of years (My) shown by the congruence of the phylogenies between symbionts (coloured) and hosts (grey). Symbiont acquisitions and replacements are indicated with differently coloured lines. Estimated time points of symbiont acquisitions based on fossil records of the hosts.

Facultative symbionts in insects

Facultative (secondary) symbionts are known as mutualists, parasites, reproductive manipulators. They can invade various host cells and tissues and are transmitted vertically and horizontally within and between host species. The mutualistic facultative symbionts can protect their hosts against stress and natural pathogens such as parasitoid wasps and fungi (Moran *et al.*, 2008; Scarborough *et al.*, 2005). For example, the facultative symbiont '*Ca.* H.

defensa' is maternally transmitted and spreads between diverse hosts, and is thus found sporadically in aphids, whiteflies and psyllids (Clark *et al.*, 1992; Russell *et al.*, 2003; Sandstrom *et al.*, 2001). The major role of '*Ca.* H. defensa' is the protection of its host insect against the larval growth of parasitoid wasps (Oliver *et al.*, 2003b). The efficiency of protection depends on the type of '*Ca.* H. defensa' strain and is correlated to the presence of the lambda-like bacteriophage APSE (Degnan and Moran, 2008b). On the other hand, '*Ca.* H. defensa' is unable to produce 8 of 10 essential amino acids and thus depends on the amino acid production of *B. aphidicola*, the obligate symbiont of most aphids. Hence, '*Ca*. H. defensa' is only conditionally beneficial to the host when the parasitoids are present. However, '*Ca.* H. defensa' contains more genes for cellular structures and pathways for cellular processes than obligate symbionts. The genome (2.11 Mb) is in a reduced state with respect to the closest free-living relatives, including *Yersinia* and *Serratia* species (4.6-5.4 Mb), but still contains many pathogenicity factors related to host invasion similarly to enteric pathogens. This symbiont circumvents the host immune system and is found intra- and extracellularly in the host insect. Therefore, '*Ca.* H. defensa' combines pathogenic and mutualistic mechanisms in its symbiotic lifestyle (Degnan *et al.*, 2009a).

Multiple symbiont associations are known to exist in the same host insect. This results in a co-adaptation of all partners, which are sometimes phylogeneticly distantly related (Moran, 2007). Facultative symbionts with different function, especially reproductive manipulators, are known to be widely distributed and occupy a broad host range. The study of Duron and co-workers showed that out of the 136 determined arthropod species known to harbour facultative symbionts showing reproductive parasitism, *Wolbachia pipientis* was the dominant symbiont with an infection rate of 32.4%, whereas members of the '*Candidatus* Cardinium hertigii', '*Candidatus* Arsenophonus arthropodicus' and *Spiroplasma* sp. clades occurred in 4% to 7% of all species (Duron *et al.*, 2008). Similarly, a location-based study by Gottlieb *et al*. demonstrated the co-occurrence of facultative symbionts ('*Ca.* H. defensa', '*Ca.* A. arthropodicus', '*Ca.* C. hertigii', *W. pipientis*, and *Rickettsia* spp.) together with the obligate symbiont '*Ca.* P. aleyrodidarum' in the same bacteriocytes of the sweet tomato whitefly (*Bemisia tabaci*). This results in the assumption, that the facultative symbionts seem to 'hitch a ride' with the obligate symbiont (Gottlieb *et al.*, 2008).

Wolbachia

W. pipientis also called 'master manipulators' are intracellular alphaproteobacterial symbionts found in various arthropod and nematode hosts. *W. pipientis* plays a role in feminization, parthenogenesis, male killing, sperm-egg incompatibility, and in nutrition. Therefore, the

interaction between *W. pipientis* and their hosts ranges from reproductive manipulation to mutualism (reviewed in (Werren *et al.*, 2008)). These symbionts can be transmitted vertically from mother to offspring and affect processes that are essential for survival and reproduction of the host. *W. pipientis* can also be transferred horizontally crossing species borders resulting in a global, widespread distribution in diverse invertebrate hosts (Hosokawa *et al.*, 2010; reviewed in (Werren *et al.*, 2008)). Statistical analysis showed the association of *W. pipientis* with 66% of all insect species (Hilgenboecker *et al.*, 2008), where *W. pipientis* mainly occurs in reproductive tissues. Due to the role as reproductive manipulator, *W. pipientis* is a potential agent for pest and vector control (Bourtzis and Miller, 2006).

In contrast to *W. pipientis* strains, which act as reproductive manipulators, the bacteriocyte-associated *W. pipientis* strain of the bedbug *Cimex lectularius* has a nutritional role. Hosokawa and co-workers postulated that this mutualistic symbiont strain evolved from a facultative lifestyle (Hosokawa *et al.*, 2010). Therefore, the differentiation of symbionts of the same clade to a certain symbiotic role seems to depend on the host and environmental conditions. In summary, the global distributions of 'facultative' symbionts underline the importance of those symbionts and confirm the importance of symbiosis research for a broader understanding of these systems.

Genomics of insect symbionts

Symbionts in insects show distinct features in their genomes depending on the type of association they share with their host insects. These features are influenced by the age of infection. Based on these features, symbionts are differentiated between long-term and evolutionary young obligate or facultative symbionts (Table S1). For example, long-term associated obligate symbionts contain highly reduced genomes (~0.14 to 1.3 Mb, Table S1) compared to recently acquired facultative symbionts (~2 to ~4 Mb, Table S1).

Long-term obligate symbionts

As mentioned before, obligate symbionts are essential and beneficial for their host insects. This has been shown by a reduced longevity, reproduction rate and development after loss of the obligate symbiont using antibiotic treatment (Koga *et al.*, 2003). Some insects are feeding on unbalanced diets (xylem, phloem, vertebrate blood) harbouring symbionts, which maintain the host insect with the missing nutritional compounds. This nutritional role is reflected in the symbionts' genomes e.g. the *B. aphidicola* genomes are coding especially for genes for the biosynthesis of essential amino acids (Gosalbes *et al.*, 2008; Shigenobu *et al.*,

2000). These obligate long-term symbionts show highly reduced genomes (138-790 kb) with mostly low G + C content (13-43%) compared to other free-living or shortly associated bacteria (Table S1) (as shown in (Toenshoff *et al.*, 2012)) and archaea (McCutcheon *et al.*, 2009a; McCutcheon and von Dohlen, 2011). There are few exceptions known such as '*Ca.* H. cicadicola' (*Alphaproteobacteria*) the symbiont of cicadas, and '*Ca.* T. princeps' (*Betaproteobacteria*) the symbiont of most mealybugs, which show 58.4% and 58.8% G + C content, respectively. The cause of the higher G + C content in these genomes is not yet understood. The strict intracellular lifestyle, small population size, and frequent population bottlenecks (originating by vertical transmission from adult to progeny) result in an accumulation of deleterious mutations. Consequently, a faster sequence evolution in symbionts is observed when compared to their free-living counterparts (Moran, 1996; Mira and Moran, 2002). Additionally, the occasional asexuality of the host insect, metabolically rich and stable growth environment, and the preference in genome evolution for deletions over insertions are causes for genome reduction in obligate symbionts (Moya *et al.*, 2009; Moran *et al.*, 2008; Feldhaar and Gross, 2009; Moran *et al.*, 2009). Furthermore, the nucleotide composition bias towards A/T is based on a higher mutation rate from G/C to A/T than the other way around, but these genomes reach an equilibrium as shown for e. g. *B. aphidicola* genomes (~25% G + C content) (Moran *et al.*, 2009). The evolutionary trend towards A + T rich genomes increases the occurrence of A/T homopolymers. These become a hot-spot for small insertions or deletions (indels) due to replication slippage, and finally lead to gene inactivation. This process has been demonstrated for seven *B. aphidicola* strains from pea aphid hosts (Moran *et al.*, 2009). In general, genes from almost all cellular processes are lost in the obligate symbiont genomes, including genes for DNA recombination, repair and uptake (McCutcheon and Moran, 2010). No gene uptake, phages or mobile elements are visible in the genomes. Despite the loss and inactivation of various genes, and significant sequence evolution, genomes of long-term obligate symbionts are highly stable over millions of years (Moran *et al.*, 2008; Tamas *et al.*, 2002). This finding is reflected in a low genetic diversity in symbiont populations as shown for e. g. '*Ca.* S. muelleri' (*Bacteriodetes*), *Blochmannia pennsylvanicus* (*Gammaproteobacteria*) and *B. aphidicola* (only about 0.3% sequence divergence within estimated 20,000 years; *Gammaproteobacteria*) and represents a general trend in genome evolution of obligate symbionts (McCutcheon *et al.*, 2009b; Degnan *et al.*, 2005b; Moran *et al.*, 2009).

Genome reduction as cause for symbiont replacement?

In the last years, genomes of insect symbionts have been sequenced showing drastic genome reduction (Table S1). These small symbiont genomes were discussed to be

'evolutionary dead ends' resulting in the extinction and replacement of the symbiont (Moya *et al.*, 2009). However, there are arguments against this theory of extinction. For example, the highly reduced genome of '*Ca.* S. muelleri' has been remarkably conserved over millions of years and this symbiont clade is showing in one group of planthoppers evidence for elimination only (McCutcheon *et al.*, 2009b; Moran *et al.*, 2005a). Moreover, the mitochondrial (~69 kb) and the chloroplast (~191 kb) genomes are the most extreme examples of genome reduction having occurred to free-living bacteria. Despite the reduced genomes of both organelles, they do not show evidence for elimination. However, in either case they are defined as organelles not as self-contained bacteria. This is due to the gene transfer of certain bacterial genes to the host DNA, which resulted in dependence of the acquired bacterium on the host environment (McCutcheon, 2010; Lang *et al.*, 1997; Reith and Munholland, 1995; Gray *et al.*, 1999). But, however, based on genome analyses, the gene content of insect symbionts is different to organelles. For example, genes responsible for transcription, translation and replication are present in symbionts but mostly absent in organelles (McCutcheon, 2010). Taken together, genome reduction seems not to be the only cause of symbiont extinction.

However, it is hypothesized that various mechanisms were established to avoid symbiont elimination during evolution. For example, the selection and elimination of individuals with increased genetical mutations may decrease the rate of fixed mutations and lower the effect of symbiont extinction (Allen *et al.*, 2009). Furthermore, in the case of aphids, the symbionts' chaperone GroEL is highly over-produced in aphids' bacteriocytes, resulting in the assumption that it takes part in amelioration of non-productive proteins (Baumann *et al.*, 1996; Moran, 1996; Fares *et al.*, 2004).

Recently acquired symbionts

In contrast to long-term associated symbionts, facultative symbionts pass through a relatively short evolutionary history with their insect hosts. They furthermore have less reduced genomes, representing dynamic genomic stages underpinned by the presence of genes for mobile elements, rearrangements and bacteriophages in their genomes (Moran *et al.*, 2008). Similar genes were used to establish various bacterial symbioses ranging from pathogenic to facultative and obligate symbioses. For instance the type III secretion system, a virulence factor of pathogens, is used for insect cell invasion by *Sodalis glossinidius*, the facultative symbiont of tsetse flies. This symbiont is found intracellularly and extracellularly in its host insect (Toh *et al.*, 2006; Dale *et al.*, 2002). The recently acquired obligate symbiont *Sitophilus oryzae* primary symbiont (SOPE) of grain weevils shows comparable genomic features like

facultative symbionts such as a less reduced genome (3.0 Mb) and a similar G + C content as free-living bacteria (Heddi *et al.*, 1998). Taken together, comparison of genomes representing different evolutionary stages facilitates insights into the mechanism of establishment of bacteria/host associations and might help to understand the process of genome evolution during the initial phase.

Adelgids and their bacteriocyte-associated symbionts

Adelgids (Hemiptera: Sternorrhyncha: Adelgidae), a sister group of aphids (Aphididae) and phylloxerans (Phylloxeridae), are highly host tree (gymnosperms) specific. They feed on parenchyma cell sap (Balch, 1952; Plumb, 1953; Rohfritsch and Anthony, 1992; Young *et al.*, 1995) or phloem (Allen and Dimond, 1968; Parry and Spires, 1982; Balch, 1952; Sopow *et al.*, 2003) as is typical for most aphids, whiteflies, psyllids and mealybugs. Approximately 65 adelgid species have been identified so far. Some are known to be severe pests for forestry by feeding of nutrients and transmission of plant viruses (Blackman and Eastop, 1994; Havill and Foottit, 2007; Buczacki, 1973). Adelgids do have a complex multiple-generation life cycle consisting of a sexual cycle, characterized by gall formation on the primary host tree (*Picea* sp.) and host tree switching from primary host tree to secondary host tree (*Abies*, *Larix*, *Tsuga*, *Pseudotsuga*, *Pinus*), and an asexual cycle where the adelgids persist on the secondary tree only. However, some species lost the ability of host tree switching and remain either on the primary or on the secondary tree (Havill and Foottit, 2007). Adelgids are divided into two main genera *Adelges* and *Pineus.* There are species, which feed on the same secondary host trees resulting in a phylogenetic species grouping (Figure 6). Some adelgids species cannot easily be separated by molecular markers or morphological characters and are thus referred to as species complex (species- pair), such as *Adelges nordmannianae/piceae* and *Adelges abietis/viridis* (Figure 6; Zurovcova *et al.*, 2010; Havill *et al.*, 2007). Adelgids still have a preference for gymnosperms, whereas most aphids and phylloxerans feed on angiosperms. Adelgids and phylloxerans separated from aphids 150 million years ago in the late Jurrasic period. Based on fossil records, the divergence of adelgids with phylloxerans was >80 million years ago in the Cretaceous period (Havill *et al.*, 2007; Heie, 1987). Adelgids are still oviparous as are the phylloxerans, this is one main difference to the viviparous aphids (Heie, 1987).

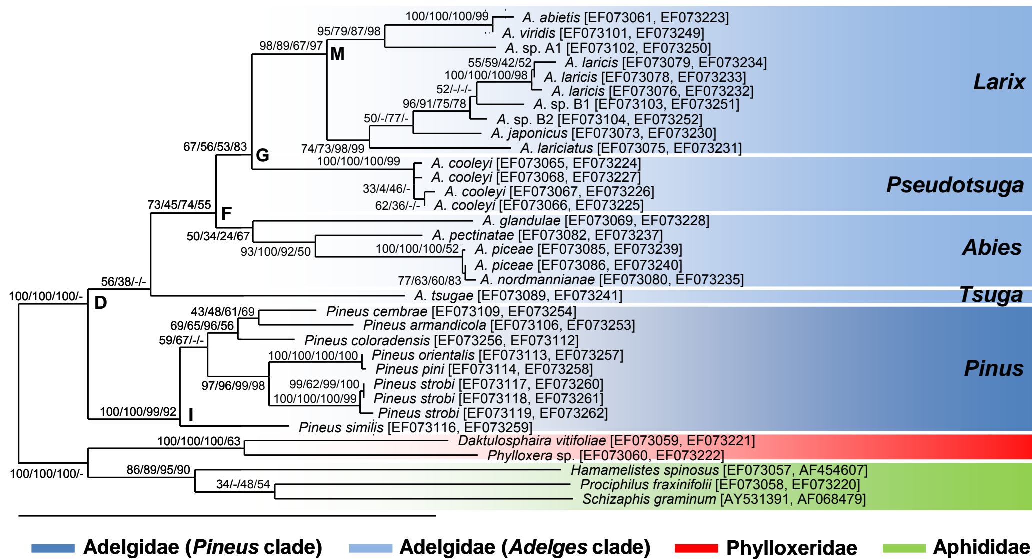

Figure 6. Phylogenetic relationship of different adelgid species to each other, and to phylloxerans and aphids. Data based on a concatenated dataset containing the cyrochrome c oxidase subunit 1 gene (*coI*) and elongation factor 1-alpha (*EF1-alpha*) of sternorrhynchan insects. A maximum likelihood tree is shown. Maximum likelihood (1000 replicates), maximum parsimony (1000 replicates) and Neighbor-joining (1000 resamplings) bootstrap values, and TREEPUZZLE support values are indicated at the internal nodes. Selected members of the Aphididae and Phylloxeridae were used as outgroup. The letters (D = 88 ± 14.09, F = 65.05 ± 12.03, G = 60 ± 11.84, I = 55 ± 11.67, M = ~40) indicate the estimated divergence times (millions of years ± standard deviation) taken from Havill *et al.* (2007). The tree topology is similar to the host phylogeny published in Havill *et al.* (2007). GenBank/EMBL/DDBJ accession numbers of *coI* and *ef1alpha* are given in square brackets. Bar, 10% estimated evolutionary distance.

Despite the importance of adelgids for forest ecosystems, our knowledge about their bacterial symbionts with respect to phylogenetic relationship and functional role is still scarce compared to the well-known bacteriocyte-associated symbionts of plant-sap feeding sternorrhynchans (aphids, whiteflies, psyllids, scale insects; Figure 7). Based on histological studies, adelgids also harbour bacterial symbionts in their bacteriocytes in the insect' abdomen (Buchner, 1953; Steffan, 1968). Since 1910, symbionts of adelgids are known, followed by studies dealing with the diversity of adelgids symbionts using the morphological differences (Buchner, 1953; Steffan, 1968; Profft, 1936).

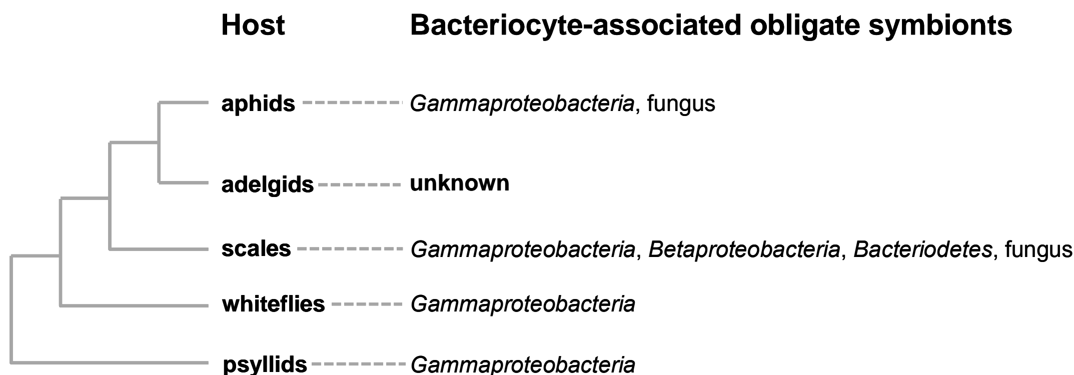

Figure 7. Bacteriocyte-associated symbionts of sternorrhynchan insects. Almost all sternorrhynchan insects contain bacteriocyte-associated symbionts, whereas the symbionts of adelgids are still unknown.

However, symbionts of most *Adelges* spp. (including the former genera *Sacchiphantes, Gilletteella*, *Dreyfusia*, and *Aphrastasia*) sp. are morphologicaly almost identical within each group, resulting in the assumption of the existence of different monophyletic symbiont clades in the Adelgidae (Table 1). Based on Proffts' study, the bacteriocyte-associated symbionts are vertically transmitted from mother to egg through the ovaries (Profft, 1936) and furthermore their nutritional role is proposed (Buchner, 1953). The elimination of *Adelges tsugae* symbionts using antibiotics resulted in the death of the host insect, revealing the symbionts' importance for the adelgid host (Shields and Hirth, 2005). Therefore, these bacterial symbionts may be potential targets for the biocontrol of these pests.

Table 1. Diversity of adelgid symbionts based on histological analyses. Adelgid species named according to the taxonomy of Steffan *et al*. (1968).

Adelgid species	Symbiont morphotypes	References
Rod-shaped symbionts		
Adelges (*Sacchiphantes*) *abietis* (Linnaeus 1758)	1-2 morphotypes (rod-shaped)	(Buchner, 1953; Steffan, 1968; Profft, 1936)
Adelges (*Sacchiphantes*) *viridis* (Ratzeburg 1843)	1 morphotype (rod-shaped)	(Profft, 1936; Steffan, 1968)
Adelges (*Sacchiphantes*) *laricifoliae* (Fitch 1856)	1 morphotype (rod-shaped)	(Steffan, 1968)
Adelges (*Sacchiphantes*) *segregis* (Steffan 1961)	1 morphotype (rod-shaped)	(Steffan, 1968)
Adelges (*Adelges*) *laricis* (Vallot 1836)	1 morphotype (rod-shaped)	(Profft, 1936; Steffan, 1968)
Adelges (*Adelges*) *tardus* (Dreyfus 1888)	2 morphotypes (rod-shaped)	(Steffan, 1968)
Adelges (*Adelges*) *strobilobius* (Kaltenbach 1843)	1 morphotype (rod-shaped)	(Buchner, 1953)
Adelges (*Adelges*) *geniculatus* (Ratzeburg 1843)	1 morphotype (rod-shaped)	(Steffan, 1968)
Adelges (*Gilletteella*) *cooleyi* (Gillette 1907)	1 morphotype (rod-shaped)	(Steffan, 1968)
Adelges (*Gilletteella*) *cummingae* (Steffan 1968)	1 morphotype (rod-shaped)	(Steffan, 1968)

Adelges (*Gilletteella*) *coweni* (Gillette 1907)	1 morphotype (rod-shaped)	(Steffan, 1968)
Pleomorphic and coccoid symbionts		
Adelges (*Dreyfusia*) *nordmannianae* (Eckstein 1890)	2 morphotypes (pleomorph, coccoid)	(Profft, 1936; Steffan, 1968)
Adelges (*Dreyfusia*) *piceae* (Ratzeburg 1844)	2 morphotypes (pleomorph, coccoid)	(Profft, 1936; Steffan, 1968)
Adelges (*Dreyfusia*) *merkeri* (Eichhorn 1957)	2 morphotypes (pleomorph, coccoid)	(Steffan, 1968)
Adelges (*Dreyfusia*) *prelli* (Grosmann 1935)	2 morphotypes (pleomorph, coccoid)	(Steffan, 1968)
Adelges (*Dreyfusia*) *todomatsui* (Inouye 1953)	2 morphotypes (pleomorph, coccoid)	(Steffan, 1968)
Adelges (*Dreyfusia*) *joshii* (Schneider-Orelli and Schneider 1959)	2 morphotypes (pleomorph, coccoid)	(Steffan, 1968)
Pleomorphic and rod-shaped symbionts		
Adelges (*Aphrastasia*) *knucheli* (Schneider-Orelli and Schneider 1954)	2 morphotypes (pleomorph, rod-shaped)	(Steffan, 1968)
Adelges (*Aphrastasia*) *ishiharai* (Inouye 1936)	2 morphotypes (pleomorph, rod-shaped)	(Steffan, 1968)
Adelges (*Cholodkovskya*) *viridana* (Cholodkovsky 1896)	2 morphotypes (pleomorph, rod-shaped)	(Steffan, 1968)
Pleomorphic and coocoid/rod-shaped symbionts		
Pineus (*Pineus*) *pini* (Macquart 1819)	2 morphotypes (pleomorph, coccoid)	(Profft, 1936; Steffan, 1968)
Pineus (*Eopineus*) *strobi* (Hartig 1837)	2 morphotypes (pleomorph, rod-shaped)	(Profft, 1936; Steffan, 1968)
Pineus (*Pineus*) *similis* (Gillette 1907)	2 morphotypes (pleomorph, coccoid)	(Steffan, 1968)
Pineus (*Pineus*) *floccus* (Patch 1909)	2 morphotypes (pleomorph, coccoid)	(Steffan, 1968)
Pineus (*Pineodes*) *pinifoliae* (Fitch 1858)	2 morphotypes (pleomorph, coccoid)	(Steffan, 1968)
Pineus (*Pineus*) *orientalis* (Dreyfus 1889)	2 morphotypes (pleomorph, coccoid)	(Profft, 1936)
Pineus (*Eopineus*) *pineoides* (Cholodkovsky 1903)	1-2 morphotypes (pleomorph, rod-shaped)	(Profft, 1936; Steffan, 1968)
Pineus (*Eopineus*) *abietinus* (Underwood and Balch 1964)	2 morhotypes (pleomorph, rod-shaped)	(Steffan, 1968)

Table S1. Genome features of selected obligate and facultative insect symbionts.
Genome features according to the NCBI Genome database.

	Sodalis glossinidius str. ´morsitans´ [NC_007712]	*Serratia symbiotica* str. Tucson [NZ_AENX00000000]	*Candidatus* Hamiltonella defensa 5AT' [NC_012751]	*Candidatus* Regiella insecticola LSR1' [NZ_ACYF00000000]	*Candidatus* Blochmannia pennsylvanicus str. BPEN' [NC_007292]	*Wigglesworthia glossinidia* [NC_004344]	*Baumannia cicadellinicola* str. Hc [NC_007984]	*Buchnera aphidicola* str. APS [NC_002528]	*Buchnera aphidicola* str. Cc [NC_008513]	*Candidatus* Carsonella rudii PV' [NC_008512]	*Candidatus* Moranella endobia PCIT' [NC_015735]	*Candidatus* Riesia pediculicola USDA' [NC_014109]	*Candidatus* Ishikawaella capsulata Mpkope'	*Candidatus* Portiera aleyrodidarum' [AY268081, AY268082]	*Candidatus* Zinderia insecticola CARI' [NC_014497]	*Candidatus* Tremblaya princeps PCIT' [NC_015736]	*Candidatus* Sulcia muelleri GWSS' [NC_010118]	*Blattabacterium* sp. str. BPLAN [NC_013418]	*Battabacterium* sp. str. Bge [NC_013454]	*Candidatus* Hodgkinia cicadicola Dsem' [NC_012960]	*Wolbachia* sp. of *Cimex lectularius* (bedbu
Phylogenetic affiliation	*Gammaproteobacteria*														*Betaproteobacteria*		*Bacteriodetes*			*Alphaproteobacteria*	
Lifesstyle	Facultative insect symbionts				Obligate insect symbionts																
Age of symbiosis[a] [my]	n.d.	~90	n.d.	n.d.	50	>40	70-100	180	150	120	n.d.	13-25	n.d.	n.d.	n.d.	n.d.	>260	150	150	190	n.d.
Genome size [bp]	4,171,146	2,573,085	2,110,331	2,035,106[b]	791,654	697,724	686,194	640,681	416,380	159,662	538,294	574,390	750,000	(33.3)[d]	208,564	138,927	245,530	636,994	636,850	143,795	1,300,000
plasmids	3	no	1	1[b]	no	1	no	2	1	no	no	1	n.d.	n.d.	no	no	no	1	1	no	n.d.
GC content [%]	54	49	40	42	29	22	33	26	20	16	43	28	n.d.	30.2[d]	13	58	22	28	27	58	n.d.
% Coding	50.9[b]	56	80	71	76	86	85	86	85	93	77	80	n.d.	71.2[d]	89	65	91	93	95	90	n.d.
Structural RNAs	91	72	85	49	44	40	46	36	37	31	46	40	n.d.	n.d.	29	19	36	47	40	19	n.d.
CDS	2432	2157	2094	1769	610	611	595	564	357	182	406	544	n.d.	n.d.	202	121	227	577	586	169	n.d.
Pseudogenes	972[b]	550	187	223	4	0	10	1	3	0/0	29	3	n.d.	n.d.	0	22	1	6	1	0	n.d.
Average CDS length [bp]	873	668	806.24	817.1	948.9	982.1	980.3	976.9	991.4	815.9	1020.9	844.7	n.d.	n.d.	918.9	746.3	984.3	1026.7	1032.4	765.8	n.d.
Average spacer length [bp]	842.1	524.9	201.6	333.7	299.7	159.9	173	159	174.9	61.4	304.9	211.2	n.d.	n.d.	113,6	401.9	97.4	77.3	54.3	85.1	n.d.

[a] (Burke & Moran 2011; Gosalbes *et al.* 2010; Moran *et al.* 2008)
[b] modified using the publication of the *Sodalis glossinidius* genome (Toh *et al*. 2006)
[c] (Degnan *et al.* 2009)
[d] (Baumann *et al.* 2003)

Outline

Previous research described morphologically different symbionts within bacteriocytes of several members of the insect family Adelgidae, but their phylogenetic relationship has remained unclear (Steffan, 1968; Buchner, 1953; Profft, 1936). Therefore, the work in this thesis aimed to reveal the phylogenetic identification and *in situ* visualization of the bacteriocyte-associated symbionts of selected adelgid species, and populations, sampled in Europe and the USA. I wanted to investigate whether adelgids harbour *Buchnera aphidicola*, the obligate symbiont of their sister group, the aphids, or whether there are other obligate symbionts in sternorrhynchan insects. Based on the different described morphologies of the adelgid symbionts, I hypothesized multiple acquirements of phylogenetically different symbionts in the insect family Adelgidae. These represent an extreme case of symbiont diversity and replacement within an insect family. In order to get first insights into genome evolution in symbionts of adelgids, we furthermore analyzed a genome fragment of one of the bacteriocyte-associated symbionts of *Adelges nordmannianae/piceae*.

Chapter I presents a general introduction to the topics of this thesis. It contains a short overview about the importance of insects and their symbionts for their ecosystem. Furthermore, it includes a summary of the current knowledge of the diversity of obligate and facultative symbionts in insects and indicates their functional role, focusing on bacteriocyte-associated symbionts in plant-sap feeding insects. Additionally, it gives insights in the genomic features of obligate and facultative symbionts and ends with a general overview about the biology and evolution of adelgids (Hemiptera: Adelgidae).

Chapter II describes the first identification of two novel phylogenetically distinct gammaproteobacterial symbionts of *Adelges nordmannianae/piceae* complex by transmission electron microscopy, fluorescence *in situ* hybridization (FISH), phylogenetic sequence analyses and genome fragment analyses. Three *Adelges nordmannianae/piceae* populations from Europe were sampled and compared. It is demonstrated that all examined populations and life stages contained phylogenetically closely related bacteriocyte-associated symbionts, which were not related to known symbionts of insects. This results in the assumption, that these symbionts were vertically transmitted from mother to offspring. Furthermore, a metagenomic library was constructed, screened and analysed demonstrating that one of the symbionts represents a rather young association with its host adelgid.

Toenshoff E. R., Penz T., Narzt T., Collingro A., Schmitz-Esser S., Pfeiffer S., Klepal W., Wagner M., Weinmaier T., Rattei T., Horn M. (2012). *Bacteriocyte-associated gammaproteobacterial symbionts of the Adelges nordmannianae/piceae complex (Hemiptera: Adelgidae)*. ISME J. 6: 384-396.

My contribution to this study: I constructed the metagenomic library, performed FISH, phylogenetic sequence and genome analyses, data-interpretation and wrote the draft manuscript. I also supervised the process of library screening that was performed by a diploma student.

Chapter III represents a study on the identification of novel bacteriocyte-associated symbionts of three adelgid complexes (*Adelges laricis/tardus*, *Adelges abietis/viridis*, *Adelges cooleyi/coweni*) by phylogenetic analyses, FISH and transmission electron microscopy. Phylogenetically closely related betaproteobacterial symbionts were found in each complex forming a monophyletic clade, as well as closely related gammaproteobacterial symbionts in two adelgid complexes out of three examined complexes. In the remaining complex a symbiont closely related to the symbiont of *Adelges nordmannianae/piceae* was found. Therefore, coevolution and symbiont replacement events are postulated. Additionally, the study contains the most likely evolutionary infection scenario to have happened in adelgids.

Toenshoff E. R., Gruber D., Horn M. (2012) *Co-evolution and symbiont replacement shaped the symbiosis between adelgids (Hemiptera: Adelgidae) and their bacterial symbionts*. Environ. Microbiol., Doi: 10.1111/j.1462-2920.2012.02712.x

My contribution to this study: I conducted the phylogenetic analyses, FISH and transmission electron microscopy, as well as the data-interpretation and wrote the draft manuscript, which was edited by all co-authors.

Chapter IV reports the identification of two novel bacteriocyte-associated symbionts in *Pineus strobi* by transmission electron microscopy, phylogenetic sequence analyses and FISH. This study is not yet finalized, but contributes to the knowledge of the diversity of adelgid symbionts. Additional PCR and cloning of the bacterial 16S rRNA genes and symbiont-specific FISH experiments are required to unterpin the present findings.

Toenshoff E. R., Gruber D., Horn M. *Pineus strobi (Hemiptera: Adelgidae) contains novel gammaproteobacterial symbionts.* Manuscript in preparation for Environ. Microbiol.

My contribution to this study: All experiments and data analyses were done by me. The draft manuscript included in this thesis was written by me.

Chapter V gives an outlook containing ideas for further projects illuminating the adelgid symbiosis.

Chapter VI gives a summary of the presented thesis in English and German.

The ***Appendix*** includes an additional publication dealing with a novel cyst-forming symbiont residing within the gills of farmed Atlantic salmon (*Salmo salar*). We investigated a population of Atlantic salmon from Norway, which displays ephitheliocysts and poliferative gill inflammation by qPCR, phylogenetic 16S rRNA gene analyses, FISH, transmission electron microscopy. This study demonstrated that cysts are caused by a novel betaproteobacterial symbiont '*Candidatus* Branchiomonas cysticola' and not by a Chlamydia-like organism.

Toenshoff E. R., Kvellestad A., Mitchell S. O., Steinum T., Falk K., Colquhoun D., Horn M. (2012). *A novel betaproteobacterial agent of gill epitheliocystis in seawater farmed Atlantic salmon (Salmo salar)*. PLoS ONE 7: e32696.

My contribution to this study: I performed FISH, phylogenetic analyses and data-interpretation. I partly wrote the manuscript, which was then edited by all co-authors.

The Appendix also contains lists of my publications, oral and poster presentations, supervision and teaching activities and ends with ***Acknowledgments*** and my **CV**.

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Chapter II

Bacteriocyte-associated gammaproteobacterial symbionts of the *Adelges nordmannianae/piceae* complex (Hemiptera: Adelgidae)

Published in *ISME J* (2012), 6: 384-396

and as supplementary online material:

http://www.nature.com/ismej/journal/v6/n2/extref/ismej2011102x1.pdf

The ISME Journal (2012) 6, 384–396
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www.nature.com/ismej

ORIGINAL ARTICLE

Bacteriocyte-associated gammaproteobacterial symbionts of the *Adelges nordmannianae/piceae* complex (Hemiptera: Adelgidae)

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Adelgids (Insecta: Hemiptera: Adelgidae) are known as severe pests of various conifers in North America, Canada, Europe and Asia. Here, we present the first molecular identification of bacteriocyte-associated symbionts in these plant sap-sucking insects. Three geographically distant populations of members of the *Adelges nordmannianae/piceae* complex, identified based on *coI* and *ef1alpha* gene sequences, were investigated. Electron and light microscopy revealed two morphologically different endosymbionts, coccoid or polymorphic, which are located in distinct bacteriocytes. Phylogenetic analyses of their 16S and 23S rRNA gene sequences assigned both symbionts to novel lineages within the *Gammaproteobacteria* sharing $<$92% 16S rRNA sequence similarity with each other and showing no close relationship with known symbionts of insects. Their identity and intracellular location were confirmed by fluorescence *in situ* hybridization, and the names '*Candidatus* Steffania adelgidicola' and '*Candidatus* Ecksteinia adelgidicola' are proposed for tentative classification. Both symbionts were present in all individuals of all investigated populations and in different adelgid life stages including eggs, suggesting vertical transmission from mother to offspring. An 85 kb genome fragment of '*Candidatus* S. adelgidicola' was reconstructed based on a metagenomic library created from purified symbionts. Genomic features including the frequency of pseudogenes, the average length of intergenic regions and the presence of several genes which are absent in other long-term obligate symbionts, suggested that '*Candidatus* S. adelgidicola' is an evolutionarily young bacteriocyte-associated symbiont, which has been acquired after diversification of adelgids from their aphid sister group.

The ISME Journal (2012) **6,** 384–396; doi:10.1038/ismej.2011.102; published online 11 August 2011
Subject Category: microbe–microbe and microbe–host interactions
Keywords: evolution; genome reduction; insects; symbiosis; *Buchnera*

Introduction

The presence of heritable bacterial endosymbionts in insects is widespread in nature (Buchner, 1953). Their roles range from obligate mutualists with essential nutritional functions and facultative mutualists necessary for host protection to parasites manipulating the host's reproductive system (Moran *et al.*, 2008). Thus, symbiosis with bacteria has a great impact on the ecology and the evolution of many insect hosts.

A group of insects comparatively well studied with respect to their symbionts are plant sap-sucking members of the suborder Sternorrhyncha including aphids, psyllids, whiteflies and mealybugs, with phloem-feeding aphids as a prime example. Most aphids harbor the gammaproteobacterial endosymbiont *Buchnera aphidicola*. *Buchnera* is located in specialized cells in the insect body cavity termed 'bacteriocytes', and is vertically transmitted from mother to offspring (Baumann, 2005; Moran *et al.*, 2008). The major function of this obligate (primary) symbiont is to convert unusable nutrition to utilizable compounds, that is, to supply its host with essential amino acids lacking in phloem sap (Shigenobu *et al.*, 2000; Moran *et al.*, 2003; Zientz *et al.*, 2004; Thomas *et al.*, 2009). *Buchnera* are highly specialized bacteria well adapted to symbiosis with their aphid hosts through $>$160 millions of years (Moran *et al.*, 1993). As obligate intracellular bacteria, they show highly

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Received 31 March 2011; revised 16 June 2011; accepted 17 June 2011; published online 11 August 2011

reduced genomes compared with free-living bacteria and lack many essential biosynthetic pathways (Moran *et al.*, 2008). In addition to *Buchnera*, many aphids harbor additional, phylogenetically distinct facultative (secondary) symbionts such as '*Candidatus* Serratia symbiotica' (Lamelas *et al.*, 2008), '*Candidatus* Hamiltonella defensa' (Degnan *et al.*, 2009) and '*Candidatus* Regiella insecticola' (Moran *et al.*, 2005; Scarborough *et al.*, 2005).

In contrast to the well-studied aphids, our knowledge about bacterial symbionts of their sister group, the adelgids, is very scarce. Adelgids are plant sap-sucking insects comprising ~65 highly host-specific species, some of which represent severe pests of various conifers especially in North America, Canada, Europe and Asia (Steffan, 1972; Blackman and Eastop, 1994; Havill and Foottit, 2007). Adelgids feed mainly on phloem or parenchyma cells and perform mostly a complex sexual life cycle consisting of multiple generations with host alternations on conifers by switching from the primary (spruce (*Picea* spp.)) to the secondary host tree (*Abies*, *Larix*, *Pseudotsuga*, *Tsuga* or *Pinus*); however, asexual reproduction has also been reported (Steffan, 1972; Havill and Foottit, 2007).

Early morphological and histological studies of adelgids have reported the occurrence of bacteria within bacteriocytes, between bacteriocytes and oenocytes and free in the hemocoel (Profft, 1936; Buchner, 1953; Steffan, 1968; Shields and Hirth, 2005). Rod-shaped, coccoid and polymorphic bacteria were observed in different adelgid species and sometimes within the same adelgid host (Buchner, 1953; Steffan, 1968). However, to our knowledge, no published sequences for the identification of these bacterial symbionts on the molecular level are available.

In this study, we investigated whether adelgids, like their aphid sister group, harbor *Buchnera*-related symbionts or whether they acquired different endosymbionts after the divergence from aphids. We analyzed adelgids of the *Adelges nordmannianae/piceae* complex. Both insects, the silver fir woolly adelgid *A. nordmannianae* (Eckstein, 1890; a.k.a. *Dreyfusia nordmannianae*) and the balsam woolly adelgid *A. piceae* (Ratzeburg, 1844; a.k.a *Dreyfusia piceae*), are a very closely related species pair with highly similar morphological and genetic characteristics (Eichhorn, 1967; Havill and Foottit, 2007; Havill *et al.*, 2007). Although *A. nordmannianae* shows a sexual life cycle including host alternation between spruce and fir trees (*Picea orientalis* and *Abies* spp. respectively), *A. piceae* has an asexual life cycle and feeds on fir trees only (Bryant, 1971; Eichhorn, 1973; Binazzi, 2000). Both are a severe pest on young firs and dreaded in the Christmas tree industry (Balch, 1952). *A. nordmannianae* primarily attacks young sprouts and needles and older branches but rarely the bark of the trunk. *A. piceae* can be located on any part of the tree depending on the host tree species (Steffan, 1972). The stylets of *A. piceae* pierce the bark tissue and can reach the phloem, but do not enter it. In contrast to aphids, these adelgids thus feed on cortical parenchyma cells and on the phelloderm, a layer of the periderm (Balch, 1952).

Using 16S rRNA sequence analysis and fluorescence *in situ* hybridization (FISH) with symbiont-specific oligonucleotide probes, we identified the bacteriocyte-associated symbionts of adelgids of the *A. nordmannianae/piceae* complex as previously unrecognized *Gammaproteobacteria* that are present in different host populations and life stages. Metagenomic analysis suggested that one of the symbionts was acquired after diversification of aphids and adelgids, recently relative to other insect symbiont lineages.

Materials and methods

Organisms

Adelgids of the *A. nordmannianae/piceae* complex were collected from silver fir (*Abies alba* Mill) and Nordmann fir (*Abies nordmanniana* (Steven) Spach) trees at three different sampling sites (Table 1). Infested branches were cut from the trees and stored at 4 °C until collection of the insects. The insects were used immediately, fixed for FISH and electron microscopy or stored in ethanol for DNA purification at a later time point.

Histology and transmission electron microscopy

Insects were prefixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4 °C and fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at room temperature. Specimens were dehydrated in ethanol and embedded in Spurr's resin (Spurr, 1969). Semi-thin sections were stained with Richardson's solution (Richardson *et al.*, 1960) and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a Zeiss EM 902 electron microscope (Carl Zeiss, Vienna, Austria) at 80 kV.

PCR, cloning, restriction fragment length polymorphism and sequencing

Intact adelgids stored in ethanol were washed in double distilled water and DNA was purified from either single or up to 30 individuals; different life stages and eggs were used for DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA was stored at −20 °C until further use for PCR. Adelgid (*coI*, *ef1alpha*) and bacterial (16S and 23S rRNA) genes were amplified in 35 PCR cycles using the primers and conditions listed in Supplementary Table S1. PCR reactions typically contained 2 µl template DNA, 50 pmol of each primer, 1 Unit of Taq DNA polymerase (Fermentas, St Leon-Rot, Germany), 10 × Taq buffer with KCl

Table 1 Adelgids and their bacteriocyte-associated endosymbionts analyzed in this study

Adelgids	*Location*	*Host plant*	*GenBank accession number (insect host)*		*GenBank accession number (symbionts)* 'Candidatus *Steffania adelgidicola*'		'Candidatus *Ecksteinia adelgidicola*'	
			coI	ef1alpha	*16S rRNA gene*	*23S rRNA gene*	*16S rRNA gene*	*23S rRNA gene*
Adelges nordmannianae/ piceae	Gosau, Austria	*Abies alba*	HQ668155	HQ668164	HQ668158	—	HQ668160	—
Adelges nordmannianae/ piceae	Grafrath, Germany	*Abies nordmanniana*	HQ668157	HQ668167	HQ668159	—	HQ668161	—
Adelges nordmannianae/ piceae	Klausen-Leopoldsdorf, Austria	*Abies alba*	HQ668156	HQ668165 HQ668166	FR872579	FR872579	HQ668162	HQ668163

and 2 mM $MgCl_2$ and 0.2 mM of each deoxynucleotide in a total volume of 50 μl. Both negative (no DNA added) and positive controls were included in all PCR reactions. PCR products were purified using the PCR purification kit (Qiagen) and were either sequenced directly or cloned using the TOPO TA cloning kit with the cloning vector pCR 2.1-TOPO (Invitrogen Life Technologies, Lofer, Austria) following the manufacturer's instructions. At least 20 16S and 23S rRNA gene clones each were screened by restriction fragment length polymorphism analysis using MspI (Fermentas). Nucleotide sequences were determined using the BigDye Terminator kit v3.1 (Applied Biosystems, Vienna, Austria) and an ABI 3130 XL genetic analyzer (Applied Biosystems).

Phylogenetic analysis

The program ARB (Ludwig *et al*., 2004) was used for phylogenetic analysis. ARB 16S and 23S rRNA databases were updated with sequences from GenBank obtained by sequence homology searches using BLASTn available at the NCBI web site (National Centre for Biotechnology Information) (Altschul *et al*., 1990). Databases for genes encoding cytochrome *c* oxidase 1 (*coI*) and elongation factor 1-alpha (*ef1alpha*), as well as a protein database RNA polymerase sigma-32 factor (RpoH) were established with representative sequences downloaded from GenBank and aligned using Mafft (Katoh *et al*., 2005). Phylogenetic trees were calculated using MrBayes and the maximum parsimony, distance matrix and TREEPUZZLE methods implemented in ARB (Huelsenbeck and Ronquist, 2001; Schmidt *et al*., 2002; Ronquist and Huelsenbeck, 2003; Ludwig *et al*., 2004). PhyML trees were calculated using the Mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py; Guindon and Gascuel, 2003). Symbiont-specific primers were designed using the probedesign/probematch tools of the ARB software package (Ludwig *et al*., 2004).

Fluorescence in situ *hybridization*

Insects were fixed in 4% paraformaldehyde for 4 h at 4 °C, crushed on a glass slide and covered with 0.2% of low melting agarose. Hybridization was performed using a standard protocol hybridization and washing buffer as described previously (Daims *et al*., 2005). The oligonucleotide probes used are given in Supplementary Table S1. Probe NONEUB (complementary to the bacterial probe EUB338-I) was used as negative control. Hybridized slides were examined using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss). Symbiont-specific probes were designed using the probedesign/probematch tools of the ARB software package (Ludwig *et al*., 2004) and deposited at probeBase (Loy *et al*., 2007). Optimal hybridization conditions for symbiont-specific probes were determined in a series of hybridization experiments with increasing formamide concentrations in the hybridization buffer.

Fosmid library construction and screening

High molecular weight DNA for the construction of a fosmid library was purified from freshly collected insects and eggs from Klausen-Leopoldsdorf. To enrich bacterial symbionts, whole insects and eggs were homogenized using a Dounce tissue grinder (Wheaton, Millville, NJ, USA) in buffer A (35 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose, pH 7.5; Ishikawa, 1982) and filtered sequentially through 53, 30 and 10 μm meshes (Eckert, Waldkirch, Germany). The remaining suspension containing both symbionts was centrifuged at 7000 r.p.m. The pellet was resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and DNA was obtained using an SDS-based DNA purification method including 1% hexadecylmethylammonium bromide, 1.5% polyvinylpyrrolidone and proteinase K (Sigma-Aldrich, Vienna, Austria) in the extraction buffer (Zhou *et al*., 1996). DNA was quality checked by gel electrophoresis and stored at −20 °C until further use. A large insert fosmid library was constructed using the CopyControl Fosmid Library Production

Kit (Epicentre, Madison, WI, USA). A total of 14 208 fosmid clones were picked, incubated and stored in 96 MicroWell plates containing LB medium, 7% glycerol and 12.5 μg ml^{-1} chloramphenicol at −80 °C.

Fosmids prepared from up to 96 pooled clones served as template for the screening of the fosmid library by PCR using symbiont-specific 16S and 23S rRNA gene-targeted primers and PCR conditions described above but with 42 PCR cycles (Supplementary Table S1). PCR products from single, positive fosmid clones were sequenced directly, and the size of the inserted DNA was estimated by pulsed field gel electrophoresis. In brief, a CHEF-DR III system (Bio-Rad, Vienna, Austria) and 1% agarose gel were used at 6V cm and 14 °C with a linear switching time (0.1–5 s) for 8.4 h. Positive fosmids were end sequenced, and checked for overlapping regions using restriction fragment length polymorphism with EcoRI (Fermentas). Fosmids were sequenced using a shotgun clone library and the Sanger sequencing method by a company (LGC Genomics, Berlin, Germany). A coverage of 4 × was achieved and sequence gaps were closed by primer walking.

Annotation and comparative genome analysis

The genome fragment of '*Candidatus* Steffania adelgidicola' was automatically annotated and analyzed using the PEDANT software platform (http://pedant.gsf.de/; Frishman *et al*., 2001; Walter *et al*., 2009). Protein-coding sequences were predicted by combining intrinsic predictions from GeneMarkS (Besemer *et al*., 2001) and Glimmer (Delcher *et al*., 2007) and extrinsic information from a BLAST search against the NCBI RefSeq database (Wheeler *et al*., 2008). All gene models were additionally manually verified by pairwise alignments with homologous proteins found in the UniProt Knowledgebase (Consortium, 2010). Prediction of tRNA genes was performed using tRNAscan-SE (Lowe and Eddy, 1997). Pseudogene analysis was performed using a house-internal pipeline for the identification of neighboring open-reading frames with identical homologs in NCBI RefSeq. All candidates for pseudogenes were subsequently inspected manually. The NCBI COG database was used for functional classification of proteins (Tatusov *et al*., 2001). UniProt and the Multi-Genome Browser of the Biocyc database collection were used for synteny analysis (Karp *et al*., 2005; Consortium, 2010). Biochemical pathway prediction was performed using KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa and Goto, 2000). Presence/absence analysis was performed by comparison with representative genomes of insect symbionts and free-living *Gammaproteobacteria* using the predicted protein sequences and BLASTp with an *E*-value cutoff of 10^{-9} (Altschul *et al*., 1990). DNAPlotter was used for generating linear DNA maps (Carver *et al*., 2009).

Nucleotide sequence accession numbers

Gene sequences of symbionts and their insect hosts and the genome fragment obtained from '*Candidatus* S. adelgidicola' were deposited at the DDBJ/EMBL/GenBank databases under the accession numbers listed in Table 1.

Results and Discussion

Identification of adelgid hosts

Three adelgid populations found on *Abies* spp. in Austria (Gosau, Klausen-Leopoldsdorf) and Germany (Grafrath) were sampled and tentatively identified as members of the *A. nordmannianae/piceae* complex based on their morphology, their host plants *A. alba* and *A. nordmanniana*, as well as their location on trees. For molecular characterization of the three adelgid populations, genes encoding the cytochrome *c* oxidase subunit 1 (*coI*) and the eukaryotic elongation factor 1-alpha (*ef1alpha*) were partially amplified (639 nt for *coI* and 664 nt for *ef1alpha*) and sequenced from DNA extracted from up to 30 individuals. Both genes are considered useful molecular markers for inferring the phylogenetic relationships of eukaryotes, and they were used previously for analysis of adelgids (Havill *et al*., 2007; Foottit *et al*., 2009; Zurovcova *et al*., 2010).

Phylogenetic analyses of concatenated *coI* and *ef1alpha* data sets using neighbor joining, maximum parsimony and maximum likelihood methods confirmed the morphology-based identification of these insects and showed consistently their affiliation with the family Adelgidae in which they formed a stable, well-supported monophyletic group with *A. nordmannianae* and *A. piceae* (Supplementary Figure S1). Within this group, the concatenated data set and *coI* alone failed to differentiate between the species *A. piceae* and *A. nordmannianae*, which is consistent with previous studies (Havill *et al*., 2007; Foottit *et al*., 2009; Zurovcova *et al*., 2010). A closer inspection of the *ef1alpha* alignment revealed three alignment positions that differentiate *A. nordmannianae* from *A. piceae* (Supplementary Table S2). If these positions were taken as indicators for species delineation, adelgids sampled in Gosau represent *A. nordmannianae* and adelgids from Grafrath belong to *A. piceae*. Two different *ef1alpha* variants containing the signatures of *A. nordmannianae* and *A. piceae*, respectively, were observed for adelgids sampled in Klausen-Leopoldsdorf, which might indicate a mixed population of *A. piceae* and *A. nordmannianae* at this sampling site as observed previously on other occasions (Steffan, 1972). However, the presence of only three single-nucleotide polymorphisms in the comparatively small *ef1alpha* data set and inconclusive information from *coI* sequence data with respect to these adelgid species demonstrate that current molecular markers are not able to reliably differentiate the highly related and co-occurring

species *A. piceae* and *A. nordmannianae.* This is consistent with the highly similar morphology of both insect groups (Blackman and Eastop, 1994). In concordance with previous reports, we thus refer to the adelgids investigated in this study as members of the *A. nordmannianae/piceae* complex.

Two morphologically different bacteriocyte-associated symbionts

Staining of semi-thin sections of insects from the *A. nordmannianae/piceae* population sampled in Gosau revealed two differentially stained types of bacteriocytes in the adelgids' body cavity, which is consistent with previous studies (Profft, 1936; Buchner, 1953; Steffan, 1968). The size of the bacteriocytes increased from larval to adult stages with an average size between 30 and 50 μm in adults. In the first instar stage, the bacteriome consisted of loose-fitting cell aggregates, whereas the adult stages (apterous exulis and winged sexupara) contained a compact bacteriome (Figure 1). Similar to the situation in aphids, the bacteriome extended in two strands from the last thoracic to the seventh abdominal segment extending alongside the gut. The anterior parts of the bacteriome strands were connected to each other by bacteriocytes, ventral to the gut. In cross-sections, the bacteriome formed an H-like structure (Figure 1).

Electron microscopy confirmed the presence of two distinct types of bacteriocytes seen in histological sections. One type of bacteriocytes has single nuclei and contains coccoid, electron-dense bacteria, which divide by binary fission and are between 1 and 4 μm in diameter (Figures 2a and b). The second type of bacteriocytes is multinucleated and harbors less electron-dense, polymorphic bacteria, between 1.4 and 7 μm in size (Figures 2a and f). Both symbionts showed a Gram-negative type cell wall and were enclosed by a symbiosome membrane (Figures 2c–e). Multilamellar bodies, but no other bacterial forms, were found inside the bacteriocytes. Other tissues such as sheath cells or the hemocoel did not contain bacterial symbionts. Taken together, the association of *A. nordmannianae*/*piceae* with bacterial symbionts is very similar to the situation in aphids and other insects, which frequently contain two different kinds of bacteriocyte-associated symbionts, sometimes referred to as primary and secondary symbionts (Baumann, 2006).

Novel gammaproteobacterial symbionts

Sequencing and analysis of 16S rRNA genes amplified from up to 30 host individuals from 3 different adelgids populations revealed 2 distinct 16S rRNA sequences showing highest similarity with members of the *Gammaproteobacteria*. Both sequences, presumably representing the two symbiont morphotypes, were only moderately similar to each other (90.1–91.2%) and to other gammaproteobacterial sequences (∼95%). Sequence type 1 showed highest

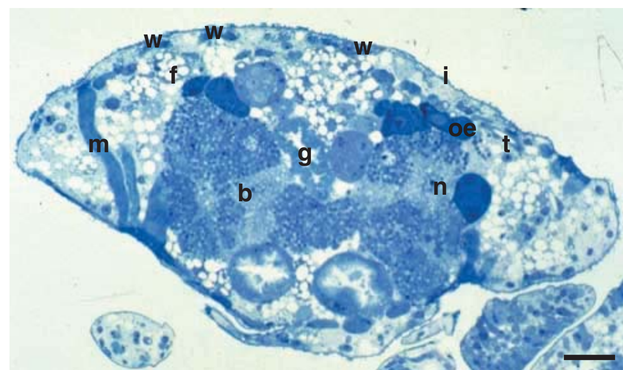

Figure 1 The bacteriome of *Adelges nordmannianae/piceae*. A semi-thin cross-section through the abdomen of a sexupara (adult winged life stage) stained with Richardson's solution (Richardson *et al*., 1960) is shown. Bacteriocytes can be readily seen in proximity to the gut. b = bacteriocytes filled with two different types of symbionts (dark and light blue), g = gut, n = nucleus, oe = oenocytes, t = tracheole, w = wax gland plate, f = fat body cell, m = muscle, i = integument; bar represents 50 μm.

similarity to *Sodalis glossinidius* str. 'morsitans', whereas sequence type 2 was most similar to *Serratia plymuthica* and *Serratia entomophila*. Both sequence types were found in all three populations and were nearly identical among the geographically distant sampling sites (99.7–99.9%). In phylogenetic trees, both sequence types established novel, deep branching lineages within the *Gammaproteobacteria* (Figure 3a). Depending on the method used for phylogeny inference, these lineages sometimes clustered together with other symbionts of insects, but overall comparison of different phylogenetic methods and bootstrap values did not provide support for proposing a specific affiliation of the two sequence types with other *Gammaproteobacteria*. The lack of phylogenetic information of rRNA genes to resolve deeply branching lineages is a well-known problem within this taxonomic clade (Williams *et al*., 2010).

Consistent with the two 16S rRNA sequence types found in *A. nordmannianae/piceae*, we also detected two different 23S rRNA gene sequences in the insect host. 23S rRNA sequence type 1 showed highest similarity (94.2%) to the *Sodalis*-related primary endosymbiont of *Sitophilus zeamais*. Sequence type 2 was most similar to '*Candidatus* Serratia symbiotica' (90.4%). Phylogenetic analysis confirmed the affiliation of both sequences with the *Gammaproteobacteria*, in which they formed novel lineages. Similar to the 16S rRNA-based phylogenetic analysis, both sequences clustered in some trees with other insect symbionts, but their relationship with other *Gammaproteobacteria* could not be unambiguously resolved (Figure 3b).

In situ *identification of* 'Candidatus *S. adelgidicola*' *and* 'Candidatus *E. adelgidicola*'

The two bacterial morphotypes observed by electron microscopy were readily visible using FISH and 16S

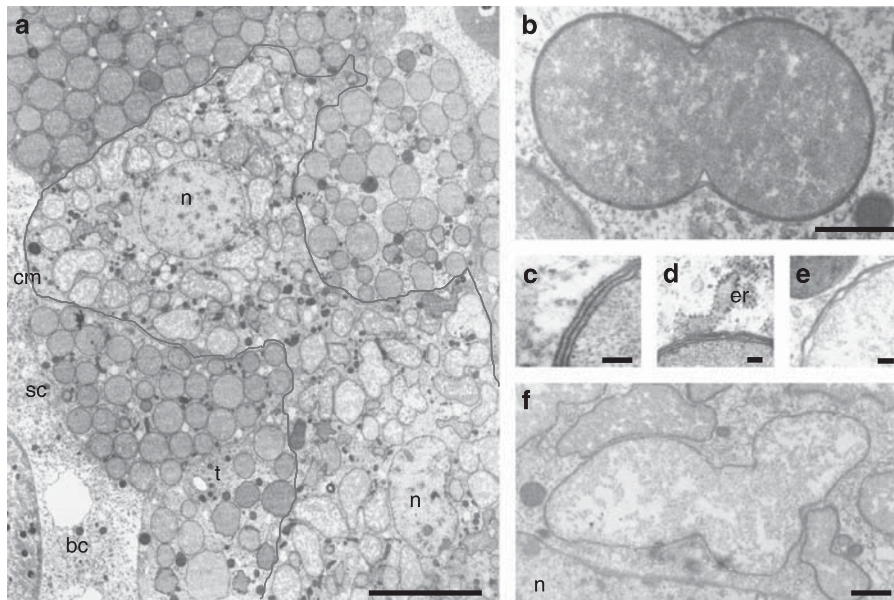

Figure 2 Ultrastructure of the bacterial symbionts of *Adelges nordmannianae/piceae*. (**a**) Electron microscopy revealed two distinct types of bacteriocytes containing different morphotypes of bacterial symbionts; bacteriocytes with electron-translucent bacteria are multinucleated; for clarity, the cell membrane of this bacteriocyte is highlighted by a gray line. (**b**) Coccoid symbiont ('*Candidatus* Steffania adelgidicola') undergoing cell division. (**c**) Detail of the coccoid symbiont ('*Candidatus* S. adelgidicola') showing its cell wall (two membranes) tightly surrounded by a third membrane, the symbiosome membrane, (**d**) which is associated with the rough endoplasmatic reticulum. (**e** and **f**) Polymorphic symbiont ('*Candidatus* Ecksteinia adelgidicola') and detail view of its cell wall (two membranes) and the symbiosome membrane. Bar in panel a represents 10 μm; bars in panels b and f represent 1 μm; bars in panels c, d and e represent 90 nm. n = nucleus, t = tracheole, sc = sheath cell, bc = body cavity, cm = cell membrane of the bacteriocyte, er = rough endoplasmatic reticulum.

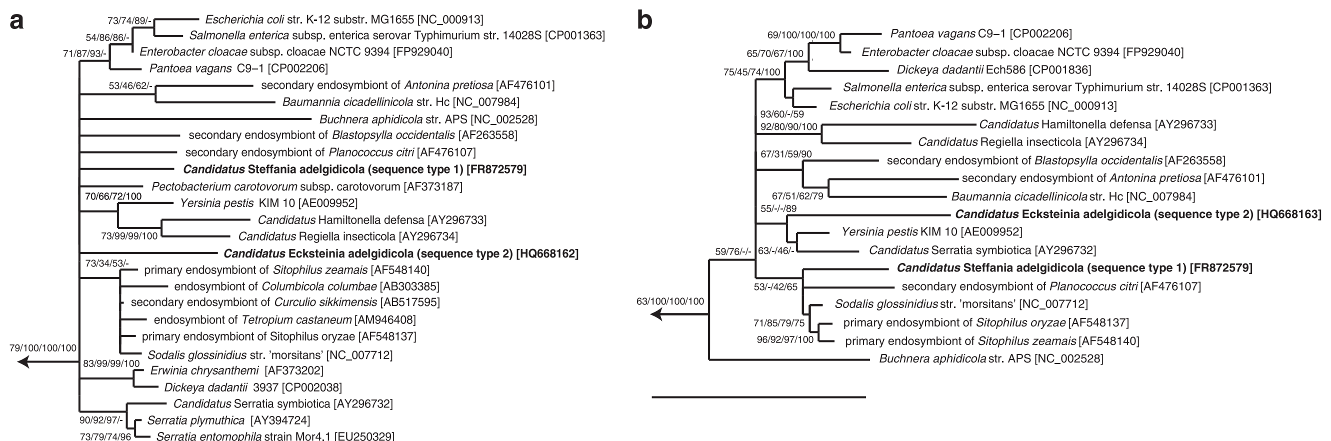

Figure 3 Phylogenetic relationships of the gammaproteobacterial symbionts of *Adelges nordmannianae/piceae*. TREEPUZZLE trees based on 16S rRNA (**a**) and 23S rRNA (**b**) genes are shown. 16S rRNA and 23S rRNA sequence types 1 represent '*Candidatus* Steffania adelgidicola'; sequence types 2 represent '*Candidatus* Ecksteinia adelgidicola'. The branching order near the root of the trees could not be resolved and differs between trees obtained with MrBayes, maximum likelihood, maximum parsimony and neighbor joining methods. TREEPUZZLE support values, bootstrap values for maximum parsimony and maximum likelihood (1000 resamplings), and posterior probabilities of MrBayes are indicated at the inner nodes. GenBank/EMBL/DDBJ accession numbers are given in square brackets. Bars, 10% estimated evolutionary distance.

rRNA-targeted probes in samples from all three adelgid populations (Figure 4a, Supplementary Figure S2). Probes specific for 16S and 23S rRNA sequence type 1 hybridized with the coccoid symbionts. Probes specific for sequence types 2 targeted the polymorphic symbionts, which showed an extremely low fluorescence signal that could be enhanced by application of three probes simultaneously (Supplementary Figure S3). All symbionts identified by FISH were located in bacteriocytes, which contained either one of the two symbionts. Combination with general bacterial probes demonstrated the absence of additional bacteria in this tissue.

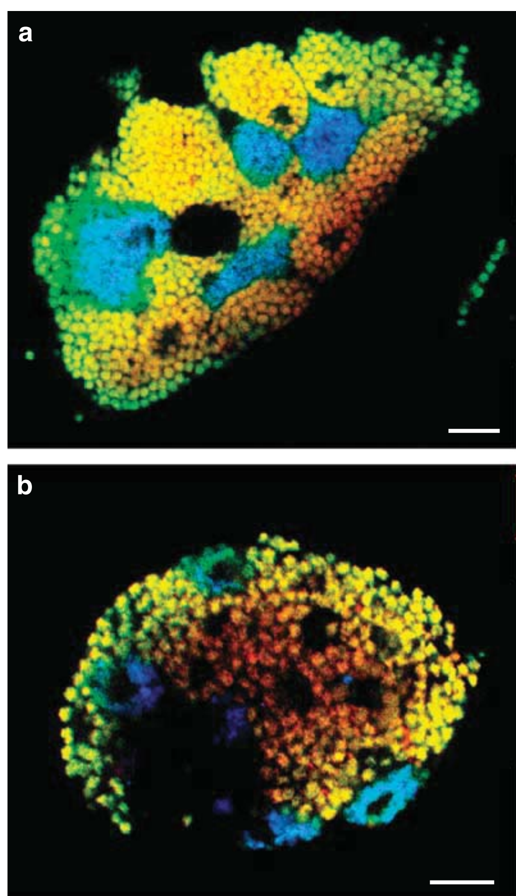

Figure 4 *In situ* identification and intracellular localization of '*Candidatus* Steffania adelgidicola' and '*Candidatus* Ecksteinia adelgidicola' in adults and eggs of *A. nordmannianae/piceae*. Bacterial symbionts were labeled by FISH using symbiont-specific 16S rRNA-targeted oligonucleotide probes together with a probe mix targeting all bacteria (Supplementary Table S1). Probes specific for the coccoid '*Candidatus* S. adelgidicola' were labeled with Cy3 (red); three probes specific for the polymorphic '*Candidatus* E. adelgidicola' were labeled with Cy5 (blue) and used simultaneously. Probes targeting all bacteria were labeled with FLUOS (green). The combined signal from bacterial and symbiont-specific probes appear yellow for '*Candidatus* S. adelgidicola' and blue–green for '*Candidatus* E. adelgidicola', respectively. (**a**) Bacteriocytes of an adult adelgid sampled in Gosau, Austria. (**b**) Both symbionts inside an adelgid egg. Bars represent 10 μm.

Taken together, we could show that *A. nordmannianae/piceae* contains two morphologically and phylogenetically distinct bacteriocyte-associated endosymbionts, which form novel evolutionary lineages within the *Gammaproteobacteria*. Notably, both symbionts are different from *Buchnera*, the obligate symbionts of most aphids which represent a sister group of the adelgids. They are also different from other known primary or secondary symbionts found in insects belonging to the order Sternorrhyncha such as mealybugs, psyllids or whiteflies (Baumann, 2006). *A. nordmannianae/piceae* has thus acquired its own gammaproteobacterial symbionts during evolution. The low degree of relationship with each other and with other *Gammaproteobacteria* justifies their taxonomic placement within two novel genera. According to Murray and Stackebrandt (1995), we propose the following provisional names.

'Candidatus *S. adelgidicola*'

'*Candidatus* S. adelgidicola' named in honor of the German entomologist August Wilhelm Steffan for his contributions to research on adelgids and their bacterial symbionts; 'adelgid-icola' meaning friend or lover of the Adelgidae. This symbiont of *A. nordmannianae/piceae* is coccoid with a cell size between 1 and 4 μm and has a Gram-negative type cell wall. It is surrounded by a symbiosome membrane and located in single-nucleated bacteriocytes. The symbiont is not cultured in cell-free medium or a cell line. '*Candidatus* S. adelgidicola' represents a novel genus within the class *Gammaproteobacteria* (phylum *Proteobacteria).* The basis of assignment is: 16S rRNA, 23S rRNA (GenBank/EMBL/DDBJ accession numbers HQ668158, HQ668159, FR872579).

'Candidatus *E. adelgidicola'*

'*Candidatus* E. adelgidicola' named in honor of the German entomologist Karl Eckstein (1859–1939) for his contributions to research on adelgids; 'adelgid-icola' meaning friend or lover of the Adelgidae. This symbiont of *A. nordmannianae/piceae* is polymorphic with a cell size between 1.4 and 7 μm and has a Gram-negative type cell wall. It is surrounded by a symbiosome membrane and is located in multinucleated bacteriocytes. The symbiont is not cultured in cell-free medium or a cell line. '*Candidatus* E. adelgidicola' represents a novel genus within the class *Gammaproteobacteria* (phylum *Proteobacteria*). The basis of assignment is: 16S rRNA, 23S rRNA (GenBank/EMBL/DDBJ accession numbers HQ668160, HQ668161, HQ668162, HQ668163).

Occurrence and vertical transmission

To further investigate the occurrence of '*Candidatus* S. adelgidicola' and '*Candidatus* E. adelgidicola' within a single adelgid population, we designed PCR assays specific for the 16S rRNA gene of each of the two symbionts (Supplementary Table S1) and screened 10 individuals from the population sampled in Gosau (Supplementary Figure S4). PCR products were obtained from all individuals and 16S rRNA sequences were nearly identical (99.5%) to the previously recovered sequences of '*Candidatus* S. adelgidicola' and '*Candidatus* E. adelgidicola', suggesting that both symbionts are present in all individuals of the investigated population. We next analyzed different life stages of the adelgid host using symbiont-specific PCR assays, and we detected '*Candidatus S. adelgidicola*' and '*Candidatus* E. adelgidicola' in both sexuparae and exules

(Supplementary Figure S5). In addition, we performed FISH on eggs of *A. nordmannianae/piceae* and could demonstrate bacteriocytes containing each of the two symbionts, respectively (Figure 4b). Taken together, these findings are strong evidence for a vertical transmission of '*Candidatus* S. adelgidicola' and '*Candidatus* E. adelgidicola' from mother to offspring, corroborating previous histological studies of *A. nordmannianae/piceae* and its bacterial symbionts (Profft, 1936; Buchner, 1953; Steffan, 1972). Vertical inheritance is a hallmark of obligate symbionts of insects which are well adapted to and essential for their hosts (Moran *et al*., 2008). Thus, '*Candidatus* S. adelgidicola' and '*Candidatus* E. adelgidicola' likely have an important role for their host's biology. This notion is consistent with the observation that the related hemlock woolly adelgid *Adelges tsugae*, for which morphologically similar symbionts were described, was inhibited in its development after treatment with antibiotics (Shields and Hirth, 2005).

*Evidence for the presence of most biosynthetic pathways in '*Candidatus *S. adelgidicola'*

Known obligate bacterial symbionts of insects mainly serve as nutrient providers complementing their hosts' diet, in some cases as the result of synergistic functions of two different symbionts (Wu *et al*., 2006; McCutcheon and Moran, 2007; Gosalbes *et al*., 2008); other symbionts can protect their hosts against parasites or heat (Montllor *et al*., 2002; Oliver *et al*., 2003; Moran *et al*., 2005; Vorburger *et al*., 2010) or they may help to expand their host's host range (Tsuchida *et al*., 2004). To get first insights into the genetic basis and the role of '*Candidatus* S. adelgidicola' and '*Candidatus* E. adelgidicola' for their insect host, we generated a metagenomic fosmid library from purified bacterial symbionts. In total, 14 208 fosmid clones were screened using 16S and 23S rRNA gene-specific primers for both symbionts. Although no clones containing rRNA genes of '*Candidatus* E. adelgidicola' were identified, eight clones containing rRNA genes of '*Candidatus* S. adelgidicola' were found. On the basis of restriction fragment length polymorphism analysis for estimation of the extent of overlap between these clones, we selected two clones containing the 16S rRNA gene (33 and 39 kb in size) and one clone containing the 23S rRNA gene (35 kb) for sequencing. Shotgun sequences of these three clones could be assembled into one continuous contig with a total size of 85 kb. This genome fragment of '*Candidatus* S. adelgidicola' includes 44 predicted coding sequences (CDS), 3 rRNA genes, 2 tRNAs and 5 pseudogenes (Supplementary Figure S6, Supplementary Table S3). No non-coding RNAs were detected using the Rfam database (Gardner *et al*., 2009).

Obligate symbionts of insects typically possess small genomes compared with free-living bacteria. As a consequence, biosynthetic pathways are frequently reduced substantially and the symbionts thus rely on the import of metabolites from their hosts (Ramsey *et al*., 2010). Interestingly, the genome fragment of '*Candidatus* S. adelgidicola' encodes for proteins involved in a wide range of biosynthetic pathways (Figure 5, Supplementary Table S3). This includes proteins required for glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle. Proteins involved in the synthesis of amino acids, in pyrimidine and purine metabolism, as well as in lipid and lipopolysaccharide biosynthesis are also encoded. In addition, genes coding for proteins involved in metabolism of cofactors and vitamins such as biotin and nicotinamide -adenine dinucleotide are present. Although it remains unknown whether '*Candidatus* S. adelgidicola' encodes the full gene complement for these pathways, the observed diversity of metabolic functions encoded on the genome fragment suggests that the genetic repertoire of this symbiont resembles that of most free-living bacteria or facultative symbionts (Toh *et al*., 2006; Degnan *et al*., 2009; Burke and Moran, 2011).

For example, the genome fragment of '*Candidatus* S. adelgidicola' contains genes for lipid A synthesis (*lpxA*, *lpxB*), a key lipopolysaccharide component, which are absent in the long-term insect-associated symbionts *Buchnera*, *Baumannia cicadellinicola* and '*Candidatus* Carsonella ruddii' but still present in the evolutionary younger obligate symbionts *Wigglesworthia glossinidia* and '*Candidatus* Blochmannia pennsylvanicus' or the facultative symbionts *S. glossinidius*, *S. symbiotica* and '*Candidatus* H. defensa' (Shigenobu *et al*., 2000; Akman *et al*., 2002; Degnan *et al*., 2005; Nakabachi *et al*., 2006; Wu *et al*., 2006; Moran *et al*., 2008).

Amino acids have a special role in many symbiotic associations between bacteria and insects. Although on the one hand, many obligate symbionts produce certain amino acids that are lacking in their hosts' diet, they possess on the other hand often only limited capabilities to synthesize other amino acids and obtain these compounds through the host metabolism or through other symbionts (Shigenobu *et al*., 2000; Zientz *et al*., 2004; Moran *et al*., 2008; McCutcheon *et al*., 2009). '*Candidatus* S. adelgidicola' encodes proteins involved in the synthesis of phenylalanine, tyrosine, tryptophan, lysine, glycine, serine, threonine, cysteine and methionine, as well as proteins for synthesis of branched-chain amino acids such as isoleucine, valine and leucine. Thus, similar to facultative symbionts like *S. glossinidius* (Toh *et al*., 2006), '*Candidatus* S. adelgidicola' might be able to generate most amino acids independent from its insect host. It is also conceivable that '*Candidatus* S. adelgidicola' has a role in essential amino acid biosynthesis for its adelgid host.

Taken together, the presence of several genes in the '*Candidatus* S. adelgidicola' genome that are also found in free-living bacteria and facultative

symbionts but that are lacking in many obligate symbionts suggests that the '*Candidatus* S. adelgidicola' is more similar to known facultative symbionts with respect to its metabolic capabilities. This might indicate that '*Candidatus* S. adelgidicola' is an evolutionarily young symbiont, and that adaptation to an intracellular lifestyle is less pronounced than in long-term obligate symbionts.

genome size
Ec Sg Ss Hd Bp Wg Bc Ba Cr
facultative obligate
C, Energy production and conversion
FumC
E, Amino acid transport, metabolism
PheA
IlvM
LysC
IlvD
IlvE
IlvL
IlvG
F, Nucleotide transport and metabolism
PyrB
TadA
G, Carbohydrate transport, metabolism
Gnd
Pgi
Pgk
Fba
H, Coenzyme metabolism
FolD
Fre
BirA
I, Lipid metabolism
AccA
FabZ
CdsA2
UppS
Dxr
J, Translation, ribosomal structure and biogenesis
RluD
RpmE
K, Transcription
CspC*
RpoH
L, DNA replication, recombination and repair
Ung
DnaE
RnhB*
PriA
M, Cell envelope biogenesis, outer membrane
RsmH
LpxB
LpxA
LpxD
Skp
YaeT
RseP
WaaL
RfaB
MurB
O, Posttransl. modification, protein turnover
ClpB*
P, Inorganic ion transport and metabolism
TrkH
R, General function prediction only
YfiO
YfbR
UbiB*
S, Function unknown
YfiH*
T, Signal transduction mechanisms
DksA
no related COG
RfaZ
WalW3

Figure 5 Affiliation of '*Candidatus* Steffania adelgidicola' proteins to NCBI COGs and occurrence of homologs in the genomes of *E. coli* and other insect symbionts. Gray boxes indicate the presence of homologs (*E*-value $<10^{-9}$, except for pseudogenes); white indicates absence. The presence of homologs correlates with genome size, with obligate symbionts lacking many of the genes found on the '*Candidatus* S. adelgidicola' genome fragment. * Indicates pseudogenes in '*Candidatus* S. adelgidicola'; it must be noted that more distant homologs of IlvL and WaaL are present in *E. coli*; Ec = *Escherichia coli* K-12 substr. MG1655 (NC_000913), Sg = *Sodalis glossinidius* str. 'morsitans' (NC_007712), Ss = *Serratia symbiotica* str. Tucson (AENX00000000), Hd = '*Candidatus* Hamiltonella defensa 5AT' (*Acyrthosiphon pisum*) (NC_012751), Bp = '*Candidatus* Blochmannia pennsylvanicus str. BPEN' (NC_007292), Wg = *Wigglesworthia glossinidia* endosymbiont of *Glossina brevipalpis* (NC_004344), Bc = *Baumannia cicadellinicola* str. Hc (*Homalodisca coagulata*) (NC_007984), Ba = *Buchnera aphidicola* str. APS (*Acyrthosiphon pisum*) (NC_002528), Cr = '*Candidatus* Carsonella ruddii PV' (NC_008512).

'Candidatus S. adelgidicola' is an evolutionarily young symbiont

Reduction of genome size occurs through deletion of genes or genome regions. Deletion may begin with the disruption of (redundant) genes or promoter regions by point mutations, frameshifts and integration of transposable elements, which leads to the formation of pseudogenes (Mira *et al*., 2001). Owing to the lack of selection, these pseudogenes are degraded gradually until they have disappeared completely. This process correlates frequently with a change in the environment or lifestyle, for example, during adaptation to intracellular symbiosis with an eukaryotic host (Moran, 2002; Klasson and Andersson, 2004). The genome of the pathogen *Mycobacterium leprae* is a well-known example for a genome in an early stage of genome erosion (Cole *et al*., 2001). It contains a high number of pseudogenes ($n = 1116$) and large intergenic spacer regions (representing strongly degraded pseudogenes, which are not recognized as such anymore). On the other end of the spectrum, the highly reduced genome of *Buchnera* contains only few pseudogenes and the lengths of intergenic regions are typical for more stable genomes (Mira *et al*., 2001). The genome fragment of '*Candidatus* S. adelgidicola' contains five pseudogenes (Supplementary Figure S6), which were formed by point mutations introducing premature stop codons and frameshifts. Three of these pseudogenes, which are absent in most obligate symbionts, are in genome regions that are syntenic with *Escherichia coli* (Figure 6). Extrapolated, the observed number of pseudogenes on the '*Candidatus* S. adelgidicola' genome fragment corresponds to

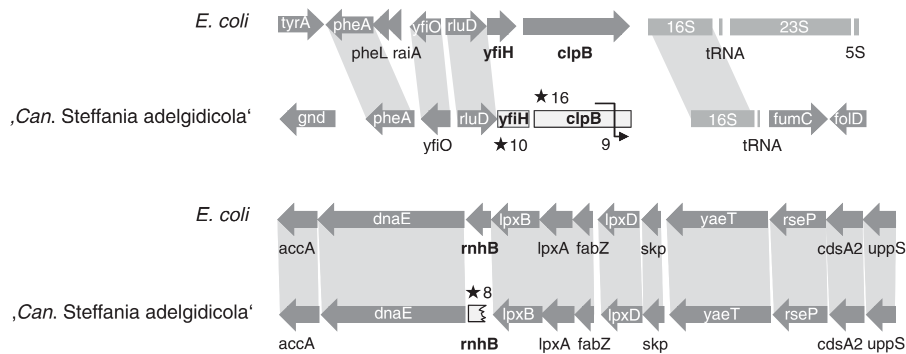

Figure 6 Pseudogene formation in '*Candidatus* Steffania adelgidicola'. The formation of pseudogenes (light grey boxes) in the '*Candidatus* S. adelgidicola' genome can be observed in syntenic regions with *E. coli* K-12 substr. MG1655. It must be noted that the genome region shown in the lower panel is also synthenic with *S. glossinidius*, '*Candidatus* Blochmannia pennsylvanicus' and other *Gammaproteobacteria* but not with *Buchnera*. The number of stop codons (star symbol) and frameshifts (black arrow) are noted. The *rnhB* gene is truncated at the 5′ end.

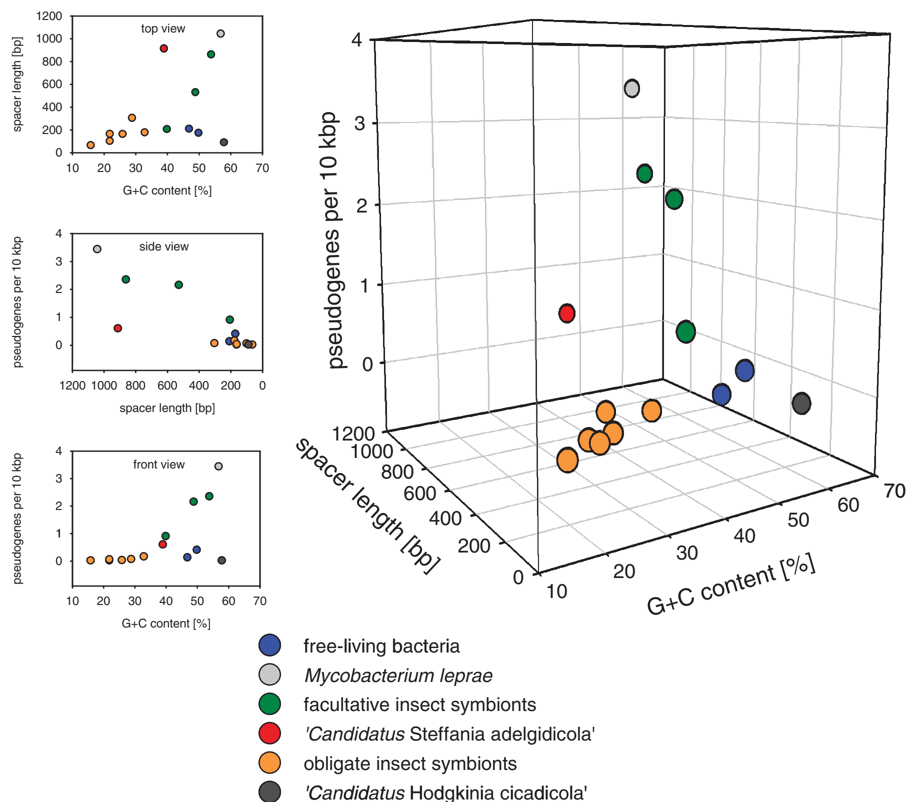

Figure 7 Correlation of bacterial lifestyle and general genome features for selected free-living bacteria and insect symbionts. The numbers of pseudogenes, genomic G + C content and average spacer length are displayed (for details, see Supplementary Table S4). Gammaproteobacterial obligate symbionts of insects share similar genome features and are clearly separated in this three-dimensional scatter plot. Signs for genome erosion can be observed in facultative symbionts to different degrees. The placement of '*Candidatus* Steffania adelgidicola' between these two groups suggests that '*Candidatus* S. adelgidicola' is an evolutionarily young symbiont the genome of which is in the process of size reduction. For comparison, '*Candidatus* Hodgkinia cicadicola', an alphaproteobacterial symbiont of cicadas is shown. Its genome is highly reduced but in contrast to the genomes of gammaproteobacterial insect symbionts shows a high G + C content.

0.584 pseudogenes per 10 kbp, which is closer to the number of pseudogenes in facultative symbionts (0.886–2.33) than in obligate symbionts (0–0.146; Figure 7, Supplementary Table S4). Consistent with this notion, the average spacer length of '*Candidatus* S. adelgidicola' (909.3 bp) is also in the range of facultative symbionts (201.6–857.6 bp) and markedly larger than that of obligate symbionts (61.4–299.7 bp; Figure 7, Supplementary Table S4). Thus, the genome of '*Candidatus* S. adelgidicola' shows

typical features of facultative symbionts and is less streamlined than the genomes of obligate symbionts.

Within the *Gammaproteobacteria*, genome reduction is also accompanied by a decrease in genomic G+C content (Moran *et al.*, 2008). The average G+C content of the genome fragment of '*Candidatus* S. adelgidicola' is 39.1%. Although the G+C content of the closest free-living relative of '*Candidatus* S. adelgidicola' is unknown, this is in the range of the genomic G+C content of other facultative symbionts of insects but notably higher than the G+C content in highly reduced bacterial genomes from obligate symbionts such as *Buchnera* and *Wigglesworthia* (Figure 7, Supplementary Table S4; Williams *et al.*, 2010). These genome features and the lifestyle of bacterial symbionts of insects reflect the age of their association with their insect hosts, with facultative symbionts being evolutionary younger symbionts than obligate symbionts (Supplementary Table S4; Moran *et al.*, 2008). Taken together, if G+C content, pseudogenes and spacer length are taken as proxies for the degree of genome reduction, lifestyle and evolutionary age of the symbiosis, '*Candidatus* S. adelgidicola' is an evolutionarily young bacteriocyte-associated symbiont and its genome might still be undergoing genome reduction.

Although phylogenetic analysis of 16S and 23S rRNA genes failed to identify the closest relative of '*Candidatus* S. adelgidicola' (Figure 3), two lines of evidence suggest an affiliation with *Sodalis*-related symbionts of diverse insect hosts (Kaiwa *et al.*, 2010). First, 42 out of 44 predicted proteins encoded on the '*Candidatus* S. adelgidicola' genome fragment show *S. glossinidius* proteins as closest homologs. Second, in phylogenetic trees based on the RNA polymerase sigma-32 factor RpoH, '*Candidatus* S. adelgidicola' formed a monophyletic group with the respective *S. glossinidius* protein (Supplementary Figure S7). *Sodalis*-related symbionts are considered evolutionary young symbionts showing less reduced genomes compared with long-term-associated obligate symbionts (Rio *et al.*, 2003), corroborating our findings from the analysis of the '*Candidatus* S. adelgidicola' genome fragment.

Conclusions

This study shows that adelgids of the *A. nordmannianae/piceae* complex harbor two novel *Gammaproteobacteria* as bacteriocyte-associated symbionts, both of which show no close relationship with known facultative or obligate symbionts of other insects. These adelgids have thus acquired their symbionts independently from members of their sister group, the aphids. The genome of one of these symbionts, '*Candidatus* S. adelgidicola', is reduced compared with the genome of free-living *Gammaproteobacteria*, but it is less streamlined than the genomes of known long-term obligate insect symbionts. The symbiosis between '*Candidatus* S. adelgidicola' and its insect host thus most likely represents an evolutionary young association, which was established after diversification of the Adelgidae. We predict that other members of this insect family have acquired their symbionts independently, a notion which is also supported by evidence for a larger morphological diversity of bacterial symbionts in this insect family (Profft, 1936; Steffan, 1968). Further molecular characterization of other adelgid symbionts is required to improve our understanding of the evolutionary history of these associations and to reveal the specific functions of these intracellular symbionts for their insect hosts.

Acknowledgements

We gratefully acknowledge Marc Mußmann for advice during fosmid library preparation, and Frank Maixner and Patrick Tischler for help with sampling and annotation, respectively. This work was partially funded by the Austrian Science Fund (FWF) grants Y277-B03 and P22533-B17.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)

Bacteriocyte-associated gammaproteobacterial symbionts of the *Adelges nordmannianae/piceae* complex (Hemiptera: Adelgidae)

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Supplementary Information

Table S1: Primer and probes used in this study.

Short name[a]	Sequence (5´-3´)	Specificity	Target site	Annealing temperature/ Formamide concentration	Reference
16S and 23S rRNA primer					
616V	AGA GTT TGA TYM TGG CTC	16S rRNA gene, most *Bacteria*	8-25	52	(Juretschko *et al.*, 1998)
1492R	GGY TAC CTT GTT ACG ACT T	16S rRNA gene, most *Bacteria* and *Archaea*	1492-1510	52	(Loy *et al.*, 2005)
TTL1-16S-70F	CAT CGG AAA GGA GTT TAC TTC	16S rRNA gene, '*Candidatus* Steffania adelgidicola'	70-90	58	This study
TTL1-16S-1267R	GAG GTC CGC TGA CCC TCA	16S rRNA gene, '*Candidatus* Steffania adelgidicola'	1267-1284	58	This study
TTL4-16S-107F	GGA CGG GTG AGT AAT ATT	16S rRNA gene, '*Candidatus* Ecksteinia adelgidicola'	107-124	58	This study
TTL4-16S-1449R	GTA AGT GCC CTC CAA TAC	16S rRNA gene, '*Candidatus* Ecksteinia adelgidicola'	1450-1466	58	This study
255f	AGT AGY GGC GAG CGA A	23S rRNA gene, *Bacteria*	241-255	50	(Lane, 1991)
1930R	CGA CAA GGA AYT TCG CTA C	23S rRNA gene, *Bacteria*	1930-1948	50	(Hunt *et al.*, 2006)
TTL1-23S-346F	GGT GTG TTA GTT GTG AGC	23S rRNA gene, '*Candidatus* Steffania adelgidicola'	347-364	64	This study
TTL1-23S-1169R	CCA GCA ACA CTC TCA TGC	23S rRNA gene, '*Candidatus* Steffania adelgidicola'	1169-1186	64	This study
TTL1-23S-347R	GCT CAC AAC TAA CAC ACC	23S rRNA gene, '*Candidatus* Steffania adelgidicola'	347-364	n.d.	This study
TTL4-23S-260F	GGG ACA GCC CAG AGC TAG	23S rRNA gene, '*Candidatus* Ecksteinia adelgidicola'	260-277	64	This study
TTL4-23S-1169R	GCA ATG CAT ATT TCA CAT	23S rRNA gene, '*Candidatus* Ecksteinia adelgidicola'	1169-1183	64	This study
Host gene targeting primer					
911	TTT CTA CAA ATC ATA AAG ATA TTG G	Mitochondrial cytochrome c oxidase subunit 1 (*col*), various eukaryotes		50	(Guryev *et al.*, 2001)
912	TAA ACT TCA GGG TGA CCA AAA AAT CA	Mitochondrial cytochrome c oxidase subunit 1 (*col*), various eukaryotes		50	(Guryev *et al.*, 2001)
AdelEF1F1	GTA CAT CCC AAG CCG ATT GT	Partial nuclear elongation factor 1-alpha (*ef1alpha*), various Hemiptera		61	(Havill *et al.*, 2007)
AdelEF1R2	CTC CAG CTA CAA AAC CAC GA	Partial nuclear elongation factor 1-alpha (*ef1alpha*), various Hemiptera		61	(Havill *et al.*, 2007)
Oligonucleotide probes					
EUB338-I	GCT GCC TCC CGT AGG AGT	16S rRNA, most *Bacteria*	338-355[b]	10-50	(Amann *et al.*, 1990)
EUB338-II	GCA GCC ACC CGT AGG TGT	16S rRNA, Bacteria not covered by probe EUB338-I, e.g. many *Planctomycetes*	338-355[b]	10-60	(Daims *et al.*, 1999)
EUB338-III	GCT GCC ACC CGT AGG TGT	16S rRNA, Bacteria not covered by probe EUB338-I, e.g. many *Verrucomicrobia*	338-355[b]	10-60	(Daims *et al.*, 1999)
TTL1-1027	GTC ACA GAG TCC CCT AAG	16S rRNA, '*Candidatus* Steffania adelgidicola'	1028-1045	30-35	This study
TTL4-608	CAC GTT AAG CGC AGG GAT	16S rRNA, '*Candidatus* Ecksteinia adelgidicola'	609-626	30-35	This study
TTL4-1264	GGT TTG CTT ACT CTT GCG	16S rRNA, '*Candidatus* Ecksteinia adelgidicola'	1265-1282	30-35	This study
TTL4-1445	GCC CTC CAA TAC ATG GTT	16S rRNA, '*Candidatus* Ecksteinia adelgidicola'	1446-1460	30-35	This study
NONEUB	ACT CCT ACG GGA GGC AGC	Control probe complementary to EUB338-I	338-355	n.d.	(Wallner *et al.*, 1993)

TTL1-1215	GCA CAC CTT ACA GTG CAC	23S rRNA, '*Candidatus* Steffania adelgidicola'	1216-1233	10-25	This study
TTL4-877	AAG CTG GGA TAG CCC CTT	23S rRNA, '*Candidatus* Ecksteinia adelgidicola'	878-895	10-25	This study
TTL4-967	GTT GTT TCC CTT TAC ACG	23S rRNA, '*Candidatus* Ecksteinia adelgidicola'	968-985	10-25	This study
TTL4-1215	GCA ATC CTC ACG AGT CAC	23S rRNA, '*Candidatus* Ecksteinia adelgidicola'	1216-1233	10-25	This study

[a] Short probe name used in this study or in the references
[b] EUB338-I, EUB338-II, and EUB338-III were applied simultaneously to target most *Bacteria*

Table S2: *ef1alpha* single nucleotide polymorphisms differentiating *A. nordmannianae* and *A. piceae* (grey background) and additional variable sites among the *A. nordmannianae/A. piceae* species complex. All available *ef1alpha* gene sequences of this complex are shown.

Organism	Sampling location	Genbank Acc. Number	Alignment position[a]									
			203	204	295	331	398	446	509	548	649	650
Adelges nordmannianae	Slovak Republic	EF073235	T	T	T	T	T	T	A	T	T	C
Adelges piceae	Owlshead; Maine; USA	EF073239	A	-	T	T	C	C	A	C	T	C
Adelges piceae	Delemont; Switzerland	EF073240	T	T	T	T	C	C	A	C	T	C
Adelges nordmannianae/piceae	Gosau; Austria	HQ668164	T	T	T	T	T	T	A	T	T	C
Adelges nordmannianae/piceae	Grafrath; Germany	HQ668167	T	-	T	T	C	C	A	C	T	C
Adelges nordmannianae/piceae Klausen-Leopoldsdorf	Klausen-Leopoldsdorf; Austria	HQ668165	T	T	T	C	T	T	A	T	T	T
Adelges nordmannianae/piceae **Klausen-Leopoldsdorf**	Klausen-Leopoldsdorf; Austria	HQ668166	T	-	C	T	C	C	G	C	A	C

[a]nucleotide position based on the *ef1alpha* gene sequence of *Adelges nordmannianae* (EF073235)

Supplementary Table S3: List of genes encoded on the genome fragment of '*Candidatus* Steffania adelgidicola' and the classification of their proteins into COG categories. Comparison of proteins of '*Candidatus* Steffania adelgidicola' to proteins of other insect symbionts and free-living bacteria.

***Candidatus* Steffania adelgidicola'**						Ec	Sg	Ss	Hd	Bp	Wg	Bc	Ba	Cr				
locus tags	**start**	**stop**	**type**	**gene discription**	**gene name**										**remarks**	**COGs**	**code**	**discription**
STA_A00010	907	1	orf	Ribosomal RNA small subunit methyltransferase H	*rsmH*	6e-109	6e-122	4e-105	4e-96	4e-90	5e-76	6e-101	1e-86	2e-04		COG0275	M	Cell envelope biogenesis, outer membrane
STA_A00020	2981	2310	orf	Uracil-DNA glycosylase	*ung*	2e-87	2e-102	7e-85	6e-79	5e-81	1e-50	3e-90	2e-73	2.6		COG0692	L	DNA replication, recombination and repair
STA_A00030	4730	3771	orf	Acetyl-coenzyme A carboxylase carboxyl transferase subunit	*accA*	5e-164	1e-176	4e-166	2e-149	3e-146	3e-144	4e-154	2.1	0.19		COG0825	I	Lipid metabolism
STA_A00040	8224	4742	orf	DNA polymerase III subunit alpha	*dnaE*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5e-122		COG0587	L	DNA replication, recombination and repair
STA_A00050	8300	8623	pseudogene	Ribonuclease HII*	*rnhB**	2e-85	7e-113	7e-89	1e-80	6e-55	3.9	0.15	0.65	2.5	1	COG0164	L	DNA replication, recombination and repair
						0.023	0.003	0.14	1.3	1.3	0.77	2.8	1.1	0.044	2			
STA_A00060	10062	8878	orf	Lipid-A-disaccharide synthase	*lpxB*	2e-154	8e-169	2e-157	2e-132	5e-135	3e-155	0.006	0.82	0.38		COG0763	M	Cell envelope biogenesis, outer membrane
STA_A00070	10820	10032	orf	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-	*lpxA*	1e-117	2e-133	2e-112	8e-86	9e-111	6e-105	2.0	0.020	0.76		COG1043	M	Cell envelope biogenesis, outer membrane
STA_A00080	11280	10825	orf	(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase	*fabZ*	7e-70	2e-79	1e-69	2e-66	5e-74	3e-08	6e-67	5e-10	7.0		COG0764	I	Lipid metabolism
STA_A00090	12452	11430	orf	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	*lpxD*	1e-153	2e-168	4e-152	3e-131	6e-138	7e-116	1e-08	5e-11	0.022		COG1044	M	Cell envelope biogenesis, outer membrane
STA_A00100	12953	12456	orf	Chaperone protein skp	*skp*	2e-39	1e-67	2e-57	2e-28	1e-45	3e-32	1e-59	1.7	6.5		COG2825	M	Cell envelope biogenesis, outer membrane
STA_A00110	15491	13065	orf	Outer membrane protein assembly factor yaeT	*yaeT*	0.0	0.0	0.0	0.0	0.0	1e-143	0.0	3e-103	0.010		COG4775, COG0729	M	Cell envelope biogenesis, outer membrane
STA_A00120	16878	15529	orf	Regulator of sigma E protease	*rseP*	1e-178	0.0	4e-04	2e-125	8e-126	4e-89	1e-139	0.025	0.15		COG0750	M	Cell envelope biogenesis, outer membrane
STA_A00130	17755	16898	orf	Phosphatidate cytidylyltransferase	*cdsA2*	5e-94	3e-108	1e-86	1e-88	2e-71	6e-38	1e-81	4.1	0.13		COG0575	I	Lipid metabolism
STA_A00140	18506	17763	orf	Undecaprenyl pyrophosphate synthase	*uppS*	7e-100	3e-117	1e-101	5e-87	1e-95	4e-95	1e-101	3e-80	2.1		COG0020	I	Lipid metabolism
STA_A00150	19902	18706	orf	1-deoxy-D-xylulose 5-phosphate reductoisomerase	*dxr*	5e-147	2e-174	1e-149	4e-145	3e-132	2e-131	2e-153	3e-122	0.62		COG0743	I	Lipid metabolism
STA_A00160	22196	20790	orf	6-phosphogluconate dehydrogenase, decarboxylating	*gnd*	0.0	0.0	0.0	0.0	0.0	0.037	0.0	0.0	2e-39		COG0362	G	Carbohydrate transport and metabolism
STA_A00170	24086	22920	orf	P-protein	*pheA*	4e-142	2e-164	9e-134	0.075	2e-69	0.40	0.007	9e-97	0.83		COG0077, COG1605	E	Amino acid transport and metabolism
STA_A00180	24957	24226	orf	UPF0169 lipoprotein yfiO	*yfiO*	8e-91	1e-101	2e-86	2e-78	1e-51	3e-25	2e-54	2e-34	2.3		COG4105	R	General function prediction only
STA_A00190	25088	26068	orf	Pseudouridine synthase	*rluD*	4e-123	6e-139	3e-123	1e-108	3e-91	2e-65	1e-98	5e-79	3e-15		COG0564	J	Translation, ribosomal structure and biogenesis
STA_A00200	26065	26796	pseudogene	UPF0124 protein yfiH*	*yfiH**	5e-145	9e-21	0.11	1.8	1.4	0.63	4.1	0.34	0.62	3	COG1496	S	Function unknown
						3e-06	0.44	2.6	0.45	0.31	0.14	9.8	1.0	5.8	4			
STA_A00210	26937	29232	pseudogene	Chaperone protein clpB*	*clpB**	0.0	0.0	0.0	0.0	0.0	1e-04	0.0	1e-05	9e-08	5	COG0542	O	Posttranslational modification, protein turnover, chaperones
						1e-160	3e-178	2e-73	7e-134	6e-60	0.71	9e-145	4.0	0.65	6			
STA_Ar0010	30685	32195	rRNA	16S rRNA gene														
STA_At0010	32250	32322	tRNA	Glu tRNA														
STA_A00220	32564	33952	orf	Fumarate hydratase class II	*fumC*	0.0	0.0	8e-86	0.0	0.0	1e-178	0.0	2e-09	1.3		COG0114	C	Energy production and conversion
STA_A00230	34852	33995	orf	Bifunctional protein folD	*folD*	9e-130	6e-142	1e-74	2e-115	2e-113	2e-109	1e-124	4e-105	0.13		COG0190	H	Coenzyme metabolism
STA_At0020	35050	35123	tRNA	Arg tRNA														
STA_A00240	36028	35444	orf	UPF0207 protein	*yfbR*	3e-80	5e-98	5e-83	2e-76	5.6	4.4	0.56	0.93	0.11		COG1896	R	General function prediction only
STA_A00250	37493	38389	orf	putative lipopolysaccharide glycosyltransferase	*rfaZ*	2e-12	3e-131	0.76	0.69	1.6	0.75	1.3	2.0	1.6		no COG		
STA_A00260	38370	39575	orf	Putative lipid A core:surface polymer ligase	*waaL*	1.2	63-139	2.2	2.1	1.2	0.32	2.3	4.7	5.6		COG3307	M	Cell envelope biogenesis, outer membrane
STA_A00270	40491	39529	orf	lipopolysaccharide biosynthesis protein	*walW3*	0.91	1e-150	7e-130	0.16	3.3	0.55	1.0	0.28	2.8		no COG		
STA_A00280	41314	42429	orf	Putative lipopolysaccharide glycosyltransferase	*rfaB*	3e-13	5e-164	1e-119	6e-07	0.46	0.89	0.17	2.2	0.076		COG0438	M	Cell envelope biogenesis, outer membrane
STA_A00290	44929	43079	orf	Dihydroxy-acid dehydratase	*ilvD*	0.0	0.0	0.0	0.028	0.0	0.81	0.33	0.0	4e-83		COG0129	E, G	Amino acid transport and metabolism [E], Carbohydrate transport and metabolism [G]
STA_A00300	45919	44987	orf	Branched-chain-amino-acid aminotransferase	*ilvE*	4e-158	5e-169	0.12	0.037	1e-135	1.3	2e-08	2.7	4e-04		COG0115	E, H	Amino acid transport and metabolism [E], Coenzyme transport and metabolism [H]
STA_A00310	46195	45965	orf	Acetolactate synthase isozyme 2 small subunit	*ilvM*	6e-20	2e-30	3e-15	1.1	4e-20	0.44	7.3	1.8	nss		COG0440	E	Amino acid transport and metabolism
STA_A00320	47841	46195	orf	Acetolactate synthase isozyme 2 large subunit	*ilvG*	0.0	0.0	5e-22	5e-04	0.0	0.15	0.051	4e-122	9e-96		COG0028	E, H	Amino acid transport and metabolism [E], Coenzyme transport and metabolism [H]
STA_A00330	48083	47982	orf	IlvGMEDA operon leader peptide	*ilvL*	nss	0.39	nss	3.4	3.8	nss	nss	8.4	4.7		no COG		
STA_A00340	50001	49042	orf	Bifunctional protein birA	*birA*	1e-115	9e-137	7e-118	4e-92	2e-68	1e-49	1e-71	0.94	0.33		COG0340 [H], COG1654 [K]	K, H	Coenzyme metabolism [H], Transcription [K]
STA_A00350	51038	49998	orf	UDP-N-acetylenolpyruvoylglucosamine reductase	*murB*	2e-116	5e-139	2e-107	4e-101	1e-100	2e-94	8e-117	2e-87	1.6		COG0812	M	Cell envelope biogenesis, outer membrane
STA_A00360	51626	52105	orf	tRNA-specific adenosine deaminase	*tadA*	1e-49	2e-62	5e-53	1e-40	5e-42	4e-40	4e-43	6e-39	3.3		COG0590	F, J	Nucleotide transport and metabolism [F], Translation, ribosomal structure and biogenesis [J]
STA_A00370	53744	52800	orf	Aspartate carbamoyltransferase	*pyrB*	4e-150	5e-162	1e-150	3e-105	2e-14	2e-120	2e-139	2e-128	0.36		COG0540	F	Nucleotide transport and metabolism
STA_A00380	56360	54996	orf	Lysine-sensitive aspartokinase 3	*lysC*	0.0	0.0	0.0	1e-43	2e-41	6e-30	0.015	4e-41	3e-15		COG0527	E	Amino acid transport and metabolism
STA_A00390	56960	58609	orf	Glucose-6-phosphate isomerase	*pgi*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5		COG0166	G	Carbohydrate transport and metabolism
STA_A00400	61738	63355	pseudogene	Probable ubiquinone biosynthesis protein ubiB*	*ubiB**	0.0	0.0	0.27	0.0	0.0	4e-164	0.033	6.2	9.4	7	COG0661	R	General function prediction only
						2e-128	1e-154	5.6	1e-98	3e-98	7e-77	3.0	4.6	0.053	8			
STA_A00410	67142	65691	orf	Trk system potassium uptake protein trkH	*trkH*	0.0	0.0	0.0	0.0	1.6	4e-167	0.001	4.9	1.4		COG0168	P	Inorganic ion transport and metabolism
STA_A00420	69171	68470	orf	NAD(P)H-flavin reductase	*fre*	3e-87	7e-106	1e-86	5e-74	2.9	0.22	1e-74	2.3	0.052		COG0543	H, C	Coenzyme metabolism [H], Energy production and conversion [C]
STA_Ar0020	70122	72680	rRNA	23S rRNA gene														
STA_Ar0030	73125	73240	rRNA	5S rRNA gene														
STA_A00430	73807	73352	orf	DnaK suppressor protein	*dksA*	2e-78	3e-80	3e-78	2e-68	3e-56	3.0	1e-71	1e-64	5.1		COG1734	T	Signal transduction mechanisms
STA_A00440	74799	74647	pseudogene	Cold shock-like protein cspC*	*cspC**	2e-34	2e-35	1e-34	4e-29	2e-34	9e-30	1e-29	3e-33	8e-19	9	COG1278	K	Transcription
						3e-05	1e-05	8e-06	7e-05	4e-06	3e-04	3e-04	5e-07	0.44	10			

STA_A00450	76088	76945	orf	RNA polymerase sigma-32 factor	*rpoH*	4e-132	1e-151	7e-143	3e-126	8e-120	9e-123	7e-130	8e-123	2e-04		COG0568	K	Transcription
STA_A00460	80510	81673	orf	Phosphoglycerate kinase	*pgk*	3e-178	0.0	7e-177	6e-163	2e-158	3e-101	3e-147	5e-123	1.1		COG0126	G	Carbohydrate transport and metabolism
STA_A00470	81781	82860	orf	Fructose-bisphosphate aldolase	*fba*	9e-163	0.0	3e-164	4e-158	2e-152	4e-131	5e-152	3e-133	1.5		COG0191	G	Carbohydrate transport and metabolism
STA_A00480	83860	83645	orf	50S ribosomal protein L31	*rpmE*	5e-28	1e-30	4e-29	4e-23	1e-25	4e-19	3e-27	1e-26	0.001		COG0254	J	Translation, ribosomal structure and biogenesis
STA_A00490	84060	85550	orf	Primosomal protein N	*priA*	1e-160	0.0	4e-170	1e-148	1.7	5e-06	7e-151	8e-74	0.078		COG1198	L	DNA replication, recombination and repair

* To search for '*Candidatus* Steffania adelgidicola' pseudogene homologs the respective *S. glossinidius* or *E. coli* proteins were used (see remarks).
Ec, *Escherichia coli* K-12 substr. MG1655 [NC_000913]
Sg, *Sodalis glossinidius* str. 'morsitans' [NC_007712]
Ss, *Serratia symbiotica* str. Tucson [AENX00000000]
Hd, '*Candidatus* Hamiltonella defensa 5AT' (*Acyrthosiphon pisum*) [NC_012751]
Bp, '*Candidatus* Blochmannia pennsylvanicus str. BPEN' [NC_007292]
Wg, *Wigglesworthia glossinidia* endosymbiont of *Glossina brevipalpis* [NC_004344]
Bc, *Baumannia cicadellinicola* str. Hc (*Homalodisca coagulata*) [NC_007984]
Ba, *Buchnera aphidicola* str. APS (*Acyrthosiphon pisum*) [NC_002528]
Cr, '*Candidatus* Carsonella ruddii PV' [NC_008512]
nss = no significant similarity

[1] blastp with RnhB protein of *Sodalis glossinidius*
[2] blastx with nucleotide sequence of ´*Candidatus* Steffania adelgidicola´
[3] blastp with YfiH protein of *E. coli*
[4] blastx with nucleotide sequence of ´*Candidatus* Steffania adelgidicola´
[5] blastp with ClpB protein of *Sodalis glossinidius*
[6] blastx with nucleotide sequence of ´*Candidatus* Steffania adelgidicola´
[7] blastp with UbiB protein of *Sodalis glossinidius*
[8] blastx with nucleotide sequence of ´*Candidatus* Steffania adelgidicola´
[9] blastp with UbiB protein of *Sodalis glossinidius*
[10] blastx with nucleotide sequence of ´*Candidatus* Steffania adelgidicola´

Table S4. Genome features of 'Candidatus Steffania adelgidicola' and selected free-living bacteria and insect symbionts.
Genome features according to the NCBI Genome database. Values for '*Candidatus* Steffania adelgidicola' are based on a 85 kb genome fragment.

	Escherichia coli str. K-12 substr. MG1655 [NC_000913]	*Yersinia pestis* KIM 10 [NC_004088]	*Mycobacterium leprae* Br4923 [NC_011896]	*Sodalis glossinidius* str. 'morsitans' [NC_007712]	*Serratia symbiotica* str. Tucson [NZ_AENX00000000]	*Candidatus* Hamiltonella defensa 5AT' [NC_012751]	*Candidatus* Steffania adelgidicola' [FR872579]	*Candidatus* Blochmannia pennsylvanicus str. BPEN' [NC_007292]	*Wigglesworthia glossinidia* [NC_004344]	*Baumannia cicadellinicola* str. Hc [NC_007984]	*Buchnera aphidicola* str. APS [NC_002528]	*Candidatus* Carsonella rudii PV' [NC_008512]	*Candidatus* Sulcia muelleri GWSS' [NC_010118]	*Candidatus* Hodgkinia cicadicola Dsem' [NC_012960]
Phylogenetic affiliation	*Gammaproteobacteria*		*Actinobacteria*	*Gammaproteobacteria*									*Bacteriodetes*	*Alphaproteob.*
Lifestyle	Free-living commensal	Free-living pathogen	Obligate intracellular pathogen	Facultative insect symbionts			Obligate insect symbionts							
Age of symbiosis[b] [my]				n.d.	~90	n.d.	**n.d.**	50	>40	100	180	120	>270	190
Genome size [bp]	4,639,675	4,600,755	3,268,071	4,171,146	2,573,085	2,110,331	**n.d.**	791,654	697,724	686,194	640,681	159,662	245,530	143,795
GC content [%]	50	47	57	54	49	40	**39.1**	29	22	33	26	16	22	58
% Coding	85	82	49	50.9[a]	56	80	**59.3**	76	86	85	86	93	91	90
Structural RNAs	175	100	50	91	72	85	**>5**	44	40	46	36	31	36	19
CDS	4145	4049	1604	2432	2157	2094	**n.d.**	610	611	595	564	182	227	169
Pseudogenes/ pseudogenes per 10 kbp	179/0.386	54/0.117	1116/3.415	972[a]/2.33	550/2.138	187/0.886	**5/0.584**	4/0.051	0/0	10/0.146	1/0.016	0/0	1/0.041	0/0
Average CDS length	941.4	931.7	998.4	857.6	668	806.24	**805**	948.9	982.1	980.3	976.9	815.9	984.3	765.8
Average spacer length	167.9	204.5	1039.1	857.6	524.9	201.6	**909.3**	299.7	159.9	173	159	61.4	97.4	85.1

[a] modified using the publication of the *Sodalis glossinidius* genome (Toh et al, 2006)
[b] (Burke & Moran 2011; Gosalbes et al, 2010; Moran et al, 2008)

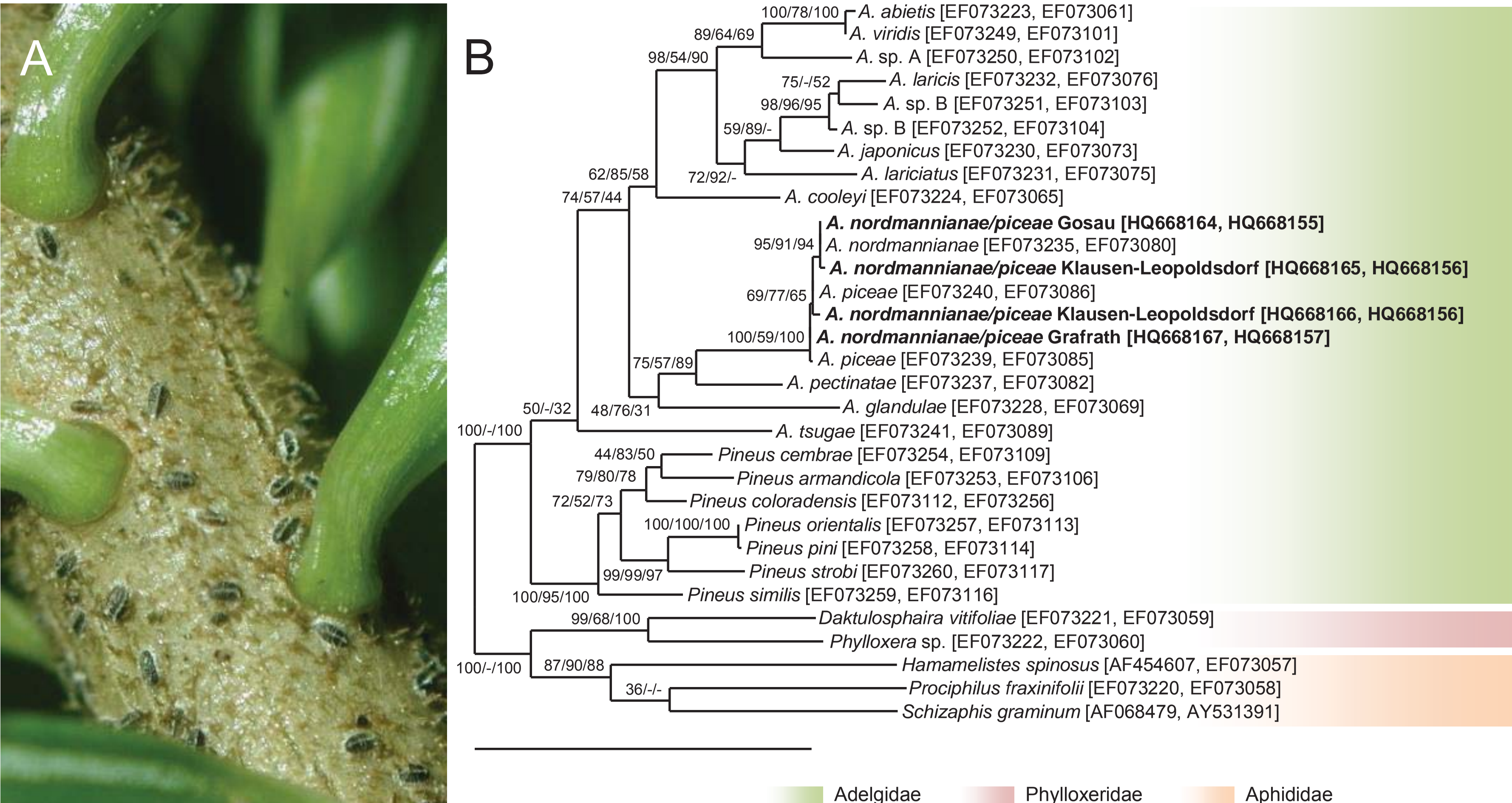

Figure S1. Adelgids analyzed in this study. (A) Adelgid exules (a wingless parthenogenetic life stage) feeding on a branch of silver fir (*Abies alba*). **(B)** Phylogenetic affiliation of the studied adelgids with the *A. nordmanniae/piceae* complex based on a concatenated dataset of *ef1alpha* and *coI*. A maximum likelihood tree (PhyML) based on nucleotide sequence alignments is shown. Maximum likelihood bootstrap values (1000 resamplings), TREEPUZZLE support values and maximum parsimony bootstrap values (1000 resamplings) are indicated at the internal nodes. Selected members of the Phylloxeridae and the Aphididae were used as outgroup. GenBank accession numbers for *ef1alpha* and *coI* genes, respectively, are given in square brackets. Bar, 10% estimated evolutionary distance.

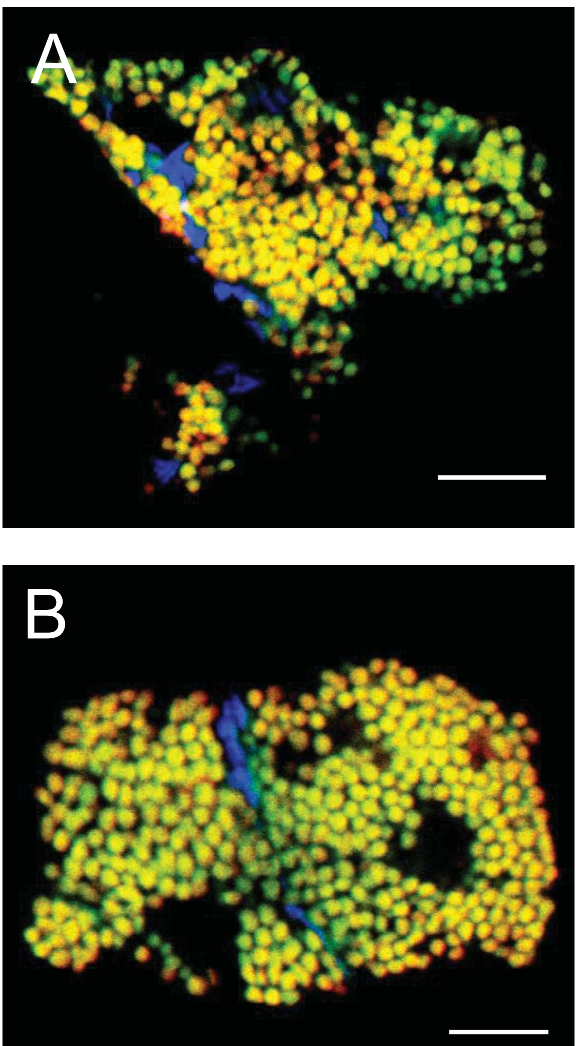

Figure S2. In situ identification of '*Candidatus* Steffania adelgidicola' and '*Candidatus* Ecksteinia adelgidicola' in bacteriocytes of further *A. nordmannianae/piceae* populations. Oligonucleotide probes targeting the 16S were used in FISH experiments for the assignment of the 16S rRNA gene sequences to the two different symbionts in the adelgid bacteriome. Probe names and sequences are listed in Supplemental Table S1. '*Candidatus* Steffania adelgidicola' labeled with a specific probe labeled in Cy3 (red), '*Candidatus* Ecksteinia adelgidicola' labeled with specific probes labeled in Cy5 (blue) and the general bacterial probe mix labeled with FLUOS (green) were used; the combined signal from bacterial probe labeled with FLUOS (green) and the '*Candidatus* Steffania adelgidicola-specific probe appears yellow and the '*Candidatus* Ecksteinia adelgidicola'-specific probes appears blue-green. to increase signal intensity three different 16S rRNA probes were applied simultaneously for '*Candidatus* Ecksteinia adelgidicola'. Adelgid populations sampled in **(A)** Grafrath, Germany and **(B)** Klausen-Leopoldsdorf, Austria are shown. Bars represent 10 µm.

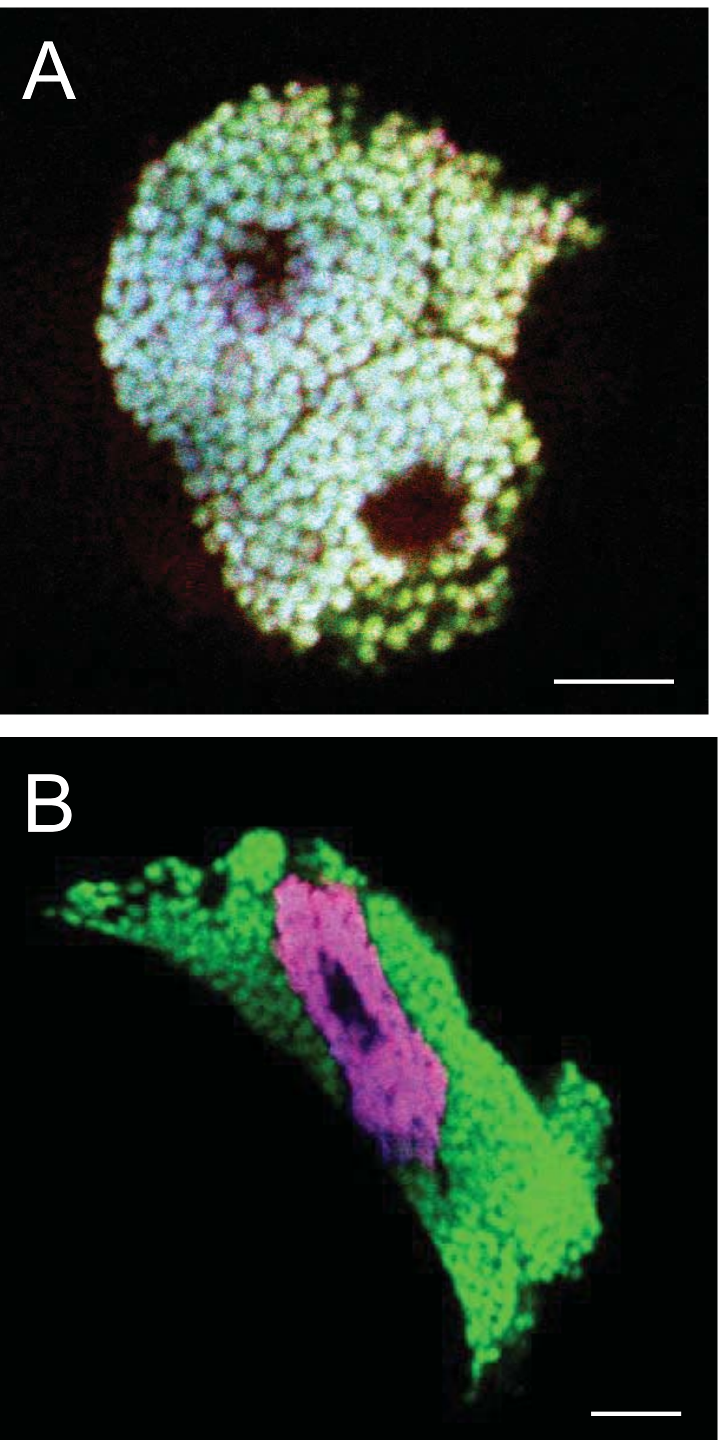

Figure S3. In situ identification of '*Candidatus* Steffania adelgidicola' and '*Candidatus* Ecksteinia adelgidicola'. Oligonucleotide probes targeting the 16S and 23S rRNA were used in FISH experiments to assign 16S and 23S rRNA sequence types to the two different symbiont populations in the adelgid bacteriome. Probe names and sequences are listed in Supplemental Table S1. **(A)** '*Candidatus* Steffania adelgidicola' labeled with specific probes targeting the 23S rRNA labeled in Cy3 (red) and the 16S rRNA labeled in Cy5 (blue); the combined signal from bacterial probe labeled with FLUOS (green) and symbiont-specific probes appears white. **(B)** '*Candidatus* Ecksteinia adelgidicola' labeled with specific probes targeting the 23S rRNA labeled in Cy3 (red) and the 16S rRNA labeled in Cy5 (blue); to increase signal intensity three different 16S or 23S rRNA probes were applied simultaneously. The green signal from the single bacterial probe is very dim, hence the combined signal from bacterial and symbiont-specific probes appears purple, '*Candidatus* Steffania adelgidicola' appears green. Bars represent 10 µm.

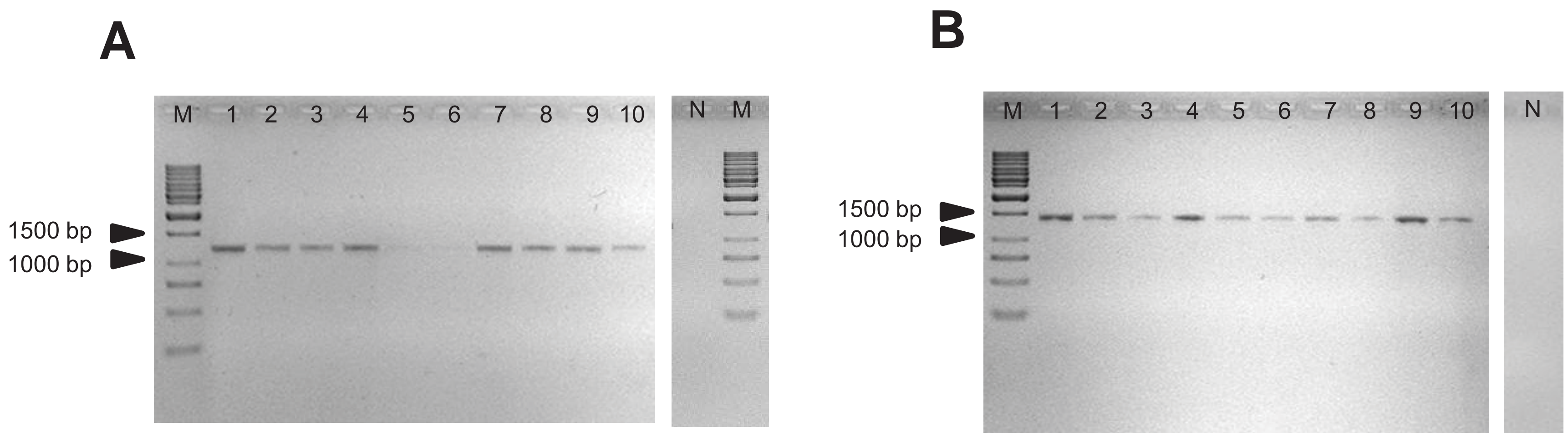

Figure S4. Screening of ten single adelgid individuals (Gosau) by PCR using symbiont specific 16S rRNA gene primers for the presence of **(A)** ‚*Candidatus* Steffania adelgidicola' and **(B)** ‚*Candidatus* Ecksteinia adelgidicola' (Supplementary Table S1). Lanes 1 to 10 indicate the presence of PCR products of the correct size (1197 bp and 1342 bp, respectively) in all of the screened individuals. N = negativ control (no DNA added), M = molecular weight marker

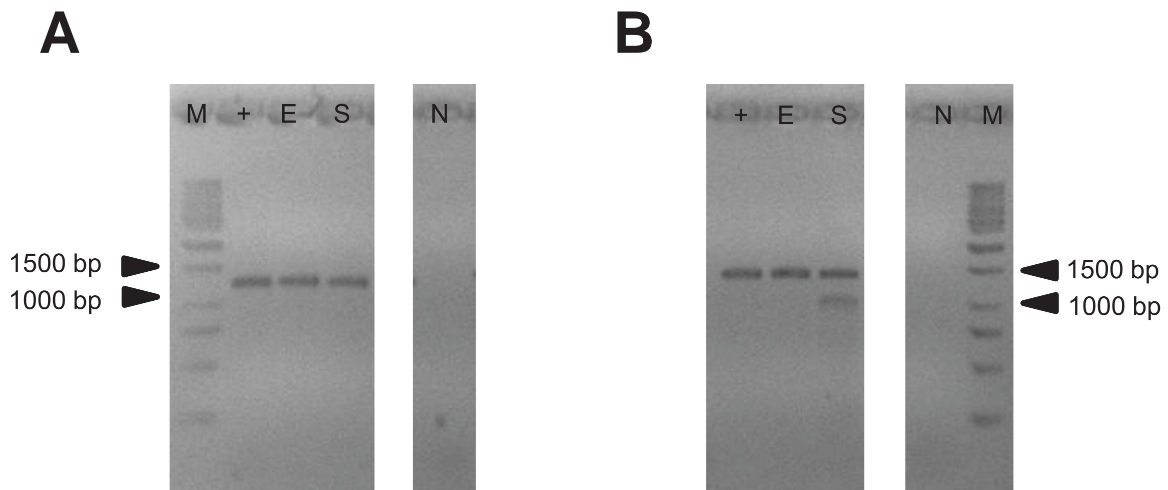

Figure S5. Screening for the presence of both symbionts in different life stages. PCR was performed using symbiont specific primer for the symbionts **(A)** ‚*Candidatus* Steffania adelgidicola‘ and **(B)** ‚*Candidatus* Ecksteinia adelgidicola‘ (Supplementary Table S1). M = molecular weight marker, E = exules, S = sexuparae, + = positive control, N = negative control (no DNA added).

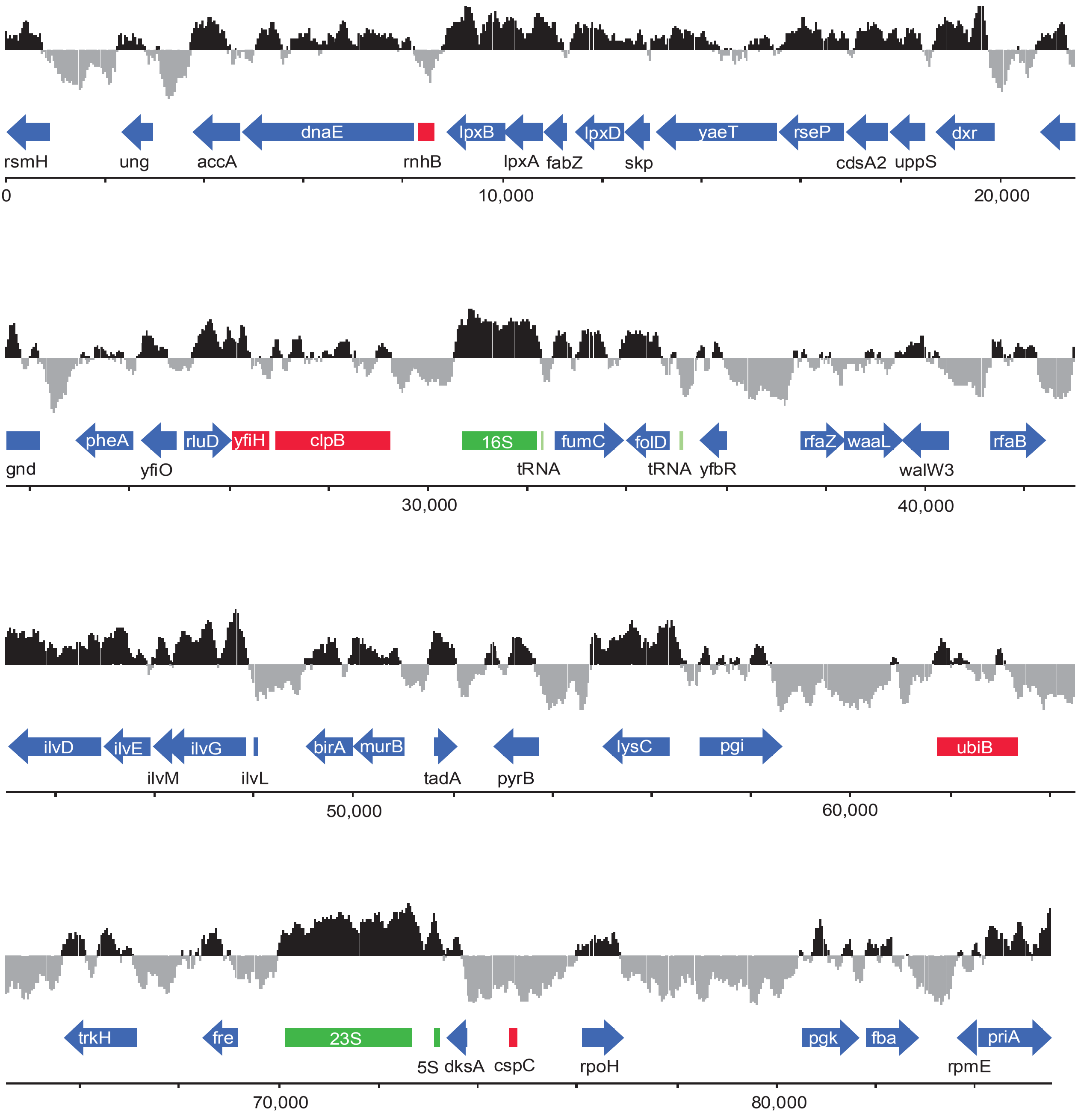

Figure S6. Genome fragment of '*Candidatus* Steffania adelgidicola'. An 85 kb contig reconstructed from three fosmid clones is shown. Predicted CDSs are indicated in blue, rRNA and tRNA genes in green, pseudogenes in red. Deviations from the average G+C content (39.1%) are displayed in black and grey, respectively. Scale bar in bp.

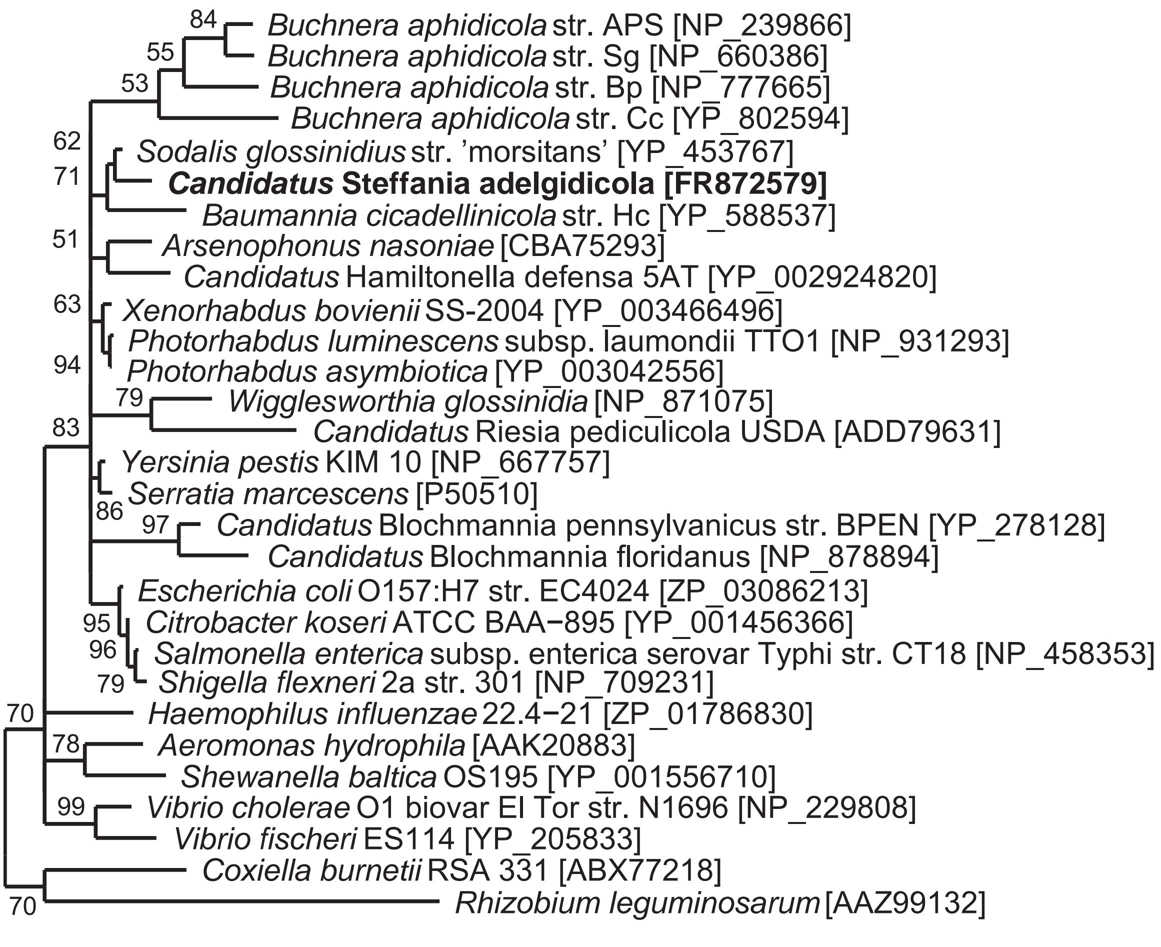

Figure S7. Phylogenetic relationships of '*Candidatus* Steffania adelgidicola' based on protein sequences of RpoH. TREEPUZZLE tree is shown. Bars, 10% estimated evolutionary distance.

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Chapter III

Co-evolution and symbiont replacement shaped the symbiosis between adelgids (Hemiptera: Adelgidae) and their bacterial symbionts

Published online in *Environ Microbiol* (2012), DOI: 10.1111/j.1462-2920.2012.02712.x

and as supplementary online material:

http://onlinelibrary.wiley.com/doi/10.1111/j.1462-2920.2012.02712.x/suppinfo

VOLUME 14 NUMBER 5 May 2012 www.env-micro.com ISSN 1462-2912

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Co-evolution and symbiont replacement shaped the symbiosis between adelgids (Hemiptera: Adelgidae) and their bacterial symbionts

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Summary

The Adelgidae (Insecta: Hemiptera), a small group of insects, are known as severe pests on various conifers of the northern hemisphere. Despite of this, little is known about their bacteriocyte-associated endosymbionts, which are generally important for the biology and ecology of plant sap-sucking insects. Here, we investigated the adelgid species complexes *Adelges laricis/tardus*, *Adelges abietis/viridis* and *Adelges cooleyi/coweni*, identified based on their *coI* and *ef1alpha* genes. Each of these insect groups harboured two phylogenetically different bacteriocyte-associated symbionts belonging to the *Betaproteobacteria* and the *Gammaproteobacteria*, respectively, as inferred from phylogenetic analyses of 16S rRNA gene sequences and demonstrated by fluorescence *in situ* hybridization. The betaproteobacterial symbionts of all three adelgid complexes (‘*Candidatus* Vallotia tarda’, ‘*Candidatus* Vallotia virida’ and ‘*Candidatus* Vallotia cooleyia’) share a common ancestor and show a phylogeny congruent with that of their respective hosts. Similarly, there is evidence for co-evolution between the gammaproteobacterial symbionts (‘*Candidatus* Profftia tarda’, ‘*Candidatus* Profftia virida’) and *A. laricis/tardus* and *A. abietis/viridis*. In contrast, the gammaproteobacterial symbiont of *A. cooleyi/coweni* (‘*Candidatus* Gillettellia cooleyia’) is different from that of the other two adelgids but shows a moderate relationship to the symbiont ‘*Candidatus* Ecksteinia adelgidicola’ of *A. nordmannianae/piceae*. All symbionts were present in all adelgid populations and life stages analysed, suggesting vertical transmission from mother to offspring. In sharp contrast to their sister group, the aphids, adelgids do not consistently contain a single obligate (primary) symbiont but have acquired phylogenetically different bacterial symbionts during their evolution, which included multiple infections and symbiont replacement.

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Introduction

Symbioses between bacteria and insects are widespread in nature. Ten per cent of all known insects, the most species-rich group of organisms in nature, contain specific symbionts, which play crucial roles for their host’s biology, ecology and evolution (Buchner, 1953; Moran *et al*., 2008; Moya *et al*., 2008). Obligate (primary) symbionts are essential for survival and reproduction by provision of nutrients. They are vertically transmitted, from mother to offspring, and generally maintained in a special insect organ, the bacteriome. This long-term association resulted in co-speciation with their insect hosts. One of the best-studied symbiosis, stable since 160 million of years, is the association between the obligate gammaproteobacterial symbiont *Buchnera aphidicola* and its aphid host (Baumann, 2005; Moran *et al*., 2008), where *B. aphidicola* provides essential amino acids missing in the insect’s diet (Shigenobu *et al*., 2000; Thomas *et al*., 2009). In contrast to obligate symbionts, facultative (secondary) symbionts are found in diverse host tissues, and they are vertically and horizontally transmitted within and between host species. Facultative symbionts can affect the host insect’s biology in several aspects, for example, by providing protection against natural enemies or heat stress (Oliver *et al*., 2010), by determining host plant specificity (Tsuchida *et al*., 2004), and by modification of the body colour (Tsuchida *et al*., 2010).

A suborder of the Sternorrhyncha that has been only poorly studied with respect to their bacterial symbionts is the Adelgidae, a sister group of the Aphididae. Adelgids (Hemiptera: Aphidoidea: Adelgidae) are plant sap feeding insects, represented by ~ 65 described species that exclusively feed on conifers, are highly host tree specific and can be severe pests (Blackman and Eastop, 1994). Most adelgids show both a holocyclic, sexual life cycle

including gall formation and an anholocyclic, asexual life cycle. The holocyclic lifestyle includes a host tree change from the primary host tree spruce (*Picea*) after three generations (sexualis, fundatrix, gallicola) to secondary host trees such as fir (*Abies*), larch (*Larix*), pine (*Pinus*), hemlock (*Tsuga*) and Douglas-fir (*Pseudotsuga*) and two further generations (exulis, sexupara). Exceptions are known, where only an anholocylic lifestyle either on the primary or secondary host tree occurs (Havill and Foottit, 2007). Adelgids feed on phloem sap (Balch, 1952; Allen and Dimond, 1968; Parry and Spires, 1982; Sopow *et al*., 2003) or on cortical parenchyma cells (Balch, 1952; Plumb, 1953; Rohfritsch and Anthony, 1992; Young *et al*., 1995).

Early studies based on histological analyses indicated the presence of bacteriomes containing morphologically different bacterial symbionts in various adelgid species (Profft, 1936; Buchner, 1953; Steffan, 1968). The role of these bacteria for their insect hosts' biology is still unknown, but their importance is evident from the effect of antibiotic treatment, which resulted in the eventual death of the hemlock woolly adelgid (*Adelges tsugae*) (Shields and Hirth, 2005). So far, only two bacterial symbionts of a single species complex of the Adelgidae have been identified and characterized partially on the molecular level (Toenshoff *et al*., 2011). *Adelges nordmannianae/piceae* harbours two morphological different gammaproteobacterial symbionts, '*Candidatus* Steffania adelgidicola' and '*Candidatus* Ecksteinia adelgidicola', which are located in distinct bacteriocytes. Both symbionts are vertical transmitted but are not closely related to any of the known obligate symbionts of aphids, mealybugs, whiteflies or psyllids (Baumann, 2005). '*Candidatus* Steffania adelgidicola' seems to represent an evolutionarily relatively young symbiont considering the reduction state of its genome compared with long-term obligate insect symbionts (Toenshoff *et al*., 2011). The situation in the adelgids might thus to be fundamentally different from their aphid sister group, where a single symbiont, *B. aphidicola*, was acquired as symbiont by an aphid ancestor more than 180 Million years ago and still represents the primary symbiont in most extant aphid species (Munson *et al*., 1991a,b; Fukatsu and Ishikawa, 1996; Martinez-Torres *et al*., 2001; Gosalbes *et al*., 2008; Moran *et al*., 2008; Lamelas *et al*., 2011).

In this study, we asked whether adelgid species other than *A. nordmannianae/piceae* also contained symbionts related to '*Candidatus* Steffania adelgidicola' and '*Candidatus* Ecksteinia adelgidicola', or whether adelgid-associated symbionts are more diverse than those of the aphids. To answer this question, we analysed three different closely related adelgid species pairs, including *Adelges laricis/tardus*, *Adelges abietis/viridis* and *Adelges cooleyi/coweni*. Electron microscopy, 16S rRNA based phylogenetic analysis and fluorescence *in situ* hybridization (FISH) confirmed the presence of bacteriocyte-associated symbionts in these insects and demonstrated that the symbionts are novel members of the *Gammaproteobacteria* and the *Betaproteobacteria* respectively. Although (with one exception) they were not related to the symbionts of *A. nordmannianae/piceae*, we found evidence for co-evolution between some of these bacteria and their insect hosts. None of the adelgids studied so far contained the aphid symbiont *B. aphidicola*. Instead, they show a range of phylogenetically different symbionts suggesting a more complex evolutionary history of association with symbiotic bacteria.

Results

Identification of adelgid hosts

Eight different adelgid populations were sampled in Austria, Germany, Italy and the USA (Fig. S1), and the genes encoding cytochrome *c* oxidase subunit 1 (*coI*) and elongation factor 1-alpha (*ef1alpha*) were used for phylogenetic analysis. These genes have been demonstrated previously to be suitable for resolving relationships among adelgids (Havill *et al*., 2007; Zurovcova *et al*., 2010). However, they cannot differentiate between very closely related adelgids species – also called species pairs or complexes – which show none or very few morphological differences and low sequence divergence (Havill *et al*., 2007; Foottit *et al*., 2009; Zurovcova *et al*., 2010). This analysis identified the members of three adelgid populations as *A. laricis/tardus*, three populations as *A. abietis/viridis*, and two populations as *A. cooleyi/coweni* (Table 1; Fig. S2). The grouping of concatenated *coI-ef1alpha* sequences in phylogenetic trees with the respective adelgid complexes was well supported by different treeing methods including neighbour joining, maximum parsimony, maximum likelihood, TREEPUZZLE and Bayesian analysis (Fig. S2).

Bacteriocyte-associated symbionts of A. laricis/tardus

Wingless (apterous) adults of *A. laricis/tardus* at the gallicola life stage (Klausen-Leopoldsdorf population) were fixed for histological and transmission electron microscopic analyses. Toluidine blue staining of semi-thin sections of the abdomen revealed the presence of bacteriocytes near and longitudinal to the gut (Fig. 1A) similar to the situation in *A. nordmannianae/piceae* and aphids (Baumann *et al*., 1995; Toenshoff *et al*., 2011). The bacteriocytes were filled with two differently stained bacteria (Fig. 1A). Electron microscopy confirmed the presence of two different types of symbionts, which were densely packed inside the same bacteriocytes (Fig. 1C). One

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Table 1. Adelgids and their bacterial symbionts analysed in this study.

Organism/location	Host plant	Life stage	GenBank Accession No. (host)		GenBank Accession No. (endosymbionts)	
			col	*ef1alpha*	16S rRNA gene	
Adelges laricis/tardus					'*Candidatus* Profftia tarda'	'*Candidatus* Vallotia tarda'
Klausen-Leopoldsdorf, Austria	*Picea* sp. (gall 3)	gallicolae	JN810892	JN810902	JN810878	JN810867
Eberndorf, Austria	*Picea* sp.	gallicolae	JN810896	JN810901	JN810882	JN810869
Tutzing, Germany	*Picea* sp.	gallicolae	JN810890	JN810900	JN810884	JN810873
Adelges abietis/viridis					'*Candidatus* Profftia virida'	'*Candidatus* Vallotia virida'
Klausen-Leopoldsdorf, Austria	*Picea* sp. (gall 2)	gallicolae	JN810894	JN810904	JN810881	JN810868
Klausen-Leopoldsdorf, Austria	*Picea* sp. (gall 3)	gallicolae	JN810893	JN810905	JN810880	JN810870
Klausen-Leopoldsdorf, Austria	*Picea* sp. (gall 1)	gallicolae	JN810895	JN810903	JN810879	JN810871
Kaltern, Italy	*Picea* sp.	gallicolae	JN810887	JN810898	JN810876	JN810866
Aschering, Germany	*Picea* sp.	eggs	JN810889	JN810907	JN810877	JN810875
Aschering, Germany	*Picea* sp.	gallicolae	JN810891	JN810906	JN810885	JN810872
Adelges cooleyi/coweni					'*Candidatus* Gillettellia cooleyia'	'*Candidatus* Vallotia cooleyia'
Kaltern, Italy	*Pseudotsuga* sp.	exules	JN810888	JN810899	JN810883	JN810865
Madison, Wisconsin, USA	*Pseudotsuga menziesii*	exules	JN810897	JN810908	JN810886	JN810874

symbiont type was electron dense and rod-shaped with a length of 0.5–2.9 μm, the other morphotype was rather electron translucent and rod-shaped with a length of 0.7–3.5 μm (Fig. 1D–F). Both symbionts showed a Gram-negative type cell wall and were surrounded by a third membrane, the so-called symbiosome membrane. Sheath cells, which were not infected by bacteria, were located in between and circumjacent the bacteriocytes (Fig. 1G).

Two abundant 16S rRNA phylotypes belonging to the *Gammaproteobacteria* and the *Betaproteobacteria*, respectively, were found in the *A. laricis/tardus* population sampled in Klausen-Leopoldsdorf, presumably representing the two different morphotypes seen with electron microscopy. Nearly identical gamma- and betaproteobacterial 16S rRNA gene sequences were detected in the other two examined populations (Eberndorf, Tutzing) by using specific PCR assays (99.8–100%, and 98.6–99.3% respectively). The betaproteobacterial sequences showed highest similarity (95.3–96.1%) to sequences belonging to symbionts located in the cytosol of the fungus *Rhizopus* spp. (Lackner *et al.*, 2009). The gammaproteobacterial sequences were most similar to the free-living enterobacteria *Hafnia alvei* (95.7%) and *Obesumbacterium proteus* (95.8%). *Hafnia* is commonly found in the gastrointestinal tract of humans and other animals; *Obesumbacterium* is frequently associated with yeasts and a well-known brewery contaminant. The gammaproteobacterial symbiont of *A. laricis/tardus* shows only low similarities to the *A. nordmannianae/piceae* symbionts '*Candidatus* Steffania adelgidicola' (89.2–89.9%) and '*Candidatus* Ecksteinia adelgidicola' (91-91.6%).

Fluorescence *in situ* hybridization with oligonucleotide probes specific for the identified gamma- and betaproteobacterial phylotypes was successfully used to visualize both symbionts (Fig. 1B, Table S1). It was not possible to decide which of the two phylotypes represents which of the two highly similar morphotypes seen with histology and electron microscopy, but this analysis confirmed that the two phylogenetically different symbionts share their bacteriocytes and are not located in separate host cells. Both symbionts were identified in individuals from all three investigated populations.

Based on the low degree of relationship to other known bacteria, we propose two new names according to Murray and Stackebrandt (Murray and Stackebrandt, 1995) for the symbionts of *A. laricis/tardus*: '*Candidatus* Vallotia tarda' for designation of the betaproteobacterial symbiont, in honour of the researcher Vallot, who described *A. laricis* in 1836, and 'tarda' referring to the species name of the adelgid host; '*Candidatus* Profftia tarda' for the gammaproteobacterial symbiont in honour of the scientist Joachim Profft and his contribution to our knowledge on symbiosis of adelgids.

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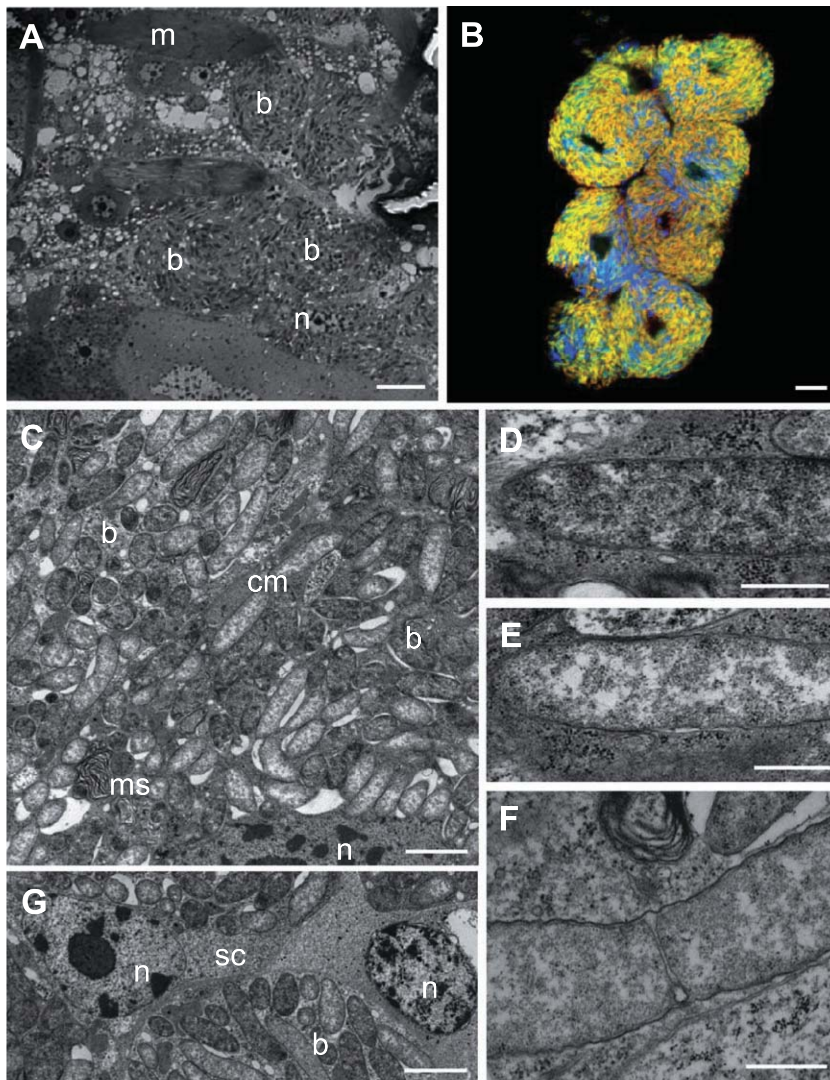

Fig. 1. Identification and ultrastructure of bacteriocyte-associated symbionts (‘*Candidatus* Vallotia tarda’, ‘*Candidatus* Profftia tarda’) of *Adelges laricis/tardus*.
A. A digital interference contrast (DIC) of a semi-thin longitudinal section of the insect abdomen of *A. laricis/tardus* (Klausen-Leopoldsdorf) stained with toluidine blue showing bacteriocytes containing two differently stained symbiont types.
B. Intracellular localization of bacteriocyte-associated symbionts of *A. laricis/tardus* (Eberndorf) by fluorescence *in situ* hybridization. A probe mix labelled with FLUOS (green) and targeting most *Bacteria*, a probe labelled with Cy3 (red) and specific for the betaproteobacterial symbiont ‘*Candidatus* Vallotia tarda’ (ValTa-1136), and a probe labelled with Cy5 (blue) and specific for the gammaproteobacterial symbiont ‘*Candidatus* Profftia tarda’ (ProTa-185) were used simultaneously. The combined signals from bacterial and symbiont-specific probes appear yellow for ‘*Candidatus* Vallotia tarda’ and blue-green for ‘*Candidatus* Profftia tarda’ respectively.
C–G. Transmission electron microscopy of ultra-thin sections of bacteriocytes in the abdomen of *A. laricis/tardus* (Klausen-Leopoldsdorf).
C. Two different morphotypes of symbionts are densely packed, co-inhabiting the same bacteriocytes.
D and E. Both symbiont types are rod-shaped with a Gram-negative type cell wall and surrounded by a symbiosome membrane, but one symbiont type is more electron-dense than the other.
F. Electron-translucent symbiont undergoing cell division.
G. Uninfected sheath cells are located between bacteriocytes. Bars in (A) and (B) represent 10 μm; bar in (C) represents 2 μm; bars in (D), (E) and (F) represent 500 nm; bar in (G) represents 2 μm. b = bacteriocytes containing symbionts, m = muscle, n = nucleus, cm = cell membrane, ms = membrane stacks, sc = sheath cell.

Bacteriocyte-associated symbionts of A. abietis/viridis

Apterous gallicola adults of *A. abietis/viridis* from two populations (Kaltern, Klausen-Leopoldsdorf) were analysed by histology and transmission electron microscopy. Similar to *A. laricis/tardus*, two different bacteriocyte-associated bacterial morphotypes were observed after toluidine blue staining, densely packed within the same bacteriocytes (Fig. 2A). Histologic and ultrastructure analyses showed the location of the bacteriocytes in proximity to the gut and confirmed the presence of two rod-shaped types of symbionts sharing the same bacteriocytes (Figs 2C–E and S3); one morphotype was electron translucent with a length of 0.8–4.8 μm, the other more electron dense with a length of 0.8–2.7 μm (Fig. 2F and G). Both symbionts possessed a Gram-negative type cell wall and were surrounded by a symbiosome membrane. Ultrastructure and location of the symbionts were identical among both *A. abietis/viridis* populations (Fig. 2C and E).

A gammaproteobacterial and a betaproteobacterial 16S rRNA gene sequence was recovered from individuals of the *A. abietis/viridis* population sampled in Kaltern. The analysis of two additional populations (Klausen-Leopoldsdorf, Aschering), including galls, gallicola adults and eggs, using specific PCR assays revealed nearly identical gamma- and betaproteobacterial sequences in all three populations and life stages. The betaproteobacterial sequences were 99.3–100% identical and showed highest similarity to the betaproteobacterial symbiont ‘*Candidatus* Vallotia tarda’ of *A. laricis/tardus* (96.5–97.5%). The gammaproteobacterial sequences shared

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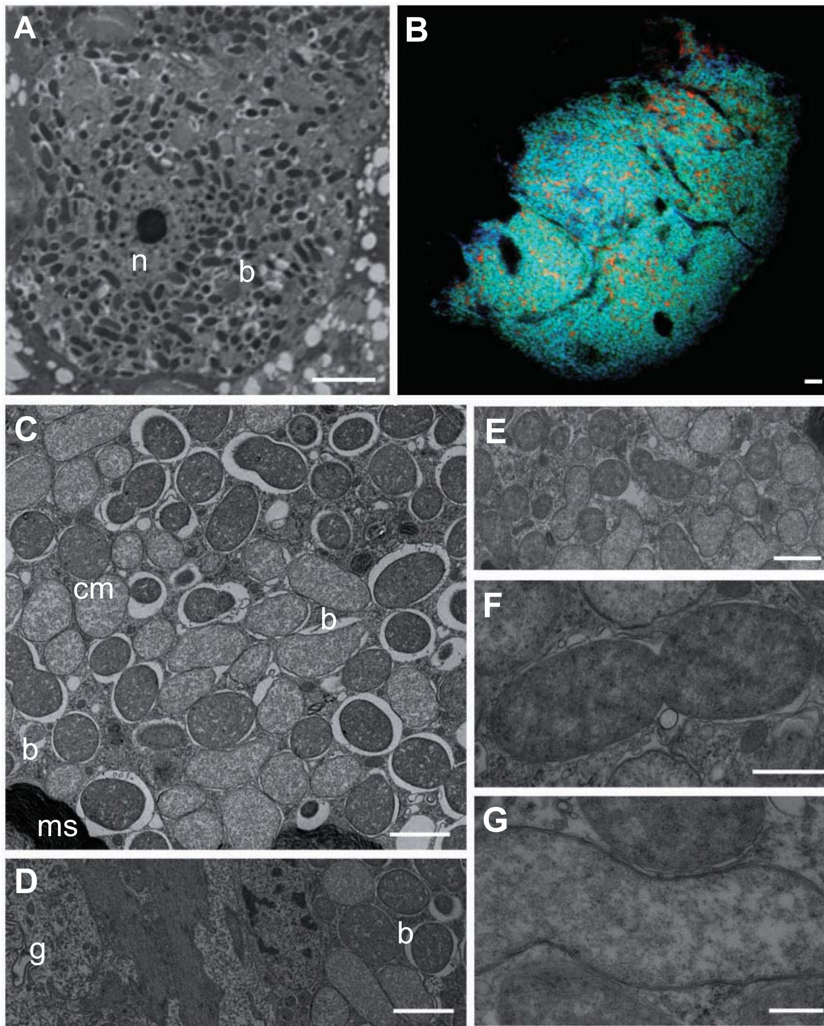

Fig. 2. Identification and ultrastructure of bacteriocyte-associated symbionts ('*Candidatus* Vallotia virida', '*Candidatus* Profftia virida') of *Adelges abietis/viridis*.
A. Semi-thin cross-section (DIC) of the abdomen of *A. abietis/viridis* (Klausen-Leopoldsdorf) stained with toluidine blue showing two differently stained types of symbionts co-inhabiting the same bacteriocytes.
B. Intracellular localization of bacteriocyte-associated symbionts of *A. abietis/viridis* (Klausen-Leopoldsdorf) by fluorescence *in situ* hybridization. A probe mix labelled with FLUOS (green) and targeting most *Bacteria*, a probe labelled in Cy3 (red) and specific for the gammaproteobacterial symbiont '*Candidatus* Profftia virida' (ProVi-177), and a probe labelled in Cy5 (blue) and specific for the betaproteobacterial symbiont '*Candidatus* Vallotia virida' (ValVi-624) were used simultaneously. The combined signal of the bacterial and the symbiont-specific probes appears yellow for '*Candidatus* Profftia virida' and blue-green for '*Candidatus* Vallotia virida' respectively.
C–G. Transmission electron microscopy analyses of ultra-thin sections of bacteriocytes within the abdomen of *A. abietis/*viridis.
C. Two symbiont morphotypes that can be distinguished based on electron density co-inhibit the same bacteriocytes found in *A. abietis/viridis* (Kaltern). Symbionts are rod-shaped and individually surrounded by a third membrane, the symbiosome membrane.
D. The bacteriocytes of *A. abietis/viridis* (Kaltern) are occasionally directly located next to the gut.
E. Symbionts of *A. abietis/viridis* (Klausen-Leopoldsdorf) are highly similar in morphology and location as the symbionts of the population sampled in Kaltern.
F. The electron dense symbiont undergoing cell division, showing its Gram-negative type cell wall (two membranes) tightly surrounded by a third so-called symbiosome membrane.
G. The more electron-translucent symbiont showing the Gram-negative type cell wall and the symbiosome membrane. Bars in (A) and (B) represent 10 μm; bars in (C), (D) and (E) represent 2 μm; bar in F represents 1 μm; bar in (G) represents 500 nm. g = gut, b = bacteriocytes containing symbionts, m = muscle, cm = cell membrane, ms = membrane stacks, n = nucleus.

99.5–100% identity and showed highest similarity to the gammaproteobacterial symbiont '*Candidatus* Profftia tarda' of *A. laricis/tardus* (95–95.5%). FISH experiments confirmed the identity of both bacterial symbionts and showed that they colocalize within the same bacteriocytes inside the body cavity of their insect hosts (Fig. 2B). Based on the close relationship to the *A. laricis/tardus* symbionts (and the commonly applied 95% 16S rRNA sequence similarity threshold for delineation of bacterial genera), we propose to tentatively classify the beta- and gammaproteobacterial symbionts of *A. abietis/viridis* as '*Candidatus* Vallotia virida' and '*Candidatus* Profftia virida' respectively ('virida' referring to the species name of the adelgid host).

Bacteriocyte-associated symbionts of A. cooleyi/coweni

Two populations of *A. cooleyi/coweni* (Kaltern, Madison) were analysed, and similar to the other two adelgids investigated here, both gamma- and betaproteobacterial 16S rRNA gene sequences were recovered. The latter were almost identical (99.5%) and similar to '*Candidatus*

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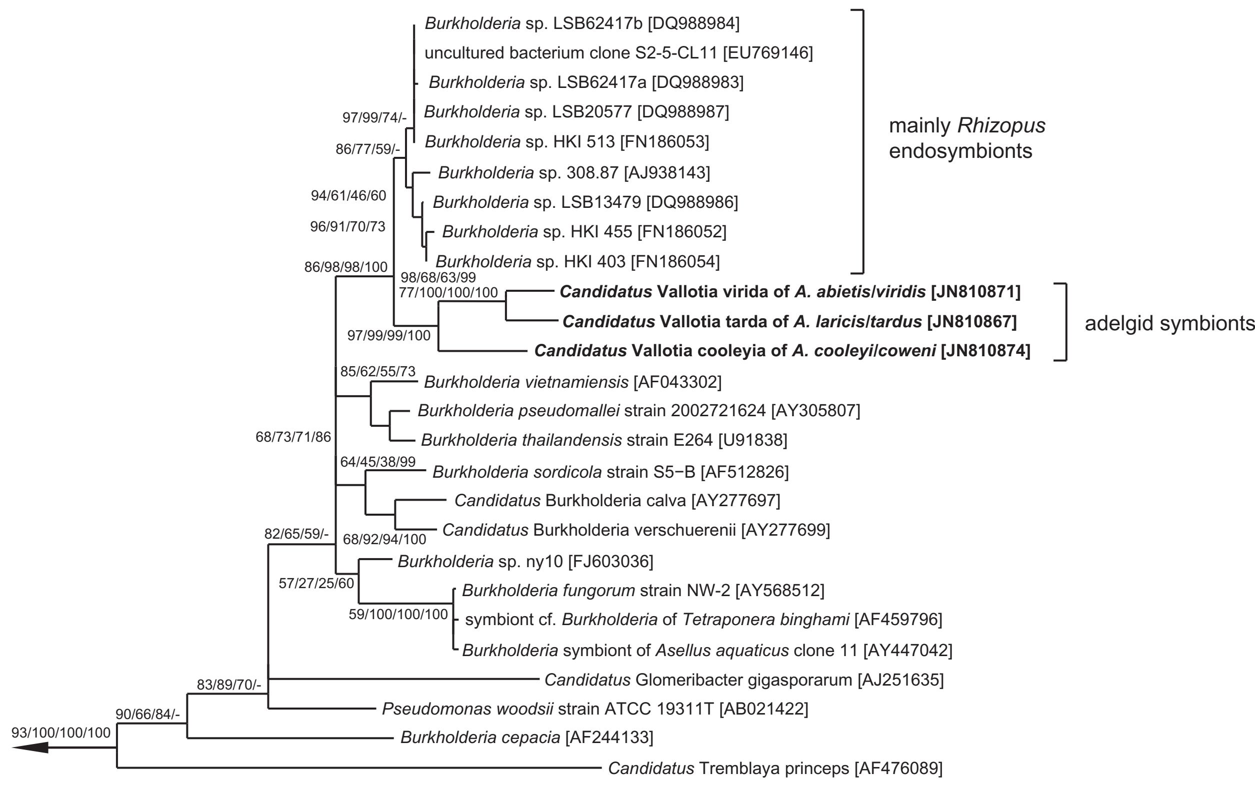

Fig. 3. Phylogenetic relationship of the betaproteobacterial symbionts of *A. laricis/tardus*, *A. abietis/viridis* and *A. cooleyi/coweni*. A 16S rRNA-based TREEPUZZLE tree is shown. TREEPUZZLE support values, maximum likelihood (1000 replicates) and maximum parsimony (1000 replicates) bootstrap values and Bayesian posterior probabilities are indicated at the inner nodes. GenBank/EMBL/DDBJ accession numbers of 16S rRNA genes are given in squared brackets. Bar, 10% estimated evolutionary distance. Selected members of the *Alphaproteobacteria* were used as out-group [NC_002678, NC_011988, NC_002978, NC_006142, NC_009883].

Vallotia tarda' (94.8–95.4%) and '*Candidatus* Vallotia virida' (95.1–95.4%). We thus propose the tentative name '*Candidatus* Vallotia cooleyia' for the betaproteobacterial symbiont of *A. cooleyi/coweni*. The gammaproteobacterial sequences detected in the two *A. cooleyi/coweni* populations were also very similar to each other (99.8%). Surprisingly, however, they were not particularly similar to the gammapoteobacterial symbionts '*Candidatus* Profftia tarda' (92.5–92.7%) or '*Candidatus* Profftia virida' (91.3–91.8%), but showed highest similarity to *Serratia* spp. (93.6–94.1%) and '*Candidatus* Ecksteinia adelgidicola' (93.2–93.3%), one of two symbionts of *A. nordmannianae/piceae*. Based on the low degree of sequence similarity with known bacteria, we suggest to tentatively classify the gammaproteobacterial symbionts of *A. cooleyi/coweni* as '*Candidatus* Gillettellia cooleyia' (in honour of the entomologist Clarence P. Gillette, 1859–1941, who first described the adelgid species *A. cooleyi* in 1907; 'cooleyia' referring to the species name of the adelgid host). The presence of both symbionts in adult individuals (exulis) of *A. cooleyi/coweni* was confirmed by FISH. The gammaproteobacterial symbiont '*Candidatus* Gillettellia cooleyia' and the betaproteobacterial symbiont '*Candidatus* Vallotia cooleyia' were both located within the same bacteriocytes (Fig. S4).

Phylogeny of bacteriocyte-associated symbionts of adelgids

Comprehensive phylogenetic analysis of all known bacterial symbionts of adelgids using different treeing methods including neighbour joining, maximum likelihood, maximum parsimony, TREEPUZZLE and Bayesian analysis demonstrated that the betaproteobacterial symbionts '*Candidatus* Vallotia virida', '*Candidatus* Vallotia tarda' and '*Candidatus* Vallotia cooleyia' form a well-supported monophyletic group (Fig. 3). This clade branches within the genus *Burkholderia* and is most closely related to *Burkholderia* sp. symbionts of *Rhizopus* fungi. Generally, rRNA-based phylogeny is poorly resolved within the *Gammaproteobacteria* (Williams *et al*., 2010). However, the gammaproteobacterial symbionts '*Candidatus* Profftia tarda' and '*Candidatus* Profftia virida' formed a well-supported monophyletic clade within the *Enterobacteriaceae*, with *H. alvei* and *O. proteus* as closest relatives (Fig. 4); while the gammaproteobacterial symbiont

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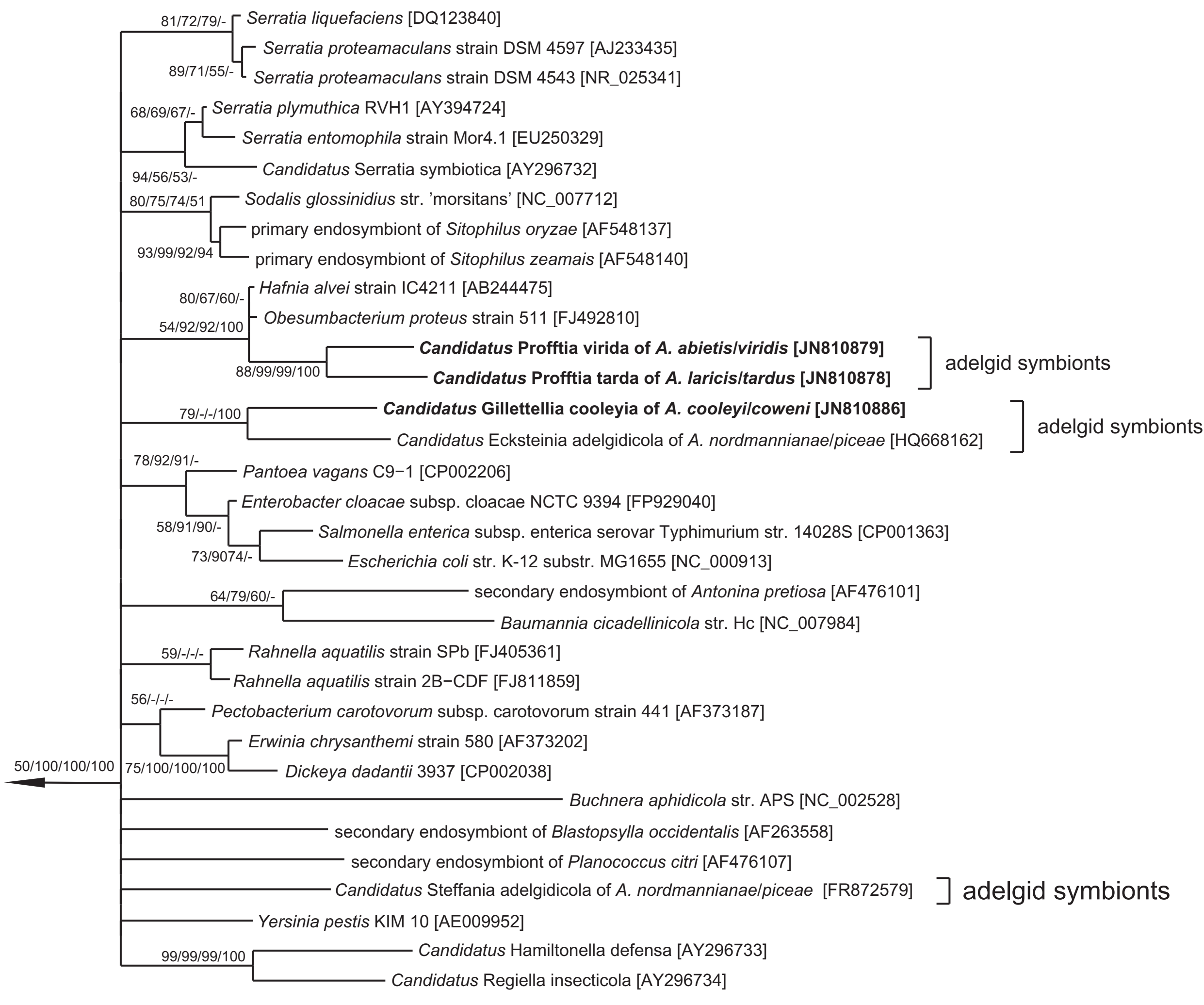

Fig. 4. Phylogenetic relationship of the gammaproteobacterial symbionts of *A. laricis/tardus*, *A. abietis/viridis* and *A. cooleyi/coweni*. A 16S rRNA-based TREEPUZZLE tree is shown. TREEPUZZLE support values, maximum likelihood (1000 replicates) and maximum parsimony (1000 replicates) bootstrap values and Bayesian posterior probabilities are indicated at the inner nodes. GenBank/EMBL/DDBJ accession numbers of 16S rRNA genes are given in squared brackets. Bar, 10% estimated evolutionary distance. Selected members of the *Alphaproteobacteria* were used as out-group [NC_002678, NC_011988, NC_002978, NC_006142, NC_009883].

'*Candidatus* Gillettellia cooleyia' formed a separate evolutionary lineage within the *Enterobacteriaceae*. Maximum likelihood and Bayesian analysis suggested that this symbiont of *A. cooleyi/coweni* shares a common ancestor with the *A. nordmannianae/piceae* symbiont '*Candidatus* Ecksteinia adelgidicola' (Fig. 4).

Discussion

So far the bacterial symbionts of in total four adelgids species pairs have been identified in this and a previous study (Toenshoff *et al.*, 2011). Consistently all adelgids contained two phylogenetically different bacteriocyte-associated symbionts affiliated with the *Betaproteobacteria* or the *Gammaproteobacteria*. None of these is closely related to known obligate (primary) symbionts of other insects of the suborder Sternorrhyncha, such as *B. aphidicola*, *Uzinura diaspididicola, Tremblaya princeps, Carsonella ruddii* or *Portiera aleyrodidarum* (Martinez-Torres *et al.*, 2001; Thao *et al.*, 2001; Thao and Baumann, 2004; Baumann and Baumann, 2005; Downie and Gullan, 2005; Gruwell *et al.*, 2007). Thus, independent from their aphid sister group and other Sternorrhyncha, the adelgids have acquired their own symbionts during evolution.

The bacterial symbionts of adelgids were present in all investigated populations confirming their importance for their host insects. Furthermore, the symbionts of *A. nordmannianae/piceae* (Toenshoff *et al.*, 2011) and *A. abietis/viridis* were detected in different life stages (adults, eggs), which strongly suggests vertical transmis-

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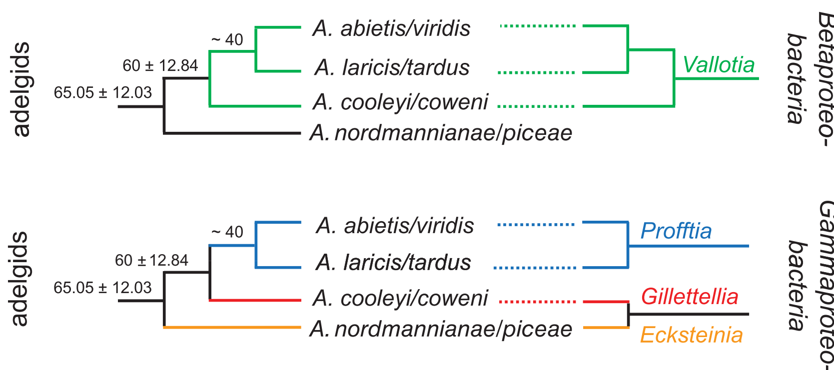

Fig. 5. Symbiont replacement and co-evolution between adelgids and their bacterial symbionts. A schematic representation of the congruence of host and symbiont phylogenies is shown; dendrograms are simplified versions of the trees from Figs 3, 4 and S2. To enhance clarity, congruent phylogenies suggesting co-evolution between bacterial symbionts and their adelgid hosts are drawn in the same colour. Estimated host divergence time points taken from Havill *et al.* (Havill *et al.*, 2007) are indicated in millions of years.

sion from mother to offspring similar to other symbionts of insects (Baumann, 2005). As all symbionts were present in all populations and life stages investigated so far it remains open whether they should be considered obligate or facultative (secondary) symbionts, or whether adelgids in fact rely on two bacterial symbionts. Such a dependency on more than one bacterial symbiont is known from several insects, including cicadas, spittlebugs, and some aphids, leafhoppers and mealybugs. In these cases both symbionts, e.g. *Buchnera* and *Serratia symbiotica*, *Sulcia muelleri* and *Hodgkinia cicadicola*, *Sulcia* and *Zinderia insecticola*, *Sulcia* and *Baumannia cicadellicola*, or *Tremblaya* and *Moranella endobia*, are required to supply their insect hosts with nutrients, which are sometimes produced through a complex interplay of both symbionts (Wu *et al.*, 2006; McCutcheon and Moran, 2007; 2010; McCutcheon *et al.*, 2009; Lamelas *et al.*, 2011; McCutcheon and von Dohlen, 2011).

Vertical transmission is often correlated with co-speciation and/or co-evolution between symbiont and host (Degnan *et al.*, 2004; Baumann, 2005), and there is evidence that this is also true for adelgids and their bacterial symbionts. The betaproteobacterial symbionts '*Candidatus* Vallotia tarda' of *A. laricis/tardus*, '*Candidatus* Vallotia virida' of *A. abietis/viridis* and '*Candidatus* Vallotia cooleyia' of *A. cooleyi/coweni* form a strongly supported monophyletic clade in 16S rRNA-based trees (Fig. 3), and the phylogeny of the symbionts and their insect hosts is congruent (Fig. 5). This demonstrates co-speciation and suggests co-evolution of '*Candidatus* Vallotia' symbionts with their host insects. There is only limited data from adelgid fossils, which enable the estimation of host divergence time points (Havill *et al.*, 2007), but based on the available information, we conclude that the symbiosis between the ancestors of '*Candidatus* Vallotia' and the adelgids *A. laricis/tardus*, *A. abietis/viridis* and *A. cooleyi/coweni* was established at least 60 million years ago (Fig. 5; Havill *et al.*, 2007).

Adelges laricis/tardus and *A. abietis/viridis* also share the monophyletic gammaproteobacterial symbionts '*Candidatus* Profftia virida' and '*Candidatus* Profftia tarda' (Fig. 4), whose phylogeny corresponds to the evolutionary history of their hosts and is thus evidence for co-evolution between both symbiosis partners (Fig. 5). Based on an estimated divergence time point for the adelgid hosts (Havill *et al.*, 2007), the symbiosis with '*Candidatus* Profftia' was established at least 40 million years ago. Interestingly, '*Candidatus* Profftia' is not present in *A. cooleyi/coweni* and *A. nordmannianae/piceae*, who instead possess two other gammaproteobacterial symbionts that form a common evolutionary lineage (in two out of four treeing methods) independent from '*Candidatus* Profftia' (Figs 4 and 5). The second gammaproteobacterial symbiont of *A. nordmannianae/piceae*, '*Candidatus* Steffania adelgidicola', did not cluster with any of the other symbionts in our phylogenetic analysis.

Different scenarios are conceivable for the evolutionary history of the symbiosis between adelgids and their bacterial symbionts. One of the most parsimonious scenarios assumes that novel infections or symbiont replacements have occurred rarely and that the adelgids required two different bacterial symbionts during their recent evolutionary history. According to this hypothesis, the ancestor of all investigated adelgids who lived some 65 million years ago was infected by the progenitors of the betaproteobacterial '*Candidatus* Vallotia' and the gammaproteobacterial '*Candidatus* Ecksteinia/'*Candidatus* Gillettellia' respectively. In the ancestor of extant *A. nordmannianae/piceae* '*Candidatus* Vallotia' was replaced by the progenitor of '*Candidatus* Steffania' at some unknown time point between 60 million years ago and today. This is consistent with genomic evidence from '*Candidatus* Steffania adelgidicola' suggesting that this is an evolutionarily rather young symbiont (Toenshoff *et al.*, 2011). The other adelgids maintained and co-evolved with '*Candidatus* Vallotia'. The progenitor of the gammaproteobacterial '*Candidatus* Ecksteinia/'*Candidatus* Gillettellia' co-speciated with *A. nordmannianae/piceae* and *A. cooleyi/coweni* (at an evolutionary rate that led to the two distinct genera observed today), but was replaced by the progenitor of '*Candidatus* Profftia' in the ancestor of *A. abietis/viridis* and *A. laricis/tardus* between 40 and 60 million years ago.

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Of course, alternative scenarios are possible, but in any case, the available data show clearly that the evolutionary history of the association between adelgids and their bacterial symbionts included several symbiont replacements and is thus not as straight forward as that of aphids and their primary symbiont *B. aphidicola* who stably coexisted for some 180 million years (Moran *et al*., 2008). Symbiont replacement is known from other insect groups, for example for obligate symbionts of weevils [Dryophthoridae; (Conord *et al*., 2008)], and there is also evidence that the obligate symbiont *B. aphidicola* can be replaced (despite of the long-term intimate association with its aphid hosts) when other symbionts are present that compete and are able to take over the function of *B. aphidicola* (Koga *et al*., 2003; Gosalbes *et al*., 2008).

Replacement of symbionts can help the insect host to adapt to changing environmental conditions and to conquer new ecological niches (Lefevre *et al*., 2004; Conord *et al*., 2008). For the adelgids, these might be novel (secondary) host trees or a novel food source (parenchyma cell sap versus phloem), although there is no obvious correlation of extant symbiont phylotypes with these factors. The function of the bacterial symbionts of adelgids, i.e. their role for the biology of the insect host, is still unknown. Similar to other bacteriocyte-associated symbionts of insects, they might be involved in host nutrition, but to date there is only some data available for the *A. nordmannianae/piceae* symbiont '*Candidatus* Steffania adelgidicola'. Despite its reduced genome, this symbiont is still able to synthesize many key metabolites, and it does not show a detrimental (parasitic) effect on its host, which suggests a role in supplementing its host's diet (Buchner, 1953; Toenshoff *et al*., 2011).

In conclusion, we provide evidence for a surprising diversity of bacterial symbionts in adelgids and for a complex evolutionary history of this symbiosis, which involved co-evolution as well as multiple infection events and symbiont replacement. This is fundamentally different from the well-studied symbiosis between bacteria and aphids, the sister group of adelgids, where a single obligate symbiont, *B. aphidicola*, is present in nearly all aphid species. The investigation of additional adelgid species and genomic analysis of the identified symbionts will help to further extend our understanding of the evolution of this symbiosis and the role of the bacterial symbionts in this association.

Experimental procedures

Collection of insects

Natural adelgid populations of *A. laricis/tardus*, *A. abietis/viridis and A. cooleyi/coweni* complexes were sampled from Norway spruce [*Picea abies* (L.) Karst.] and Douglas-fir (*Pseudotsuga* sp.), respectively, during the year (2007–2009) (Table 1). Branches were cut and stored at 4°C until collection of the insects. The insects were either used directly for PCR, stored in ethanol for DNA extraction, or fixed for FISH, histology and electron microscopy.

Histology and transmission electron microscopy

Insects were prefixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) over night at 4°C and subsequently fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at room temperature. Fixed samples were dehydrated with 2,2-dimethoxypropane and embedded in low viscosity resin (Agar Scientific). Semi-thin section were stained with toluidine blue and examined by microscopy (LSM 510 Meta, Carl Zeiss, Jena, Germany). Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a ZEISS EM 902 electron microscope at 80 kV.

FISH

Insects were fixed in 4% paraformaldehyde for 4 h at 4°C, crushed on a glass slide and covered with 0.2% low melting agarose. Hybridization was performed using the protocol, hybridization and washing buffers described elsewhere (Daims *et al*., 2005). Symbiont-specific probes were designed using the probedesign/probematch tools implemented in the ARB software package (Ludwig *et al*., 2004); oligonucleotide probe sequences and hybridization conditions are given in Table S1. DOPE-FISH using double labelled probes was applied to increase the signal intensity (Stoecker *et al*., 2010). Hybridized slides were examined using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany).

DNA extraction

The adelgids were washed in double distilled water before DNA extraction. Pooled whole adult individuals or eggs were used for DNA extraction using the DNeasy Blood & Tissue kit (Qiagen) following the manufactures instructions or a Chelex based DNA extraction method (Groot *et al*., 2005). DNA was stored at −20°C until usage for PCR.

PCR and sequencing of ef1alpha *and* coI *genes*

Partial cytochrome *c* oxidase subunit 1 gene fragments (*coI*; 639 nucleotides) and the elongation factor 1-alpha gene fragments (*ef1alpha*; 664 nucleotides) of all sampled adelgids were amplified using published primers (Folmer *et al*., 1994; Guryev *et al*., 2001; Havill *et al*., 2007); Table S1). Presence and size of amplification products were checked with agarose gel electrophoresis and SyBr-Green staining. PCR products were purified with the PCR purification kit (Qiagen) and directly used for sequencing with gene-specific primers (Table S1) on an ABI 3130 XL genetic analyser using the BigDye Terminator kit v3.1 (ABI). DDBJ/EMBL/GenBank accession numbers are given in Table 1.

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PCR and sequencing of 16S rRNA genes

DNA from up to 30 individuals of different life stages (gallicolae, exules and eggs) was purified separately, and near-full-length 16S rRNA genes of the bacterial symbionts were amplified using general bacterial or symbiont-specific primer (Table S1). Presence and size of the amplification products were checked with agarose gel electrophoresis and ethidium bromide or SyBr-Green staining. Amplified 16S rRNA genes from selected populations of *A. laricis/tardus* (Klausen-Leopoldsdorf), *A. abietis/viridis* (Klausen-Leopoldsdorf) and *A. cooleyi/coweni* (Kaltern, Madison) were cloned using the TOPO TA Cloning kit (Invitrogen Life Technologies) following the manufacturer's instructions. Fifteen to 30 clones were screened by restriction fragment length polymorphism (RFLP) analysis using *MspI* (Fermentas). Up to three clones of each dominant RFLP sequence pattern were sequenced as described above. In addition, adelgid populations of *A. laricis/tardus* (Eberndorf, Tutzing) and *A. abietis/viridis* (Klausen-Leopoldsdorf, Aschering) were screened for the respective symbionts using symbiont-specific primers designed with the probedesign/probematch tools of the ARB software package (Ludwig *et al*., 2004; Table S1). PCR products were purified using the PCR purification kit (Qiagen) and directly sequenced. DDBJ/EMBL/GenBank accession numbers are given in Table 1.

Phylogenetic analysis

The program ARB (Ludwig *et al*., 2004) was used for phylogenetic analysis. ARB 16S rRNA databases were updated with sequences from GenBank obtained by sequence homology searches using BLASTn available at the NCBI web site (National Centre for Biotechnology Information; Altschul *et al*., 1990). The alignment was refined by visual inspection taking into account secondary structure information available in ARB. 16S rRNA sequences obtained in this study were checked for chimeras using the program Pintail (Ashelford *et al*., 2005). Databases for the genes encoding cytochrome *c* oxidase 1 (*coI*) and the elongation factor 1-alpha (*ef1alpha*) were established with representative sequences downloaded from GenBank and aligned using MAFFT (Katoh *et al*., 2005). Phylogenetic trees were calculated using MrBayes and the maximum parsimony and TREEPUZZLE methods implemented in ARB (Felsenstein, 1989; Huelsenbeck and Ronquist, 2001; Schmidt *et al*., 2002; Ronquist and Huelsenbeck, 2003). PhyML trees were calculated using the Mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py) (Guindon and Gascuel, 2003) and neighbour joining analysis was performed using MEGA 4 (Tamura *et al*., 2007).

Acknowledgements

We gratefully acknowledge Thomas Penz and Stefan Pfeiffer for help with sampling of the insects, and Holger Daims for macrophotography. This work was funded by Austrian Science Fund (FWF) grants Y277-B03 and P22533-B17.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. *A. abietis/viridis* (gallicola; Aschering) feeding on the primary host tree spruce. An egg cluster is seen between the wings of the insect. Photograph by Holger Daims.

Fig. S2. Phylogenetic relationships of *A. laricis/tardus*, *A. abietis/viridis* and *A. cooleyi/coweni* with other insects based on a concatenated dataset of the cytochrome c oxidase 1 (*coI*, 634 bp) and elongation factor 1-alpha (*ef1alpha*, 616 bp) genes. A maximum likelihood tree (PhyML) is shown with selected members of the Aphididae and the Phylloxeridae as outgroup. Maximum likelihood (1000 replicates) and maximum parsimony (1000 replicates) bootstrap values, TREEPUZZLE support values and Bayesian posterior probabilities are indicated at the inner nodes. The letters (G = 60 ± 11.84, F = 65.05 ± 12.03) indicate the estimated divergence times (millions of years ± standard deviation) of the Adelgidae (Havill *et al*., 2007). Names of host trees are indicated on the right. GenBank/EMBL/DDBJ accession numbers of coI and ef1alpha genes are given in squared brackets. Bar, 10% estimated evolutionary distance.

Fig. S3. Semi-thin cross-section of the abdomen of *A. abietis/viridis* (Klausen-Leopoldsdorf) stained with toluidine blue showing the location of bacteriocytes longitudinal to the gut. Bar represents 20 μm. g = gut, b = bacteriocytes containing symbionts, s = storage tissue, m = muscle.

Fig. S4. Intracellular localization of bacteriocyte-associated symbionts of *A. cooleyi/coweni* (Madison) by fluorescence in situ hybridization. A probe mix labeled with FLUOS (green) and targeting most bacteria, a probe double labeled with Cy3 (red) and specific for the betaproteobacterial symbiont ‘*Candidatus* Vallotia cooleyia’ (ValCo-458), and a probe labeled in Cy5 (blue) and specific for the gammaproteobacterial symbiont ‘*Candidatus* Gillettellia cooleyia’ (GilCo-576) were used simultaneously. The combined signal of the bacterial and the symbiont-specific probes appears yellow for ‘*Candidatus* Vallotia cooleyia’ and blue-green for ‘*Candidatus* Gillettellia cooleyia’, respectively. Bar represents 10 μm.

Table S1. Primers and probes used in this study. Additional information on oligonucleotide probes is available at probeBase (Loy et al., 2007).

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Co-evolution and symbiont replacement shaped the symbiosis between adelgids (Hemiptera: Adelgidae) and their bacterial symbionts

Elena R. Toenshoff *et al*.

Supporting Information

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Short name[a]	Sequence (5´-3´)	Specificity	Target site[c]	Annealing temperature/ Formamide concentration	Reference
16S rRNA gene primers					
616F	AGA GTT TGA TYM TGG CTC	16S rRNA gene, most *Bacteria*	8-25	52°C	(Juretschko et al., 1998)
1492R	GGY TAC CTT GTT ACG ACT T	16S rRNA gene, most *Bacteria* and *Archaea*	1492-1510		(Loy et al., 2005)
ProVi-120F	ATG TCT GGG GAA CTG CCT	16S rRNA gene, '*Candidatus* Profftia virida' of *A. abietis/viridis*	120-137	55°C	This study
ProVi-1436R	CGA GGG TTA AGC TAC TTG		1436-1453		This study
ProTa-120F	ATG TCT GGG AAA CTG CCT	16S rRNA gene, '*Candidatus* Profftia tarda' of *A. laricis/tardus*	120-137	61°C	This study
ProTa-1436R	CGA AGG TTA AGC TAC CTG		1436-1453		This study
Vallotia-131F	CGT RTC TTA GAG TGG GGG	16S rRNA gene, genus *Vallotia* of *A. abietis/viridis*, *A. laricis/tardus*, *A. cooleyi/coweni*	131-148	62°C	This study
Vallotia-1460R	ATC CTA CCG TGG TAA CCG	16S rRNA gene, genus Vallotia of *A. abietis/viridis*, *A. laricis/tardus*, *A. cooleyi/coweni*	1460-1477	62°C	This study
Host gene primers					
Cox911	TTT CTA CAA ATC ATA AAG ATA TTG G	Mitochondrial cytochrome c oxidase subunit 1 (*col*), various eukaryotes		50°C	(Guryev et al., 2001) (Folmer et al., 1994)
Cox912	TAA ACT TCA GGG TGA CCA AAA AAT CA	Mitochondrial cytochrome c oxidase subunit 1 (*col*), various eukaryotes			(Guryev et al., 2001) (Folmer et al., 1994)
AdelEF1F1	GTA CAT CCC AAG CCG ATT GT	Partial nuclear elongation factor 1-alpha (*ef1alpha*), various Hemiptera		61°C	(Havill et al., 2007)
AdelEF1R2	CTC CAG CTA CAA AAC CAC GA	Partial nuclear elongation factor 1-alpha (*ef1alpha*), various Hemiptera			(Havill et al., 2007)
16S rRNA targeted oligonucleotide probes					
EUB338-I[b]	GCT GCC TCC CGT AGG AGT	16S rRNA, most *Bacteria*	338-355	10-60%	(Amann et al., 1990)
EUB338-II[b]	GCA GCC ACC CGT AGG TGT	16S rRNA, Bacteria not covered by probe EUB338-I, e.g. many *Planctomycetes*	338-355	10-60%	(Daims et al., 1999)
EUB338-III[b]	GCT GCC ACC CGT AGG TGT	16S rRNA, Bacteria not covered by probe EUB338-I, e.g. many *Verrucomicrobia*	338-355	10-60%	(Daims et al., 1999)
NONEUB	ACT CCT ACG GGA GGC AGC	Control probe complementary to EUB338-I	338-355	n.d.	(Wallner et al., 1993)
ProVi-177	GGT CTA AAG ACG TCA TGC	16S rRNA, '*Candidatus* Profftia virida' of *A. abietis/viridis*	177-194	30%	This study
ProTa-186	CCC CAC TTT GGT CCT AAG	16S rRNA, Gammaproteobacterial symbiont '*Candidatus* Profftia tarda' of *A. laricis/tardus*	186-203	30%	This study
GilCo-576	ACA GGC CGC CTG CGT GCT	16S rRNA, Gammaproteobacterial symbiont '*Candidatus* Gillettellia cooleyia' of *A. cooleyi/coweni*	576-593	30%	This study
ValTa-1136	TCC CTA AAG TGC TCT TGC	16S rRNA, '*Candidatus* Vallotia tarda' of *A. laricis/tardus*	1136-1155	30-35%	This study

ValVi-624	CAC TGA TGC GGT TCC CGG	16S rRNA, '*Candidatus* Vallotia virida' of *A. abietis/viridis*	624-641	30-35%	This study
ValCo-458	ACC TAG GTA TTT GCC AGG	16S rRNA, '*Candidatus* Vallotia cooleyia' of *A. cooleyi/coweni*	458-475	30%	This study

[a] Short name used in this study or in the references.
[b] EUB338-I, EUB338-II, and EUB338-III were applied simultaneously to target most *Bacteria.*
[c] target site according to *Escherichia coli* 16S rRNA gene numbering.

Figure S1. ***A. abietis/viridis*** **(gallicola; Aschering) feeding on the primary host tree spruce**. An egg cluster is seen between the wings of the insect. Photograph by Holger Daims.

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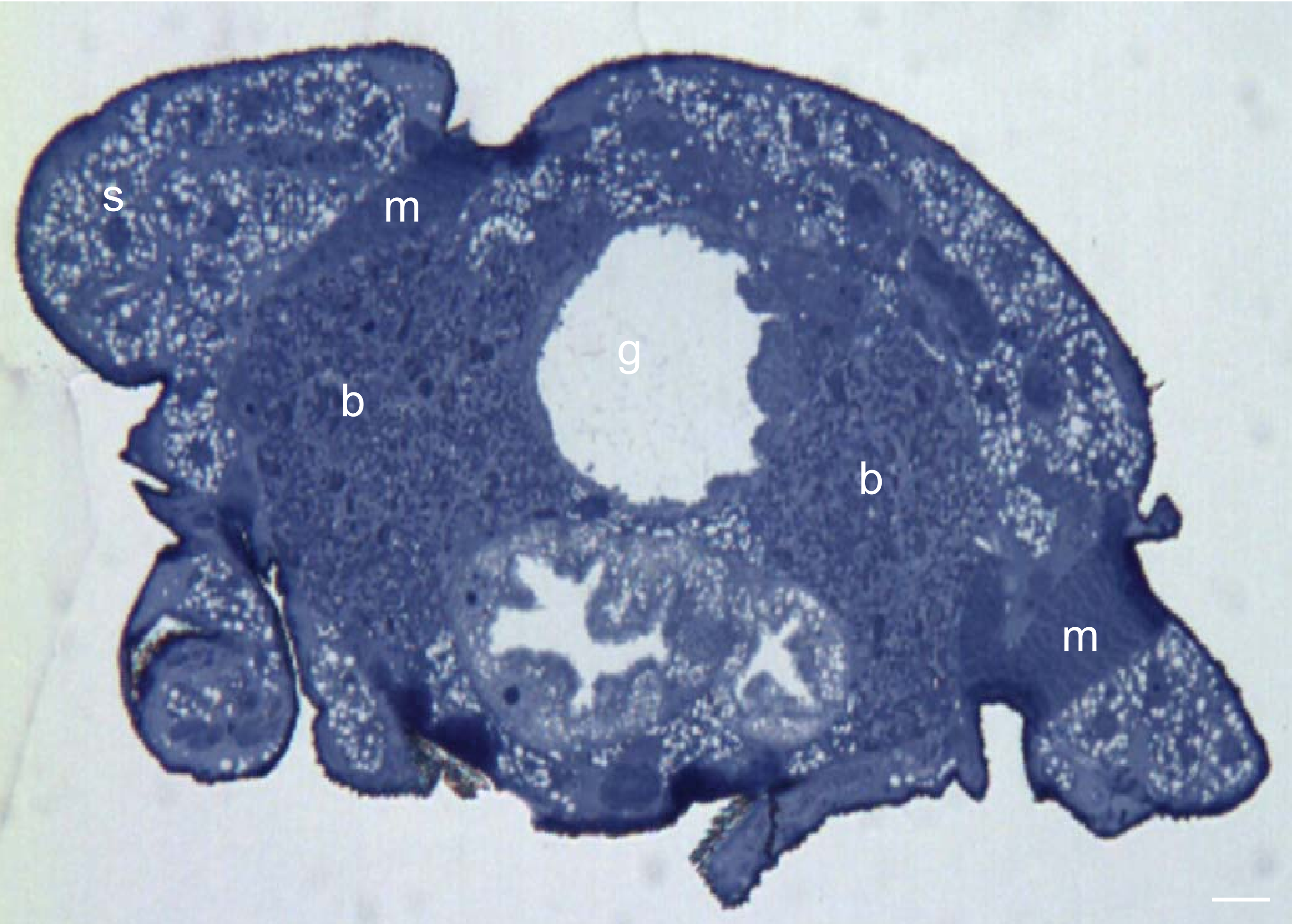

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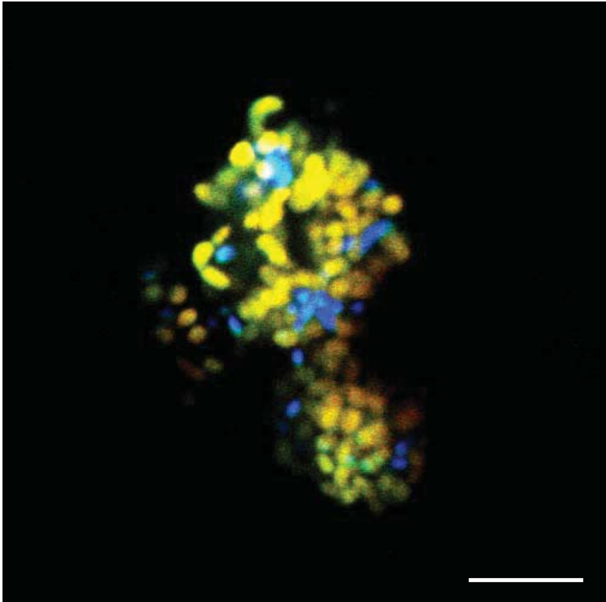

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Chapter IV

Pineus strobi (Hemiptera: Adelgidae) contains novel gammaproteobacterial symbionts

In preparation for *Environ Microbiol*

Pineus strobi (Hemiptera: Adelgidae) contains novel gammaproteobacterial symbionts

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Running title: novel gammaproteobacterial symbionts in adelgids

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Summary

Adelgids (Insecta: Hemiptera: Adelgidae), a small group of insects, are known as severe pests of various conifers in the northern hemisphere. They harbour diverse bacteriocyte-associated symbionts that belong to the *Gammaproteobacteria* and *Betaproteobacteria* and are vertically transmitted from mother to offspring. The Adelgidae acquired phylogenetically different symbionts during their evolution indicating multiple symbiont acquisition and replacement events. Additionally, members of the *Adelges* clade show evidence for co-evolution with their respective symbionts over millions of years. In this study, we investigated the bacteriocyte-associated symbionts of *Pineus strobi*, the first examined member of the insect family Adelgidae of the *Pineus* clade, as identified based on *coI* and *ef1alpha* genes. *Pineus strobi* harboured two morphologically different symbiont types that were surrounded by a symbiosome membrane and located in distinct bacteriocytes. 16S and 23S rRNA gene sequence analyses assigned both symbionts to the *Gammaproteobacteria*, but none of these symbionts was directly related to other known symbionts of adelgids of the *Adelges* clade or of any other insects. Fluorescence *in situ* hybridization confirmed the affiliation of the obtained rRNA gene sequences to the bacteriocyte-associated bacterial morphotypes.

Therefore, we propose the names '*Candidatus* Annandia pinicola' and '*Candidatus* Boerneria pinicola' for the coccoid and polymorphic symbionts, respectively.

Introduction

Symbiotic associations between bacteria and insects are widespread in nature. Ten percent of all investigated insects harbour bacterial symbionts which have an essential function in supplying nutrients to the host (Buchner, 1953; Moran *et al.*, 2008; Moya *et al.*, 2008; Akman Gündüz and Douglas, 2009). These obligate symbionts are vertically transmitted (e. g. passed from mother to offspring) and contained by specialized cells, the bacteriocytes. The symbionts form long-term associations with their hosts, leading to co-speciation. One of the best-studied symbionts is *Buchnera aphidicola*, the obligate (primary) symbiont of most aphids (Hemiptera: Aphididae). They are associated with each other for more than 180 million years (Munson *et al.*, 1991a; Munson *et al.*, 1991b; Moran *et al.*, 2008). *B. aphidicola* provides its insect partner with essential amino acids missed in the deficient host diet, the phloem (Sandström and Moran, 1999; Shigenobu *et al.*, 2000; Thomas *et al.*, 2009). Moreover, facultative (secondary) symbionts co-residing in the bacteriocytes or located in various other tissues were recognized. They support the obligate symbionts by e. g. providing protection against heat stress and natural enemies (Oliver *et al.*, 2010) and partaking in the nutrition of the host. Secondary symbionts are known for various insects such as spittlebugs, cicadas, leafhoppers, mealybugs, and some aphids (Wu *et al.*, 2006; McCutcheon and Moran, 2007; McCutcheon *et al.*, 2009; McCutcheon and Moran, 2010; Lamelas *et al.*, 2011; McCutcheon and von Dohlen, 2011).

Adelgidae (Insecta: Hemiptera: Adelgidae), a relatively small group of insects with ~65 known species, can be severe pests in forest ecosystems and for the Christmas tree industry (Blackman and Eastop, 1994). Based on phylogenetic analyses, they separated from phylloxerans (Phylloxeridae) and aphids (Aphididae) ~120 million years ago and divided into the two major clades *Adelges* and *Pineus* ~88 million years ago (Havill *et al.*, 2007). Adelgids are host tree specific and live exclusively on various conifers where they feed on parenchyma cell sap (Balch, 1952; Plumb, 1953; Rohfritsch and Anthony, 1992; Young *et al.*, 1995) or phloem (Balch, 1952; Allen and Dimond, 1968; Parry and Spires, 1982; Sopow *et al.*, 2003). Adelgids pass through a complex lifecycle with a host tree switch between the primary host *Picea* spp. and a secondary host, which can be a *Pinus*, *Larix*, *Tsuga*, *Pseudotsuga,* or *Abies* (Havill and Foottit, 2007).

Histological studies demonstrated a high diversity of symbionts in adelgids (Profft, 1937; Buchner, 1953; Steffan, 1968). The function of these symbionts in still unknown, but they are

expected to play a role in host nutrition (Buchner, 1953; Toenshoff *et al.*, 2012b). The antibiotic treatment of *Adelges tsugae* resulted in degradation of the symbionts and the eventual death of the host. This demonstrates the importance of the symbionts for their host and they thus might be potential targets for biocontrol of these pests (Shields and Hirth, 2005).

To date, only the bacteriocyte-associated symbionts of adelgids of the *Adelges* clade were investigated on the molecular level. These adelgids harbour symbionts belonging to the *Gammaproteobacteria* and *Betaproteobacteria*, which are located in bacteriocytes in the insect's abdomen. The symbionts are vertically transmitted from mother to offspring and underwent co-speciation and co-evolution with their adelgid hosts for 40 to 65 million years. Furthermore, '*Candidatus* Steffania adelgidicola', a symbionts of *Adelges nordmannianae/piceae*, seems to be an evolutionarily relatively young symbiont as inferred from its genome structure and reduction state (Toenshoff *et al.*, 2012a; Toenshoff *et al.*, 2012b). The diversity of symbionts in adelgids is remarkably larger than in their sister group, the aphids (Aphididae). They mostly harbour one single symbiont named *B. aphidicola*, which infected an ancestor of the modern aphids 180 million years ago (Moran *et al.*, 2008). Consequently, the scenario in adelgids is fundamentally different.

The pine bark adelgid *Pineus strobi* (Hartig 1837) belongs to the *Pineus* clade, is widely distributed in North America, and one of the most serious pests of the eastern white pine (*Pinus strobus*). On their secondary host tree *Pinus*, *Pineus strobi* is anholocyclic (Havill and Foottit, 2007) and is feeding on the outer tissue of the phloem (Raske and Hudson, 1964). Histological studies of *Pineus strobi* demonstrated two morphological different symbiont types, coccoid and polymorphic, which are located in single and multinucleated bacteriocytes, respectively (Profft, 1937; Steffan, 1968).

In this study, we wanted to examine whether members of the adelgids belonging to the *Pineus* clade harbour symbionts that are phyllogenetically related to the symbionts of adelgids of the *Adelges* clade ('*Candidatus* Steffania adelgidicola', '*Candidatus* Ecksteinia adelgidicola', '*Candidatus* Vallotia virida/tarda/cooleyia', '*Candidatus* Profftia virida/tarda', '*Candidatus* Gillettellia cooleyia') or whether they acquired distinct symbionts during evolution. Transmission electron microscopy, phylogenetic analyses of 16S and 23S rRNA gene sequences, and fluorescence *in situ* hybridization uncovered two morphological and phylogenetically different gammaproteobacterial symbionts located in distinct bacteriocytes of *Pineus strobi*. None of these newly found symbionts was related either to known adelgid symbionts or to other symbionts of insects. Thus, this small insect group is highly diverse in

respect to their bacteriocyte-associated symbionts and may provide an opportunity to study events like multiple acquisition, co-evolution, and genome adaptation of their symbionts.

Results and discussion

Host insect identification

Two adelgid populations (Kaltern, Italy; Christmas decoration) were sampled from *Pinus* sp. (Table 1, Supporting information Fig. S1). DNA extraction, PCR and sequencing were done as described elsewhere (Toenshoff *et al.*, 2012a). Comparative analyses of the *coI* and *ef1alpha* gene sequences indicated that the adelgids from both populations sampled were affiliated to *Pineus strobi* of the insect family Adelgidae. Phylogenetic analysis confirmed the affiliation to published *Pineus strobi* sequences, which formed a monophyletic group within the clade *Pineus* of the Adelgidae (Fig. S2).

Ultrastructural analysis of bacteriocyte-associated symbionts

Transmission electron microscopy was conducted for individuals of both populations (Fig. 1). Bacteriocytes harbouring bacterial symbionts were located in proximity to the gut in the insect abdomen (Fig. 1A). Two morphologically different symbiont types, polymorphic and coccoid, were located within distinct bacteriocytes (Fig. 1A, B) as known for the bacteriocyte-associated symbionts of *Adelges nordmannianae/piceae* (Toenshoff *et al.*, 2012b). Both bacterial morphotypes were surrounded by a typical Gram-negative type cell wall and by a host-derived membrane, the so called symbiosome membrane (Fig. 1C, D). The polymorphic symbiont type had a length of 1.8 to 5.2 µm (Fig. 1D). The coccoid symbiont type had a size of 0.9 - 3.6 µm (Fig. 1C). Additionally, membrane vesicles were present between the outer membrane of the coccoid symbiont type and the symbiosome membrane (Fig. 1C). Membrane vesicles (MVs) are conserved among 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, as vehicles for bacterial toxins, for cell-cell communication, nutrient acquisition, and inhibition of phagosome-lysosome fusion and immune recognition (Kuehn and Kesty, 2005; Deatherage *et al.*, 2009; Kulp and Kuehn, 2010). The MVs seen in the symbiosome are therefore likely to play a role in the interaction between symbiont and host living in such a close symbiotic association.

The two novel gammaproteobacterial symbionts

For the identification of the bacterial symbionts of *Pineus strobi*, DNA was isolated and the bacterial 16S and 23S rRNA genes were amplified, cloned and sequenced as described previously (Toenshoff *et al.*, 2012b). Sequence analysis revealed various 16S rRNA gene sequences of bacteria belonging to different phyla (*Proteobacteria*, *Acidobacteria*,

Bacteriodetes). Only one 16S rRNA gene sequence type (1) that was similar to 16S rRNA gene sequences of other gammaproteobacterial insect symbionts was found in both *Pineus strobi* populations (Kaltern, Christmas decoration). These sequences were highly similar to each other (99.3-99.9%) and presumably represent one of the bacteriocyte-associated symbionts of *Pineus strobi* (Fig. 1, Fig. 2A). This phylotype (1) has the highest similarity to an enterobacterial 16S rRNA gene sequence found in rhizosphere (EF151985) with 95.7-95.8% sequence similarity and to *Providencia rettgeri* (95.5-95.6%), a free-living and cultivable bacterium associated with the coral *Acropora digitifera* from Gulf of Mannar (EU660316). It showed only low similarities to the *Adelges nordmannianae/piceae, A. abietis/viridis, A. laricis/tardus,* and *A. cooleyi/coweni* symbionts '*Candidatus* Steffania adelgidicola' (89.3-89.5%) and '*Candidatus* Ecksteinia adelgidicola' (90.7-90.9%), '*Candidatus* Profftia virida' (90-90.4%), '*Candidatus* Profftia tarda' (90-90.1%), and '*Candidatus* Gillettellia cooleyia' (89.2-89.4%), respectively. Phylogenetic analyses demonstrated an affiliation of the sequence type 1 to *Arsenophonus nasoniae,* a symbiont in parasitoid wasps, to *Moellerella wisconsensis* found in human stool specimens and to *Providencia rettgeri* within the *Gammaproteobacteria*. No close relationship is given to other symbionts of adelgids and sternorrhynchan insects (Fig. 2A). Therefore, the sequence type 1 represents a new symbiont lineage within the *Gammaproteobacteria*.

Among the 23S rRNA gene sequences, sequence analyses revealed one sequence type (2) related to other insect symbionts, as well as free-living bacteria within the *Gammaproteobacteria*. It showed highest similarity to '*Candidatus* Purcelliella pentastirinorum', a bacteriome-inhabiting symbiont in cixiid planthoppers (FN428803) (82.3%), and similarities of 80.4% and 79.8% to the *Adelges nordmannianae/piceae* symbionts '*Candidatus* Steffania adelgidicola' and '*Candidatus* Ecksteinia adelgidicola', respectively. The phylotype 2 presumably represents the second of the two symbionts of *Pineus strobi*. Phylogenetic analyses using different treeing methods confirmed the affiliation of the phylotype 2 to '*Candidatus* Purcelliella pentastirinorum', but due to inconsistent results of the treeing methods applied, no certain affiliation can be assigned to this phylotype (Fig. 2B). We therefore propose a new lineage within the *Gammaproteobacteria*.

In situ *hybridization of '*Candidatus *Annandia pinicola' and '*Candidatus *Boerneria pinicola'*

The two bacterial morphotypes observed by transmission electron microscopy (Fig. 1) were observable by FISH with a 16S rRNA targeted oligonucleotide probe specific for the sequence type 1 and a 23S rRNA targeted probe for the sequence type 2. The obtained 16S and 23S rRNA sequences (see above) could thus be assigned to the coccoid and the polymorphic symbionts, respectively, and showed that they are located in distinct

bacteriocytes (Fig. 3). All bacteria within the bacteriocytes are stained with either of the symbiont-specific probes, demonstrating the absence of further bacteria. Both symbionts were detected in the exulis life stage (Fig. 3A) as well as in eggs (Fig. 3B), suggesting the vertical transmission from mother to offspring as known for other adelgid symbionts (Toenshoff *et al.*, 2012a; Toenshoff *et al.*, 2012b).

Taken together, we could show that *Pineus strobi* contains two morphologically different symbionts located in distinct bacteriocytes, which formed novel evolutionary lineages within the *Gammaproteobacteria*. They were directly related neither to known symbionts of adelgids or *B. aphidicola*, the obligate symbiont of the sister group aphids, nor to other symbiont of sternorrhynchan insects such as psyllids, scale insects and whiteflies (Baumann, 2005). The low degree of phylogenetic relation to other bacteria within the *Gammaproteobacteria* positions these symbionts in two novel genera. We thus propose two novel tentative names according to Murray and Stackebrandt (1995).

‘*Candidatus* Annandia pinicola’

‘*Candidatus* Annandia pin-icola’ is named in honor to the entomologist P. N. Annand for his contribution to the systematics of Adelgidae (Annand, 1928); pin-icola means friend or lover of pine. This symbiont 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 located in a single bacteriocyte. ‘*Candidatus* Annandia pinicola’ represents a novel genus within the class *Gammaproteobacteria* (phylum *Proteobacteria*). The basis of assignment is: 16S rRNA (GenBank/EMBL/DDBJ accession number [*to be added*]).

‘*Candidatus* Boerneria pinicola’

‘*Candidatus* Boerneria pin-icola’ is named in honor to the entomologist C. V. Börner for his contribution to the classification of Adelgidae; pin-icola means 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 located in a single bacteriocyte. ‘*Candidatus* Boerneria pinicola’ represents a novel genus within the class *Gammaproteobacteria* (phylum *Proteobacteria*). The basis of assignment is: 23S rRNA (GenBank/EMBL/DDBJ accession number [*to be added*]).

Multiple infection events and symbiont replacement

Two novel gammaproteobacterial symbionts (‘*Candidatus* Boerneria pinicola’ and ‘*Candidatus* Annandia pinicola’) that are distantly related to known symbionts of adelgids of the *Adelges* clade were found in *Pineus strobi*, the first investigated member of the *Pineus*

clade (Fig. 2, 4). This indicates that *Pineus strobi* has acquired its own symbionts during evolution. The ancestor of these symbionts infected *Pineus strobi* sometime within the last 30 million years as indicated by their presence in both populations (Fig. 4; Havill *et al.*, 2007). This study expands the number and diversity of known adelgid symbionts (Fig. 4) and thus adds support to the statement that symbiont replacement shaped the symbiosis of adelgids (Toenshoff *et al.*, 2012a). Multiple replacement events of obligate symbionts are known for other insect groups, e. g. the weevils of the family Dryophthoridae (Conord *et al.*, 2008). Similarly, novel and independent symbiont acquisition is known for aphids, where a former facultative symbiont partly took over the nutritional function of the long-term associated symbiont *B. aphidicola* (Koga *et al.*, 2003; Gosalbes *et al.*, 2008). The acquisition of novel symbionts by adelgids might help in invading new niches and expanding the host range (Tsuchida *et al.*, 2004) and thus might assure survival and allow flexibility to switch between different food sources (phloem and parenchyma cell sap) and host trees.

In conclusion, the insect family Adelgidae contain a surprisingly high number of phylogenetically different symbionts compared to the aphids, which usually harbour mostly one single obligate symbiont named *B. aphidicola* (Moran *et al.*, 2008). The adelgids seem to have a complex evolutionary history involving co-evolution of the host species and their symbionts, as well as multiple symbiont acquisition and replacement events. Additional studies identifying unknown symbionts as well as genome analyses of known symbionts will bring further insights into the evolution of these symbioses and the role of the symbionts in the association with their hosts. The Adelgidae may function as a model system for in depth studies of symbiont replacement and multiple acquisition events, and also for illuminating the processes leading to bacterial genome reduction as they harbour evolutionary relatively young symbionts.

Acknowledgements

We gratefully acknowledge Stefan Pfeiffer for support with sampling of the adelgids. This work was founded by Austrian Science Fund (FWF) grants P22533-B17 and Y277-B03.

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Figure legends

Fig. 1. Ultrastructure of bacteriocyte-associated symbionts of *Pineus strobi* (Kaltern). **(A, B)** Ultra-thin sections of the adelgid abdomen showing two morphotypes of symbionts located in distinct bacteriocytes. **(C)** The coccoid and **(D)** the polymorphic symbiont type show a typical Gram-negative type cell wall and are surrounded by the symbiosome membrane. **(C)** The coccoid symbiont type is dividing by binary fission. Vesicles are present between its outer and the symbiosome membrane. Specimens were processed as described previously (Toenshoff *et al.*, 2012a). One individual for each population (Kaltern, Christmas decoration) was examined with a ZEISS EM 902 electron microscope at 80 kV. Bars in A and B represent 2 µm; bar in C represents 1 µm; bar in D represents 500 nm. b = bacteriocytes filled with two different types of symbionts, s = symbiont located within a bacteriocyte, sm = symbiosome membrane, cm = cell membrane, ms = membrane stacks, v = vesicles.

Fig. 2. Phylogenetic relationship of the bacteriocyte-associated symbionts of *Pineus strobi* within the *Gammaproteobacteria*. **(A)** A 16S rRNA-based and **(B)** a 23S rRNA-based TREEPUZZLE tree are shown. Tree calculations were performed using the maximum parsimony and TREEPUZZLE methods implemented in ARB (Ludwig *et al.*, 2004). PhyML and Neighbour-joining trees were calculated using the Mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py; Guindon and Gascuel, 2003) and MEGA (Tamura *et al.*, 2011), respectively. TREEPUZZLE support values, maximum likelihood (1000 replicates), maximum parsimony (1000 replicates) and Neighbour-joining (1000 replicates) bootstrap values are indicated. GenBank/EMBL/DDBJ accession numbers of the 16S and 23S rRNA gene sequences are given in squared brackets. Bars represent 10% estimated evolutionary distance. 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.

Fig. 3. *In situ* hybridization of '*Candidatus* Annandia pinicola' and '*Candidatus* Boerneria pinicola' from different life stages (exulis, egg) of *Pineus strobi* (Christmas decoration). Bacterial symbionts were labelled by FISH using symbiont-specific 16S and 23S rRNA-targeted oligonucleotide probes together with the general bacterial probe (EUB338I, 5'- GCT GCC TCC CGT AGG AGT -3') double-labelled with FLUOS (green). The probe specific for the 23S rRNA of the polymorphic symbiont '*Candidatus* Boerneria pinicola' (BoePi-1439, 5'- CGC TCT CTT GGC CAA CTT -3') was labelled with Cy3 (red). The probe specific for the 16S rRNA of the coccoid symbiont '*Candidatus* Annandia pinicola' (AnnPi-265, 5'- TCG TTG CCT AGG GGA GCC -3') was labelled with Cy5 (blue). All probes were used simultaneously. The combined signal from bacterial and symbiont-specific probes appear yellow for

‘*Candidatus* Boerneria pinicola‘ and blue-green for ‘*Candidatus* Annandia pinicola‘. **(A)** Bacteriocytes of an adelgid from the exulis life stage. **(B)** Symbionts inside a *Pineus strobi* egg. Fluorescence *in situ* hybridization was done as described previously with 35% formamide in the hybridization buffer (Toenshoff *et al.*, 2012b) and further examined using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany) equipped with two helium-neon-lasers (543 nm and 633 nm) and an argon laser (458-514 nm). Image analysis was performed with the standard software delivered with the instrument (version 3.2). Bars represent 10 µm.

Fig. 4. Schematic diagram depicting the multiple acquisition and replacement events of gammaproteobacterial bacteriocyte-associated symbionts in the insect family Adelgidae. The chronogram of the host phylogeny in dark grey is based on a concatenated data set of *mtDNA* and *ef1alpha* according to Havill *et al.* (2007). Coloured lines represent the known gammaproteobacterial symbionts of adelgids and aphids. Symbiont phylogeny is based on 16S and 23S rRNA analyses and was partly taken from Toenshoff *et al.* (2012a). The letters indicate the estimated divergence times (D = 88 ± 14.09, F = 65.05 ± 12.03, G = 60 ± 11.84, I = 55 ± 11.67; millions of years ± standard deviation) of the Adelgidae (Havill *et al.*, 2007). Dotted lines represent anticipated symbiont lineages.

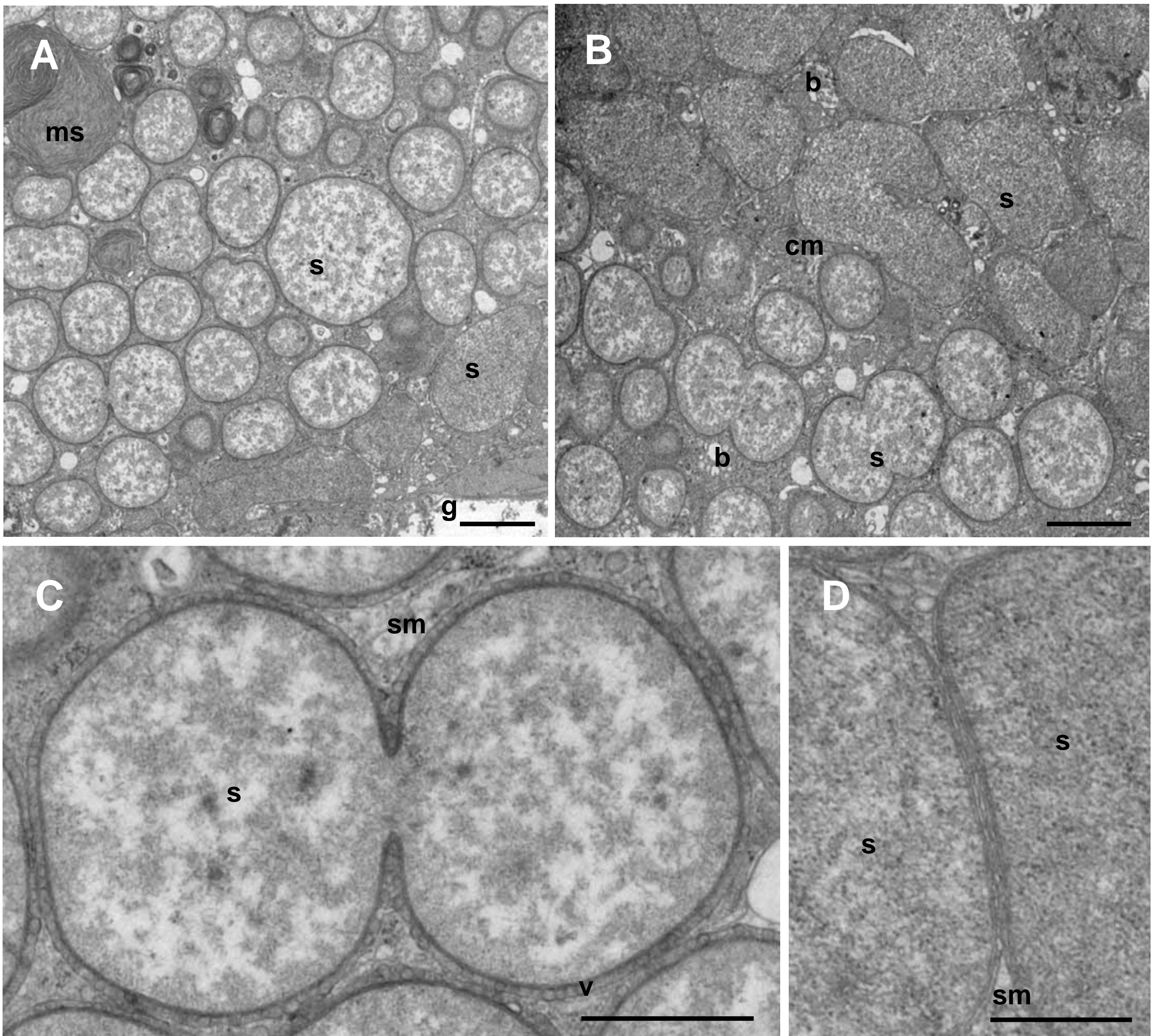

Fig. 1

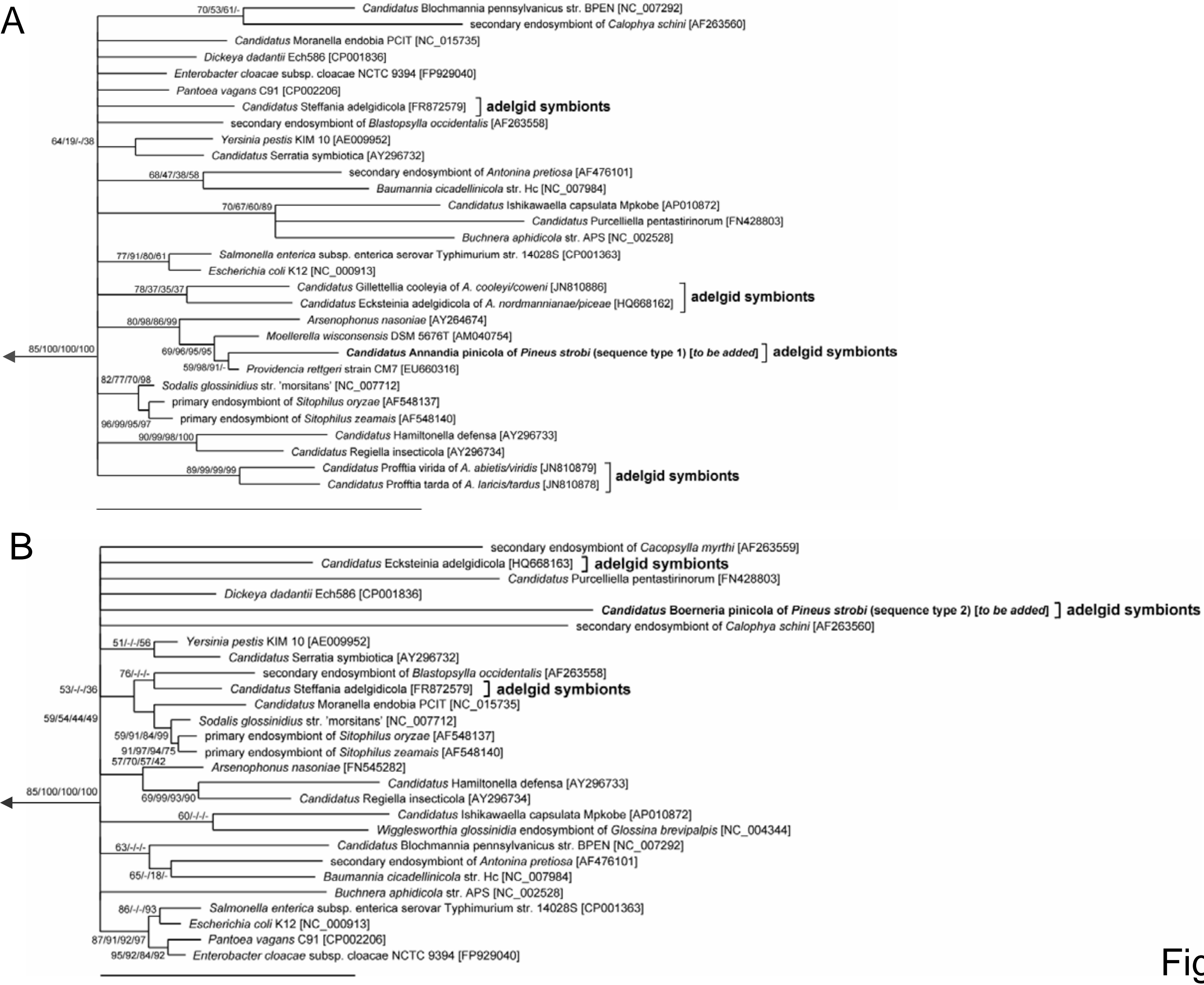

Fig. 2

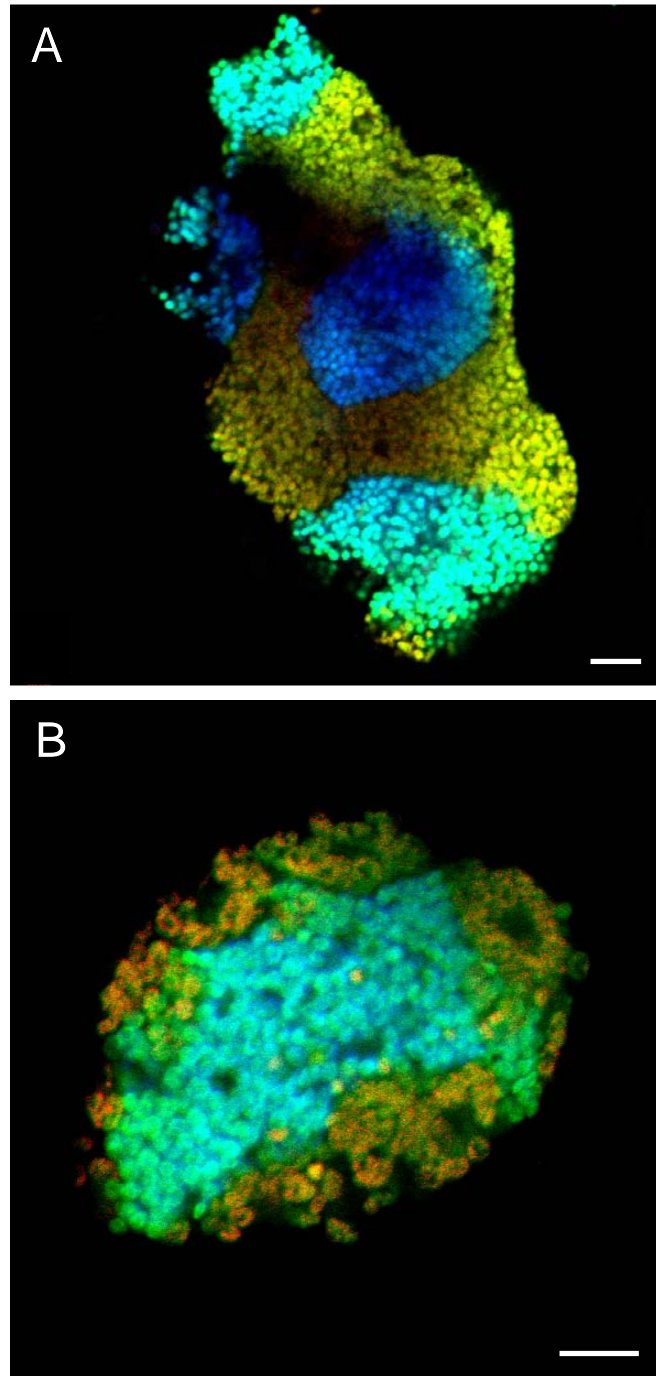

Fig. 3

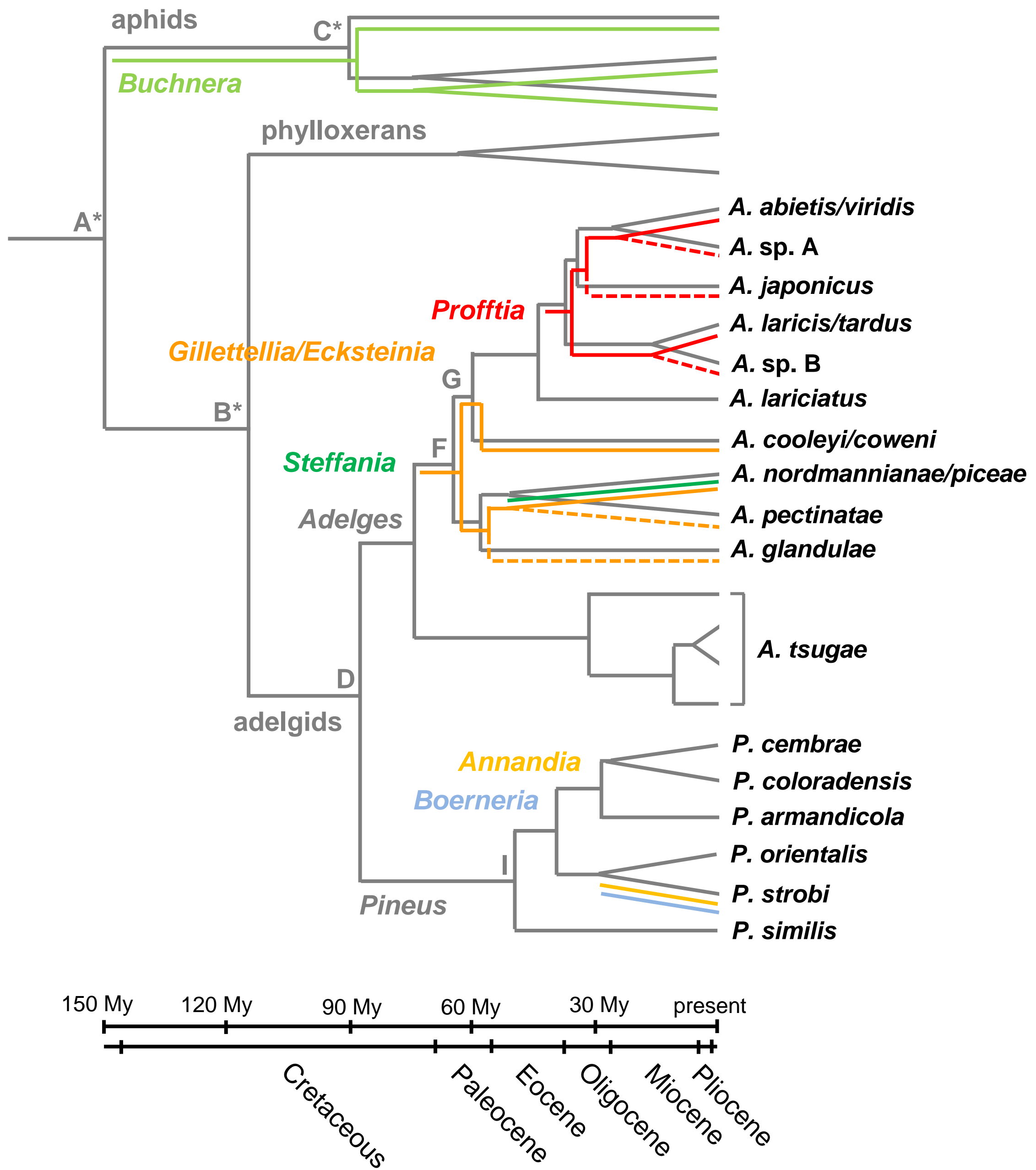

Fig. 4

Table 1. Studied adelgids and their symbionts

Organism	Location (year)	Host plant	GenBank accession numbers (insect host)		GenBank accession numbers (symbionts)	
			col	***ef1alpha***	**16S rRNA gene of '*Candidatus* Annandia pinicola' (coccoid)**	**23S rRNA gene of '*Candidatus* Boerneria pinicola' (polymorphic)**
Pineus strobi (Hartig 1837)	Kaltern, Italy (2006)	*Pinus strobus* L. (eastern white pine)	*to be added*	*to be added*	*to be added*	-
Pineus strobi (Hartig 1837)	Christmas decoration (2011)	*Pinus sp.*	*to be added*	*to be added*	*to be added*	*to be added*

Pineus strobi **(Hemiptera: Adelgidae) contains novel gammaproteobacterial symbionts**

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Supporting Information

Supplementary figure legends

Fig. S1. *Pineus strobi* (exules) sampled in Kaltern is feeding on the secondary host tree pine (*Pinus strobus*). The adelgids recognizable as white dots (grey rectangle) were located around the needles on the branch.

Fig. S2. Phylogenetic affiliation of *Pineus strobi* to the insect family Adelgidae based on a concatenated dataset of the cytochrome c oxidase subunit 1 (*coI*, 624 nuc) and elongation factor 1-alpha (*ef1alpha*, 616 nuc) genes. Tree calculations were performed using maximum parsimony and TREEPUZZLE implemented in the ARB software package (Ludwig *et al.*, 2004). PhyML trees were calculated using the Mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py; (Guindon and Gascuel, 2003)) and Neighbour-joining trees using MEGA (Tamura *et al.*, 2011). A maximum likelihood tree is shown. Maximum likelihood (1000 replicates), maximum parsimony (1000 replicates) and Neighbor-joining (1000 resamplings) bootstrap values, and TREEPUZZLE support values are indicated at the internal nodes. Selected members of the Aphididae and Phylloxeridae were used as outgroup. The letters (D = 88 ± 14.09, I = 55 ± 11.67) indicate the estimated divergence times (millions of years ± standard deviation). GenBank/EMBL/DDBJ accession numbers of *coI* and *ef1alpha* are given in square brackets. Bar, 10% estimated evolutionary distance.

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Fig. S1

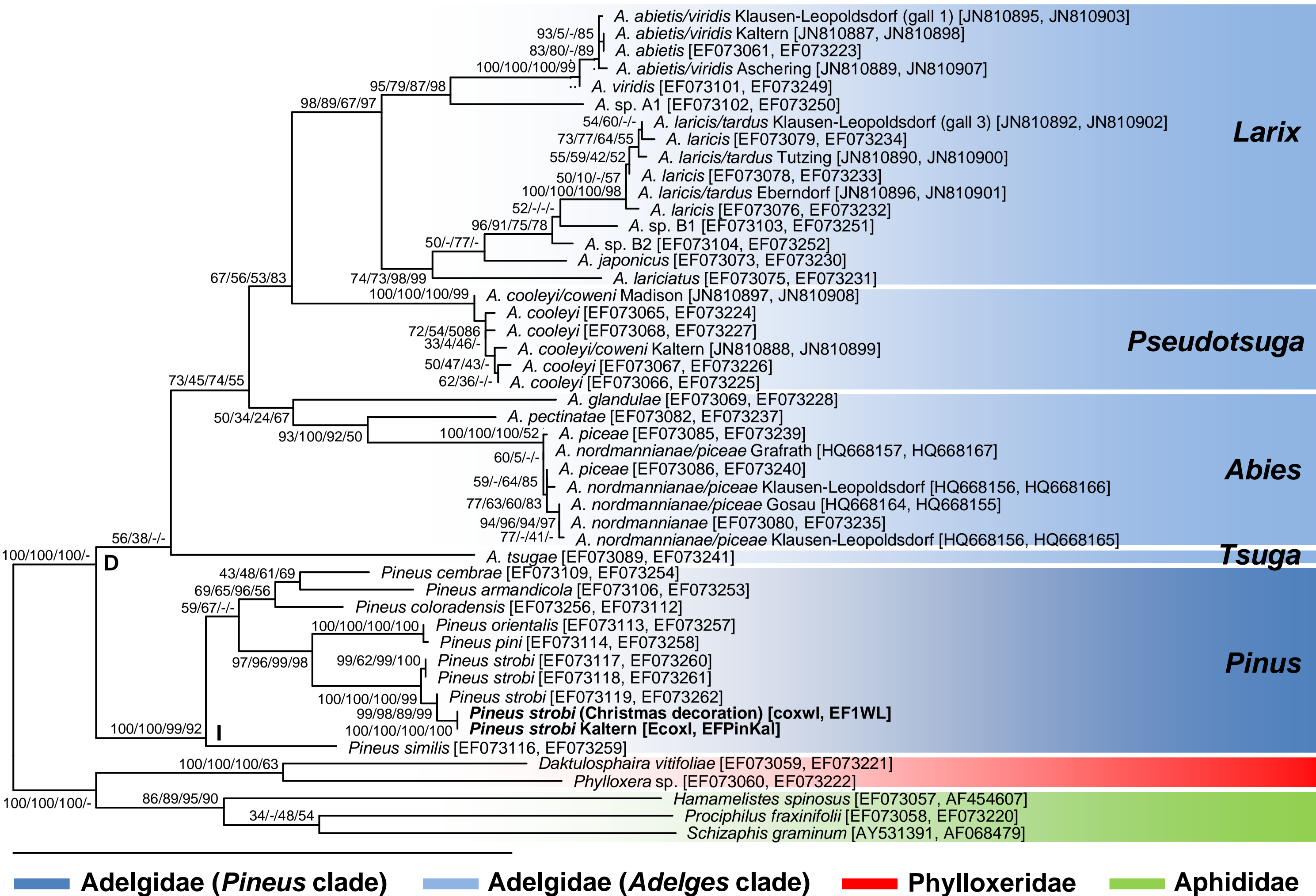

Fig. S2

Chapter V

Outlook

Outlook

In this thesis the symbiosis between adelgids and their bacterial symbionts was investigated for the first time on a molecular level. The examined adelgids contain bacteriocyte-associated symbionts belonging to the *Gammaproteobacteria* and *Betaproteobacteria*, which are distantly related to known obligate symbionts of other plant-feeding sternorrhynchan insects. Six novel bacterial genera operating as symbionts were identified in all examined adelgids species or complexes (Figure 8; Toenshoff *et al.*, 2012a; Toenshoff *et al.*, 2012b; Chapter IV). Additionally, due to their presence in various life stages, a vertical transmission from mother to offspring is postulated for these symbionts. Interestingly, symbionts of the clades '*Ca.* Vallotia', '*Ca.* Profftia' and '*Ca*. Gillettellia/ *Ca*. Ecksteinia' co-diversified and co-evolved with their respective host adelgids (Figure 8; Toenshoff *et al.*, 2012a).

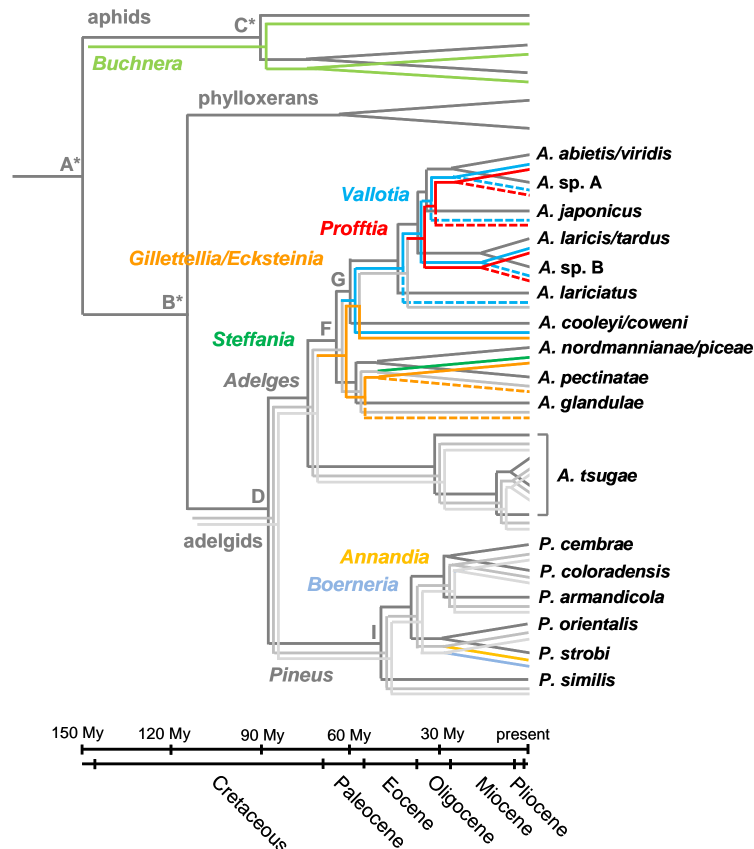

Figure 8. Schematic diagram of multiple acquisition and replacement of phylogenetically different bacteriocyte-associated symbiont in the insect family Adelgidae. A chronogram of the adelgid phylogeny (dark grey) based on a concatenated data set of *mtDNA* and *ef1alpha* is shown. Estimated time points are based on fossil insect records. The letters (D = 88 ± 14.09, F = 65.05 ± 12.03, G = 60 ± 11.84, I = 55 ± 11.67) indicate the estimated divergence times (millions of years ± standard deviation) of the Adelgidae. Chronogram and data points were taken from Havill *et al*. (2007). Phylogenetically distantly related symbionts based on 16S and 23S rRNA gene sequences are indicated with different colored lines. Doted lines are assumed symbiont infections.

These symbioses represent relatively short-term associations (between ~65 and ~40 million years) (Toenshoff *et al.*, 2012a) as corroborated by genome structure and composition of '*Ca.* Steffania adelgidicola', a bacteriocyte-associated symbiont of *Adelges nordmannianae/piceae* (Toenshoff *et al.*, 2012b). Therefore, these symbionts seem to have been acquired by adelgids after the separation from their aphid sister group. However, multiple symbiont acquisitions and replacement events shaped the symbiosis between adelgids and their bacterial symbionts. The acquisition of novel symbionts might have enabled the adelgids to occupy specialized niches, habitats and trees, which were perhaps otherwise unavailable. Symbiotic associations thus might have been the driving force for the diversification of the Adelgidae.

To further illuminate the biology, ecology, the essential mechanisms for interaction, and the evolutionary aspect of the symbiosis between bacteria and adelgids, further in-depth analyses should be done. Today, various novel single cell techniques such as cell sorting and Laser Microdissection (LMD) microscopy allow the separation of single cells from a mixture. These cells can then be used further for downstream analysis such as whole genome amplification and PCR (amplification of single target genes) and followed up with next generation sequencing (454, Illumina) (Woyke *et al.*, 2010).

I suggest examining a larger number of adelgid species (well distributed within the family Adelgidae) and their bacteriocyte-associated symbionts to generate a more complete picture of the diversity and evolution of symbiosis in adelgids. Genome sequencing of a number of phylogenetically distantly related symbionts and genome comparison will help to understand the role of these symbionts in these multiple partner systems. We may furthermore gain new insigths of the way these kinds of symbiotic associations are established, which might provide useful clues with respect to the control of these pests. Additionally, transcriptomic and proteomic studies of symbionts and hosts will allow to underpin the analyses of the genomic data and might bring insights into the regulation of this symbiosis (Vinuelas *et al.*, 2007; Bermingham *et al.*, 2009; Poliakov *et al.*, 2011). Moreover, the establishment of an adelgid lab culture would facilitate studies on symbiont replacement using symbiont free adelgids and inocula containing symbionts of free-living adelgids. Studies on the metabolic interactions between symbionts and hosts would also be possible through the use of labelled substrates and e.g. NanoSIMS-based methodologies (reviewed in (Musat *et al.*, 2012; Wagner, 2009)). Furthermore, the genome sequencing of the host adelgids might illuminate the fundamental question of the ability of multiple infections and replacements by various symbionts. For example, this could be analysed by using the genomic differences between adelgids and aphids hosting mostly one main symbiont (The International Aphid Genomics

Consortium, 2010). The analysis of the differences between the immune systems of adelgids and aphids might help to understand how the immune system controls the infection, and the establishment of the adelgids symbiosis (Douglas *et al.*, 2011; Gerardo *et al.*, 2010). Additionally, the host genome would help to elucidate the metabolic complementarities of host and symbionts (The International Aphid Genomics Consortium, 2010).

Taken together, the insect family Adelgidae, with its high number of phylogenetically diverse symbionts, represents a highly interesting system to study symbiont replacement and complementation events.

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Chapter VI

Summary

Zusammenfassung

Summary

Symbioses between bacteria and insects are widespread in nature. Estimated ten percent of all insects harbour bacteriocyte-associated symbionts in their body cavity. These heritable symbionts provide essential nutrients, which are absent in the hosts' diet; therefore, they are often essential for their host insect. These symbionts are distributed over the whole kingdom *Bacteria,* and many bacteriocyte-associated symbionts cluster into the phylum *Proteobacteria*. The symbiotic associations are often stable over million of years and this relatively constant environment of the host insect leads to the adaptation of the symbiont genome. Therefore, the age of the symbiosis is reflected in the symbiont genome showing typical genomic features such as pseudogenization, genome shrinkage and extreme G + C content. Some insect families harbour one single obligate symbiont, whereas others acquired further symbiotic bacteria during evolution and either replaced the present symbiont or built a complex symbiotic consortium.

Adelgids (Insecta: Hemiptera: Adelgidae), members of a relatively small insect family (~ 65 described species), are mainly distributed in the northern hemisphere and contain species that represent severe pests for the forest ecosystem. The adelgids are a sister group of the phloem-feeding aphids, scale insects, whiteflies and psyllids, which often harbour one single obligate symbionts. *B. aphidicola* is a prime example as the obligate symbiont of most aphids. Knowledge about bacteriocyte-associated symbionts in adelgids is still scarce and thus these symbiotic associations were examined in this thesis. My main work encompasses three tightly linked projects that deal with the diversity of bacteriocyte-associated symbionts, the acquisition and replacement of the symbionts and their genome adaptation.

First, I investigated two bacteriocyte-associated symbionts of the *Adelges nordmannianae/piceae* complex (species- pair). The host insects were identified by *coI* and *ef1alpha* gene sequencing and based on the obtained sequences assigned to the Adelgidae. I identified two novel symbionts provisionally classified as '*Candidatus* Ecksteinia adelgidicola' and '*Candidatus* Steffania adelgidicola' that represent to the best of our knowledge the first symbionts of Adelgidae identified by molecular methods. Each of the two symbionts constitutes a novel lineage within the *Gammaproteobacteria* based on phylogenetic analyses of their 16S and 23S rRNA gene sequences. No close phylogenetic relationship was detected to other insect symbionts. Light and transmission electron microscopy uncovered two morphological different symbionts, one coccoid and the other polymorphic, located in distinct bacteriocytes. Furthermore, fluorescence *in situ* hybridisation (FISH) confirmed their identity and intracellular location. Both symbionts were detected using PCR and FISH in all investigated life stages including eggs suggesting vertical transmission from mother to offspring. Additionally, a genome fragment (85 kb) of '*Ca*. S. adelgidicola' was

obtained by generating a metagenomic library from purified symbionts and further analysed. The frequency of pseudogenes, the average length of intergenic spacer regions and the presence of several genes that are absent in long-term associated symbionts indicated that '*Ca*. S. adelgidicola' is an evolutionary young symbiont. Most likely, this symbiont was acquired after the diversification of the adelgids from aphids.

Furthermore, I conducted a study to elucidate the diversity of adelgids symbionts. I investigated three additional adelgid complexes (*Adelges laricis/tardus*, *Adelges abietis/viridis*, *Adelges cooleyi/coweni*) that were identified based on their *coI* and *ef1alpha* gene sequences. All examined complexes contained two phylogenetically different symbionts belonging to *Gammaproteobacteria* as well as *Betaproteobacteria.* These symbionts were identified based on their 16S rRNA gene sequences and by FISH. The betaproteobacterial symbionts ('*Ca*. Vallotia tarda', '*Ca*. Vallotia virida' and '*Ca*. Vallotia cooleyia', respectively) of all three examined complexes co-evolved with their respective host adelgids as shown by the phylogenetic congruence of their and their respective host sequences. Similarly, the gammaproteobacterial symbionts of *Adelges laricis/tardus* and *Adelges abietis/viridis* ('*Ca*. Profftia tarda' and '*Ca*. Profftia virida', respectively) co-diversified with their respective host insect as reflected in their phylogeny. On the other hand, *Adelges cooleyi/coweni* contained a gammaproteobacterial bacteriocyte-associated symbiont ('*Ca*. Gillettellia cooleyia') that is not directly related to '*Ca*. Profftia tarda' and '*Ca*. Profftia virida', but moderate related to '*Ca*. Ecksteinia adelgidicola', the symbiont of *A. nordmannianae/piceae*. All newly obtained symbionts were present in all investigated populations and life stages as demonstrated by a symbiont-specific PCR assay. This supports a vertical transmission from mother to offspring as already shown for the symbiont- *A. nordmannianae/piceae* symbiosis. Based on estimated divergence time points for the examined adelgids, the symbioses between the symbionts and all three examined complexes were established between ~65 and ~40 million years ago. Taken together, the adelgids contain a surprisingly high number of phylogenetically different symbionts compared to aphids, which harbour mostly one single bacteriocyte-associated symbiont named *B. aphidicola*. Moreover, the adelgids symbiosis reflects a complex evolutionary history including multiple symbiont acquisitions and replacements.

In the last project, I investigated the bacteriocyte-associated symbionts of *Pineus strobi*, the first examined member of the *Pineus* clade in the Adelgidae (subdivided in *Adelges* clade and *Pineus* clade), identified based on their *coI* and *ef1alpha* genes. Transmission electron microscopy demonstrated two morphologically different symbionts, one coccoid and the other polymorphic, residing in distinct bacteriocytes. Phylogenetic analyses based on 16S and 23S rRNA analyses assigned both obtained symbiont sequences to the

Gammaproteobacteria, and their identity was confirmed by FISH. None of these two newly obtained symbionts ('*Ca*. Annandia pinicola' and '*Ca*. Boerneria pinicola') were directly related to symbionts of adelgids belonging to the *Adelges* clade (Adelgidae) or other known insect symbionts. Therefore, this study supports the postulated hypothesis that multiple symbiont acquisition and replacement shaped the symbiosis between adelgids and their symbionts.

In summary, adelgids harbour various vertically transmitted bacteriocyte-associated symbionts representing relatively recent symbiotic associations. Co-evolution between symbiont and host, and symbiont replacements were identified that define this symbiosis. Hence, adelgids acquired their own symbionts during evolution after the separation from their aphid sister group. Future studies of adelgid symbionts diversity and their genomes will bring a deeper understanding of this symbiosis. The insect family Adelgidae could serve as a case study for symbiont replacement and multiple acquisition events.

Zusammenfassung

In der Natur sind Symbiosen zwischen Insekten und Bakterien weit verbreitet. In der Körperhöhle beherbergen ~ 10% aller Insekten Bakteriozyten-assoziierte Symbionten. Für Wirtsinsekten sind diese, durch direkte Vererbung weitergegebenen Symbionten, oft von essentieller Bedeutung. Diese Bakterien stellen Nährstoffe zur Verfügung, die in der Wirtsnahrung abwesend sind. Aus phylogenetischer Sicht sind bakterielle Symbionten über das ganze Königreich *Bacteria* verteilt, jedoch fällt ein großer Teil der Symbionten in das Phylum *Proteobacteria*. Diese symbiontischen Gemeinschaften sind oft über Millionen von Jahren stabil. Während diesem Zeitraum und unterstützt durch die relativ stabile Umgebung im Wirt, durchlief das Symbiontengenom vielfältige Anpassungen. Typische genomische Charakteristika dieser Bakterien sind Pseudogenisierung, Genomschrumpfung und ein meist extrem niedriger GC-Gehalt. Einige Insektenfamilien beherbergen einen alleinigen obligaten Hauptsymbionten, wohingegen andere Insektenfamilien über die Zeit weitere symbiontische Bakterien erworben haben. Entweder ersetzt das Wirtsinsekt den Hauptsymbionten oder diese bilden ein komplexes symbiontisches Konsortium.

Die Adelgiden (Insecta: Hemiptera: Adelgidae) sind eine relativ kleine Insektenfamilie, welche aus ~ 65 beschriebene Arten besteht. Sie sind hauptsächlich in der nördlichen Hemisphäre verbreitet und stellen für das Ökosystem Wald teilweise schwerwiegende Schädlinge dar. Die Adelgiden sind eine Schwesterngruppe der Phloem-saugenden Blattläuse, Schildläuse, weiße Fliegen und Blattflöhe, welche oft einen alleinigen Hauptsymbionten beherbergen. Die Blattläuse enthalten beispielsweise als obligaten Symbionten meist *Buchnera aphidicola*. Das Wissen über Bakteriozyten-assoziierte Symbionten von Adelgiden ist immer noch spärlich, deshalb wird in dieser Dissertationsarbeit diese Symbiose untersucht. Meine Dissertationsarbeit beinhaltet drei zusammenhängende Projekte, in denen die Diversität der Bakteriozyten-assoziierten Symbionten, deren Erwerb und Austausch und des weiteren ihre Genomadaptierung untersucht wurden.

Das erste Projekt beinhaltet die Identifizierung zweier Bakteriozyten-assoziierten Symbionten des *Adelges nordmannianae/piceae* Komplexes (Spezies-Paar) und die Analyse eines Genomfragmentes von einem der beiden Symbionten. Die Wirtsinsekten wurden anhand zweier Gene (Cytochrom C Oxidase Untereinheit 1 (*coI*) und Elongationsfaktor 1 Alpha (*ef1alpha*)) identifiziert. Die neu identifizierten Symbionten wurden ‚*Candidatus* Ecksteinia adelgidicola' und ‚*Candidatus* Steffania adelgidicola' benannt. Diese beiden Symbionten repräsentierten unserem Wissen nach, die ersten molekular untersuchten Bakterien in den Adelgiden. Anhand ihrer 16S und 23S rRNS Gensequenzen eröffneten beide Symbionten zwei neue phylogenetische Linien innerhalb der *Gammaproteobacteria* und der *Betaproteobacteria*. Des Weiteren zeigte keiner der beiden Symbionten eine nahe

Verwandtschaft zu anderen Insektensymbionten. Mittels Licht- und Transmissionselektronenmikroskopie konnten zwei morphologisch unterschiedliche Symbionten (kokkoid und polymorph), die in unterschiedlichen Bakteriozyten lokalisiert waren, gezeigt werden. Ihre Identität und intrazelluläre Lage im Insekt wurde darüber hinaus mit der Fluoreszenz *in situ* Hybridisierung (FISH) bestätigt. Beide Symbionten wurden mittels PCR und FISH in allen Entwicklungsstadien, einschließlich der Eier, nachgewiesen. Dieses Ergebnis wies auf eine vertikale Übertragung beider Symbionten von der Mutter auf die Nachkommen hin. Basierend auf aufgereinigter symbiontischer DNS und der daraus hergestellten metagenomischen Klonbank, wurde ein Genomefragment (85 kb) von ‚*Ca*. S. adelgidicola' untersucht. Die Frequenz der Pseudogene, die durchschnittliche Länge der intergenischen Regionen und die Anwesenheit einiger Gene, die in langzeit-assoziierten Symbionten abwesend sind, deuteten an, dass ‚*Ca.* S. adelgidicola' ein evolutionär junger Symbiont ist. Dieser Symbiont wurde sehr wahrscheinlich, nach der Abspaltung von den Blattläusen, von den Adelgiden aufgenommen.

Des Weiteren untersuchte ich, um die Diversität der Adelgidensymbionten zu studieren, drei weitere Adelgidenkomplexe (*Adelges laricis/tardus*, *Adelges abietis/viridis*, *Adelges cooleyi/coweni*). Diese Wirtsinsekten wurden ebenso anhand von *coI* und *ef1alpha* identifiziert. Basierend auf der phylogenetischen Analyse der bakteriellen 16S rRNS Sequenzen, enthielten alle betrachteten Komplexe zwei phylogenetisch unterschiedliche Symbionten. Diese gehören zu den *Gammaproteobacteria* und *Betaproteobacteria*. Untermauert wurden die vorausgegangenen Ergebnisse durch FISH. Die Übereinstimmung der Symbionten- und Wirtsstammbäume zeigte auf, dass die betaproteobakteriellen Symbionten ('*Ca*. Vallotia tarda', '*Ca*. Vallotia virida' und '*Ca*. Vallotia cooleyia'), die in allen drei Komplexen gefunden wurden, mit ihren entsprechenden Wirtsinsekten co-evolvierten. Ebenso co-evolvierten die gefundenen gammaproteobakteriellen Symbionten ('*Ca*. Profftia tarda' und '*Ca*. Profftia virida') mit ihren entsprechenden Wirtsinsekten (*Adelges laricis/tardus* und *Adelges abietis/viridis*). Im Gegensatz zu den zuvor genannten Symbionten, beinhaltete *Adelges cooleyi/coweni* einen gammaproteobakteriellen Symbionten ('*Ca*. Gillettellia cooleyia'), der nicht direkt verwandt zu '*Ca*. Profftia tarda' und '*Ca*. Profftia virida' war. Für diesen Symbionten konnte jedoch eine mäßige Verwandtschaft zu ‚*Ca*. Ecksteinia adelgidicola', dem Symbionten von *A. nordmannianae/piceae*, gezeigt werden. Eine Symbionten-spezifische PCR-Untersuchung zeigte die Anwesenheit dieser Symbionten in allen untersuchten Populationen und Entwicklungsstadien. Dieses Resultat ließ auf eine vertikale Übertragung von der Mutter auf die Nachkommen schließen. Die maternale Übertragung wurde auch in der Symbionten-*A. nordmannianae/piceae* Gemeinschaft gezeigt. Anhand der geschätzten Divergenzzeitpunkte der untersuchten Adelgiden, bildeten sich vor ca. 65 - 40 Millionen Jahren diese Symbionten-Adelgiden Gemeinschaften. Im

Vergleich zu den Blattläusen, die meist nur einen alleinigen Hauptsymbionten mit dem Namen *B. aphidicola* beherbergen, wiesen die Adelgiden eine große Anzahl an phylogenetisch unterschiedlichen Symbionten auf. Die Aufnahme unterschiedlicher Symbionten in die verschiedenen Adelgiden und unser postulierte Symbiontaustausch spiegelt eine komplexe evolutionäre Geschichte wieder.

In meinem letzten Projekt untersuchte ich die Bakteriozyten-assoziierten Symbionten von *Pineus strobi*. Dieser ist der erste molekular untersuchte Vertreter aus dem Stamm *Pineus* (Adelgidae). Diese Art wurde anhand der *coI* und *ef1alpha* Gene identifiziert. Die Transmissionselektronenmikroskopie zeigte zwei morphologisch unterschiedliche Symbionten (kokkoid und polymorph), die in unterschiedlichen Bakteriozyten wohnten. Phylogenetische Analysen, basierend auf der 16S und 23S rRNS, wiesen beide Symbiontensequenzen den *Gammaproteobacteria* zu. Dieses Resultat wurde mit Hilfe von FISH bestätigt. Keiner der beiden Symbionten ('*Ca.* Annandia pinicola' und '*Ca.* Boerneria pinicola') waren direkt mit Symbionten aus dem Stamm *Adelges* (Adelgidae) oder anderen bekannten Insektensymbionten verwandt. Diese Studie untermauert aus diesem Grund die von mir aufgestellte Hypothese, dass Symbiontenaufnahme und Austausch die Adelgidensymbiose formte.

Zusammengefasst kann festgehalten werden, dass Adelgiden verschiedene vertikal übertragene, Bakteriozyten-assoziierte Symbionten besitzten, die in dieser Arbeit zum ersten Mal identifiziert werden konnten. Die Symbiose zwischen diesen Bakterien und ihren Wirtsinsekten ist evolutionsgeschichtlich vergleichsweise jung. Meine Untersuchungen zeigen, dass Adelgiden ihre Symbionten erst nach der Abspaltung von ihrer Schwestergruppe, den Blattläusen, erworben haben. Anschließend prägten dann sowohl Coevolution zwischen Symbionten und Wirtsinsekten als auch das Ersetzen eines Symbionten durch einen anderen, die Entwicklung dieser Symbiose. Weiterführende Studien über die Diversität bakterieller Symbionten der Adelgiden und deren Genome werden helfen, diese Symbiose und deren Evolution besser zu verstehen. Aufgrund der Diversität ihrer bakteriellen Symbionten, stellt die Familie der Adelgiden ein hervorragendes Modellsystem zur Untersuchung der Dynamik der Evolution der Symbiose zwischen Bakterien und Insekten dar.

Appendix

A novel betaproteobacterial agent of gill epitheliocystis in seawater farmed Atlantic salmon (*Salmo salar*)

Published *PLoS ONE* 7: e32696

and as supplementary online material:

http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0032696#pone.0032696.s002

OPEN ACCESS Freely available online

PLoS one

A Novel Betaproteobacterial Agent of Gill Epitheliocystis in Seawater Farmed Atlantic Salmon (*Salmo salar*)

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Abstract

Epitheliocystis, a disease characterised by cytoplasmic bacterial inclusions (cysts) in the gill and less commonly skin epithelial cells, has been reported in many marine and freshwater fish species and may be associated with mortality. Previously, molecular and ultrastructural analyses have exclusively associated members of the *Chlamydiae* with such inclusions. Here we investigated a population of farmed Atlantic salmon from the west coast of Norway displaying gill epitheliocystis. Although '*Candidatus* Piscichlamydia salmonis', previously reported to be present in such cysts, was detected by PCR in most of the gill samples analysed, this bacterium was found to be a rare member of the gill microbiota, and not associated with the observed cysts as demonstrated by fluorescence *in situ* hybridization assays. The application of a broad range 16 S rRNA targeted PCR assay instead identified a novel betaproteobacterium as an abundant member of the gill microbiota. Fluorescence *in situ* hybridization demonstrated that this bacterium, tentatively classified as '*Candidatus* Branchiomonas cysticola', was the cyst-forming agent in these samples. While histology and ultrastructure of '*Ca.* B. cysticola' cysts revealed forms similar to the reticulate and intermediate bodies described in earlier reports from salmon in seawater, no elementary bodies typical of the chlamydial developmental cycle were observed. In conclusion, this study identified a novel agent of epitheliocystis in sea-farmed Atlantic salmon and demonstrated that these cysts can be caused by bacteria phylogenetically distinct from the *Chlamydiae*.

Citation: Toenshoff ER, Kvellestad A, Mitchell SO, Steinum T, Falk K, et al. (2012) A Novel Betaproteobacterial Agent of Gill Epitheliocystis in Seawater Farmed Atlantic Salmon (*Salmo salar*). PLoS ONE 7(3): e32696. doi:10.1371/journal.pone.0032696

Editor: David M. Ojcius, University of California Merced, United States of America

Received November 29, 2011; **Accepted** January 29, 2012; **Published** March 12, 2012

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Funding: This study was funded by Austrian Science Fund (FWF) grant Y277-B03 and The Research Council of Norway, grant no. 164771/S40, 163427/S40 and National Veterinary Institute, project no. 2010038. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts. SOM is employed by a privately owned non-affiliated Fish Veterinary company (Vet Aqua International). There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors..

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Introduction

With an increasing demand for fish and overfishing of the oceans, intensive aquaculture production has increased rapidly in recent decades. In 2008, 37% of the global total production of fish, crustaceans and molluscs were obtained through aquaculture, corresponding to an economic value of over 98 billion USD (ftp://ftp.fao.org/FI/STAT/summary/YB_Overview.pdf; 8.7.2011)., Despite relatively intensive research, infectious diseases continue to represent a major challenge to aquaculture production, and much remains to be discovered relating to the aetiology and pathogenesis of infectious diseases.

The term epitheliocystis has been widely used to describe cytoplasmic membrane-bound inclusions containing Gram-negative bacteria found in gill, and less commonly, skin epithelial cells of fish. Epitheliocystis has been observed in more than 50 freshwater and marine wild and cultured fish species [1,2,3]. Extensive host tissue reactions and mortality due to such infections have however, only been reported in farmed fish [4,5,6,7,8,9,10,11]. The presence of these cysts has also been implicated in proliferative gill inflammation (PGI) in sea-farmed Atlantic salmon, although the aetiology of PGI is not well understood [12,13]. Several ultrastructural studies have shown the bacterial agents associated with epitheliocystis to represent a wide range of morphological forms including, in some fish species, an array of morphotypes similar to chlamydial reticulate, intermediate and elementary bodies [4,5,8,14,15,16,17,18].

Using molecular, cultivation-independent methods, epitheliocystis in farmed salmonids has been associated recently with several novel *Chlamydiae*. These include '*Candidatus* Piscichlamydia salmonis' in Atlantic salmon and Arctic char in sea- [12,19] and freshwater [20] respectively, *Neochlamydia*-like bacteria found in Arctic char in freshwater [21], and '*Candidatus* Clavochlamydia salmonicola' found in Atlantic salmon in freshwater [17,22]. Such molecular studies have contributed to an increased understanding of the genetic diversity and wide host range of *Chlamydiae* [18]. However, the ability to cause epitheliocystis might not be restricted to the *Chlamydiae*. During a recent study of salmon populations from seawater displaying PGI, a significant discrepancy between the number of histologically observed cysts and the occurrence of '*Ca.* P. salmonis' estimated by quantitative PCR was registered [12]. As samples were also negative for '*Ca.* Clavochlamydia

salmonicola', it was suggested that other as yet unidentified bacteria were responsible for many of the observed inclusions in these fish. In the present study we identified a novel betaproteobacterium in gill cysts of seawater farmed Atlantic salmon displaying PGI. The name '*Candidatus* Branchiomonas cysticola' is proposed for this novel cyst-forming agent.

Materials and Methods

Tissue sampling

All samples were taken by a qualified veterinarian as part of a disease diagnostic investigation. Sampled fish were euthanized humanely prior to sampling. No permit is required for such diagnostic work in Norway. Gill samples were taken from a population of seawater farmed Atlantic salmon, affected by PGI, in south-western Norway, during the autumn of 2007. Tissues from the ventral parts of the second and third gills were directly fixed for histology and fluorescence *in situ* hybridisation (FISH). For transmission electron microscopy and DNA isolation, tissues were freshly frozen, or collected in RNAlater (Ambion) and stored at −80°C.

Histological examination

Dissected gills were fixed in 10% neutral phosphate-buffered formalin for three days at room temperature and subsequently embedded in paraffin using a standard protocol, sectioned and stained with haematoxylin and eosin (HE) according to standard histological techniques [23]. Single sections including gill filaments and lamellae from each fish were examined by light microscopy. Selected sections were also Gram-stained. Each fish was examined with respect to pathological changes to investigate the severity of PGI and to count the number of cysts within gill tissues using a grading system [slight/low numbers (1), moderate/moderate numbers (2), severe/large numbers (3)].

Transmission electron microscopy

Gills from three fish displaying epitheliocystis were examined. Tissues were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and stored at 4°C, washed in 0.1 M sodium cacodylate buffer at pH 7.4, postfixed in a mixture of 2% (w/v) osmium tetroxide and 1.5% (w/v) potassium ferri hexacyanide in cacodylate buffer, washed, passed through a graded ethanol series and propylene oxide, and embedded in Lx-112 medium (Ladd Research Industries, Inc., Burlington, Vermont, UK). Ultra-thin sections were contrasted with uranyl acetate and lead citrate, and examined with a Philips EM 208 S electron microscope at 60 kV.

Enrichment of gill-associated bacteria and DNA purification

Gills were homogenised and suspended in buffer A (35 mM Tris-HCl, 25 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose, pH 7.5) [24] containing 2 mg/ml Pronase E (Sigma), incubated for 35 min at 37°C and subsequently centrifuged for 10 min at 6,000 rpm at 4°C. The pellet was resuspended in buffer A containing 250 mM ETDA and again homogenized with a Dounce tissue grinder (Wheaton) and filtered through a 5 µm syringe filter. The suspension was centrifuged as before, the pellet washed twice with buffer A and then resuspended in buffer A containing 10 units DNase I. The sample was incubated for 1 h at 4°C followed by DNase inactivation with 50 mM EDTA. The suspension was centrifuged, the pellet washed with buffer A containing 250 mM EDTA and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). DNA was purified using a sodium dodecyl sulphate (SDS)-based method including 1% hexadecylmethylammonium bromide (CTAB) and 200 µg/ml Proteinase K (Roche Applied Science) in the extraction buffer [25]. DNA was stored at −20°C until further use.

PCR, cloning, RFLP, and sequencing

Partial 16 S rRNA gene sequences were amplified by PCR and sequenced as described in Table S1. Novel primers specific for '*Ca.* P. salmonis' were designed using the probedesign/probematch tool implemented in the ARB software package [26](Table S1). PCR reactions consisted of 2 µl template DNA, 1 unit of Taq DNA Polymerase (Fermentas), 10× Taq buffer with KCl and 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide and 50 pmol of each primer in a total volume of 50 µl. Negative (no DNA added) and positive controls were included in all PCR reactions. The presence and size of amplicons were checked by gel electrophoresis and ethidium bromide or Syber Green staining. PCR products were purified using a PCR purification kit (Qiagen) and either directly sequenced or cloned using the TOPO TA Cloning® kit (Invitrogen Life Technologies) according to the manufacturer's instructions. Fifteen to 30 clones were screened and analyzed by restriction length polymorphism (RFLP) analysis using the enzyme *MspI* (Fermentas). PCR products or clones were sequenced using the BigDye Terminator kit v3.1 and an ABI 3130 XL Genetic Analyzer.

Quantitative PCR (qPCR)

Approximately 17.5 mg of gill soft tissues (preserved in RNAlater) were homogenized with a Roche MagNA lyser (Roche Ltd., Basel, Switzerland). DNA was extracted from half the homogenate volume using the Roche High Pure PCR Template Preparation kit (Roche) according to the manufacturer's instruc-

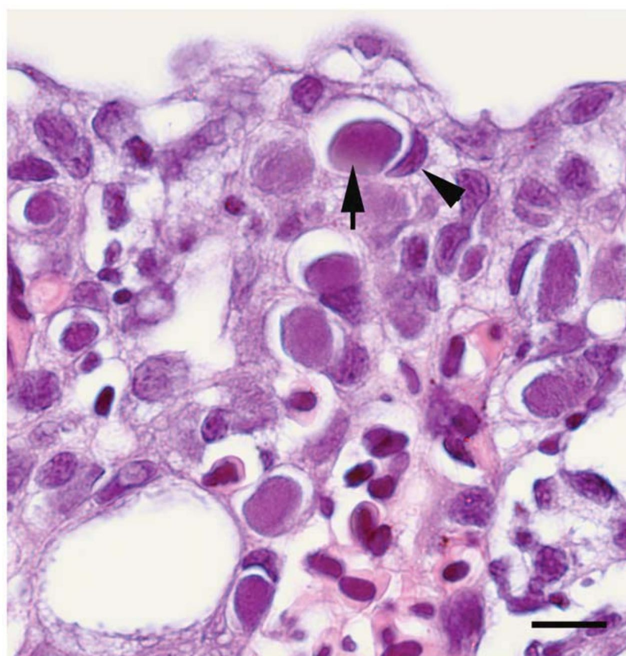

Figure 1. Epitheliocystis in gills of Norwegian seawater farmed Atlantic salmon (*Salmo salar* L.). Haematoxylin and eosin stained sections from formalin-fixed and paraffin embedded gill tissues. The cysts (arrow) appeared in epithelial cells as regular rounded to oval, granular, basophilic (blue) and well-circumscribed cytoplasmic inclusions occupying most of the cell volume. The host cell nuclei were flattened and displaced (arrowhead). Scale bar represents 10 µm.
doi:10.1371/journal.pone.0032696.g001

tions. The suitability of the purified DNA for qPCR was verified with an elongation factor alpha 1 PCR assay and the samples examined for the presence of '*Ca.* P. salmonis' as described previously [12].

Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization was performed either on sections of gill tissues prepared for histological analysis, or on fresh, frozen gill tissues fixed separately. For the latter, gill samples were squashed and fixed in 4% paraformaldehyde at 4°C for 1 h and subsequently washed in phosphate buffered saline. An aliquot of this suspension was then dropped on a glass slide, dried at 46°C and used for FISH. Standard hybridization conditions, hybridization and washing buffers were used [27]. Oligonucleotide probes used are given in Table S1. New probes were designed using the probedesign/probematch tools of the ARB software package [26] and deposited at probeBase [28]. Probe NONEUB was used as negative control. Optimal hybridization conditions for the newly designed probes were determined in a series of hybridization experiments with increasing formamide concentrations in the hybridization buffer. Slides were examined using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss) equipped with two helium-neon-lasers (543 nm and 633 nm) and an argon laser (458–514 nm). Standard software delivered with the instrument (version 3.2) was used for image acquisition.

Clone-FISH

Clone-FISH was performed to evaluate the '*Ca.* P. salmonis' specific oligonucleotide probe (Table S1). The vector pCR®2.1-TOPO® (Invitrogen Life Technologies) containing the amplified '*Ca.* P. salmonis' 16 S rRNA gene fragment was transformed into *E. coli* JM109 (DE3) cells and the insert was *in vivo* transcribed to generate target-rRNA as described [29]. *E.coli* cells were fixed with 4% paraformaldehyde for 30 min at RT and used for FISH following the standard protocol [27].

Phylogenetic sequence analysis

The ARB program package [26] was used for phylogenetic analysis. An in-house 16 S rRNA sequence database updated using blastn homology search for the newly obtained sequences with sequences deposited in the GenBank database provided by the NCBI (National Centre for Biotechnology Information) was used [30]. Trees were calculated using TREEPUZZLE with the HKY evolutionary model of substitution and maximum parsimony (1000 replicates) implemented in ARB [31,32]. PhyML trees (HKY85, 1000 replicates) were calculated using PHYML 3.0

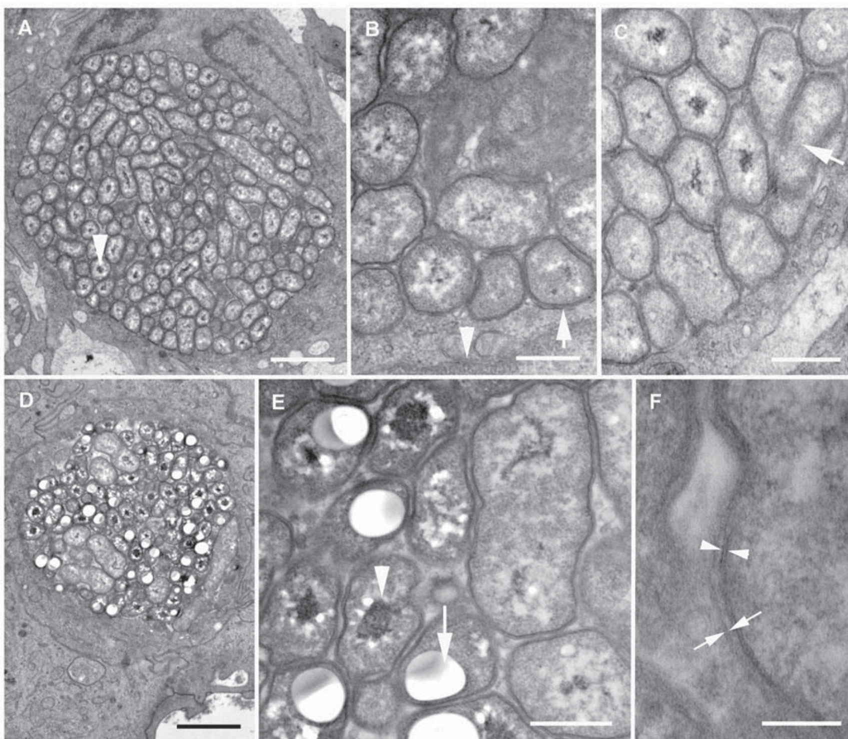

Figure 2. Cyst and bacterial ultrastructure. Ultra-thin sections of gill tissue examined by transmission electron microscopy. The micrograph depicts cyst morphology in fish gill tissues negative for the presence of '*Ca.* P. salmonis' by qPCR. (**A**) The cysts were densely packed with polymorphic bacteria of round, coccoid or short to long rod-shaped morphologies, with or without nucleoids (electron dense material in the centre of the bacterium, arrowhead). Scale bar represents 2 µm. (**B**) Cysts were limited by a membrane formed by the host cell (arrow). Plasma membrane of the host cell (arrowhead). Electron dense nucleoids were observed in many bacterial cells. Scale bar represents 500 nm. (**C**) Nucleoids were apparently absent in a low number of bacteria, resembling morphotypes described as chlamydial reticulate bodies in previous studies (arrow). Scale bar represents 500 nm. (**D**) Other cysts contained more bacterial cells with distinct nucleoids resembling morphotypes previously described as chlamydial intermediate bodies. Scale bar represents 2 µm. (**E**) A high number of the cyst associated bacteria contained large nucleoids (arrowhead) and vesicles (arrow), which may have artefactually expanded during tissue processing, compressing the surrounding structures. Scale bar represents 500 mn. (**F**) All bacteria were limited by a double membrane of which the inner (arrowhead) was clearly trilaminar while the outer (arrows) was less distinct. Scale bar represents 100 nm.
doi:10.1371/journal.pone.0032696.g002

provided by the Mobyle portal (http://mobyle.pasteur.fr/cgi-bin/portal.py) [33]. The program MEGA [34] was used for the distance method Neighbor-Joining (Jukes-Cantor correction, 1000 bootstrap replicates).

Nucleotide sequence accession numbers

The obtained 16 S rRNA gene sequences of '*Ca.* B. cysticola' and '*Ca.* P. salmonis' were deposited at GenBank/EMBL/DDBJ under the accession numbers JN968376 and JQ065095/JQ065096 respectively.

Results

Histology and electron microscopy

A population of seawater farmed Atlantic salmon showing signs of respiratory distress was investigated. Of the 15 sampled fish, 14 displayed pathological changes consistent with PGI, briefly; circulatory disturbance, inflammation, epithelial cell-death and hyperplasia [13], while cysts containing Gram-negative bacteria were observed in 12 (Figure 1).

Electron microscopy was performed on gill tissues from three fish, displaying cysts and low loads of '*Ca.* P. salmonis'. The cysts appeared as large membrane-bound cytoplasmic inclusions containing pleomorphic bacterial cells with a Gram-negative type cell wall (Figure 2A–F). Rounded to elongated forms, approximately 0.2–0.4 (diameter)$\times \leq 2$ µm (length) were observed, with a number of the rounded type containing small vesicles possibly consisting of storage compounds. Most of the observed morphotypes resembled the intermediate- while few resembled the reticulate- developmental forms of *Chlamydiae* described in Atlantic salmon [16,19] and warm-blooded animals [35]. Elementary body-like morphotypes were not observed.

'Ca. P. salmonis' not present in cysts

Samples from all 15 fish were examined by qPCR with a 'Ca. P. salmonis'-specific assay [12]. Twelve fish were positive and gave Ct values between 26.9 and 35.5 (median 31.6) suggesting a typical load similar to the estimated $\leq$1360 cells mg^{-1} soft tissue previously reported [12]. Only one sample in the present study was considered as having a high 'Ca. P. salmonis' load (Ct value 26.9) and notably no cysts could be histologically observed in this fish. Although this study has examined too few samples to conclude on the association between 'Ca. P. salmonis' and cysts, no or moderate loads were detected by qPCR in gills of eight of the 12 fish displaying both severe PGI and large numbers of cysts. Thus our results support the previously reported lack of association between 'Ca. P. salmonis' load and cyst number [12].

This suggests the presence of another agent of epitheliocystis in these fish. Yet, '*Ca.* P. salmonis' was clearly present in most of the samples. To further investigate, we analysed a fish with severe PGI, large numbers of cysts and a high Ct value (35.5), reflecting a low load of '*Ca.* P. salmonis', using a *Chlamydiae*-specific PCR assay combining a general chlamydial- and a universal 16 S rRNA gene-targeted primers (SigF2/Univ1390R; Table S1). Cloning of the obtained PCR product and RFLP analysis of 30 clones revealed 14 different RFLP patterns. One representative clone of each RFLP type was sequenced. We recovered one 16 S rRNA gene sequence (represented by three of 30 clones analyzed) identical to '*Ca.* P. salmonis' from a farmed Atlantic salmon population from Norway (99.9–100% sequence similarity) [19]. The remaining sequences were related to *Planctomycetes* commonly found in sea water; no other sequence related to known members of the *Chlamydiae* was found. Additional analysis of four other fish using *Chlamydiae*-specific (primers SigF2/SigR2) and '*Ca.* P. salmonis' specific (primers Pisci211F/Pisci1353R, Table S1) PCR assays confirmed these findings. Identical '*Ca.* P. salmonis' 16 S rRNA genes, but none similar to other *Chlamydiae*, were detected in all specimens. Taken together, the available evidence suggests that '*Ca.* P. salmonis' was a rare member of the microbial community associated with the gills of the studied fish, and that no other bacteria related to known *Chlamydiae* were present.

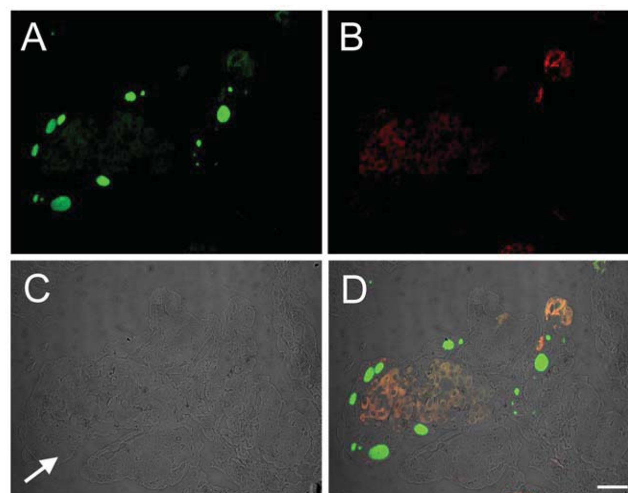

Figure 3. Absence of '*Ca.* Piscichlamydia salmonis' in cysts. Fluorescence *in situ* hybridization of sections of gill tissues using (**A**) a general bacterial probe mix labelled with Fluos (green), and (**B**) the '*Candidatus* P. salmonis' specific probe Psc-523 labelled with Cy3 (red) simultaneously. The faint red signal represents autofluorescence of the gill tissues. (**C**) Digital interference contrast image showing cysts (arrow) in the epithelial cells. (**D**) The overlay of all three images demonstrates the presence of bacteria in the cysts, which hybridize with the bacterial probe mix but not with the '*Ca.* Piscichlaymdia salmonis' specific probe and thus appear green. Additional staining with the DNA stain 4′,6-diamidino-2-phenylindole (DAPI) confirmed that all bacteria present in the gill tissues hybridized with the general bacterial probe mix. Scale bar represents 20 µm.
doi:10.1371/journal.pone.0032696.g003

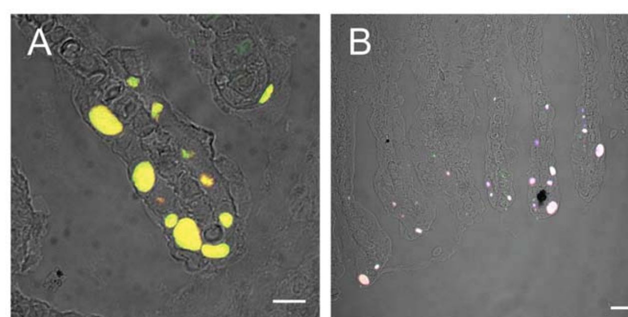

Figure 4. *In situ* identification and localization of '*Ca.* Branchiomonas cysticola' within cysts. Overlays of digital interference contrast (DIC) and fluorescence images of gill tissue sections are shown. (**A**) Hybridization with the '*Ca.* B. cysticola' specific probe BraCy-129 labelled with Cy3 (red) in combination with a bacterial probe mix targeting most *Bacteria* labelled in Fluos (green). The combined fluorescence signals of both probes appear yellow. Scale bar represents 10 µm. (**B**) Hybridization with the '*Ca.* B. cysticola' specific probe BraCy-129 labelled in Cy3 (red), the bacterial probe mix labelled in Cy5 (blue), and probe BTWO23A targeting a subset of the *Betaproteobacteria* labelled in Fluos (green). The combined fluorescence signals of all three probes appear white. Scale bar represents 20 µm.
doi:10.1371/journal.pone.0032696.g004

To verify our PCR-based analysis we performed fluorescence *in situ* hybridization (FISH). For the specific detection of '*Ca.* P. salmonis' we designed a novel oligonucleotide probe (Psc-523) and used the Clone-FISH method [29] to show that this probe successfully detects '*Ca.* P. salmonis' 16 S rRNA and to determine its optimal hybridization conditions (Table S1, Figure S1). We then analysed three of the fish for which we had qPCR and PCR evidence for the presence of '*Ca.* P. salmonis'. Simultaneous application of the '*Ca.* P. salmonis' specific probe and a general bacterial probe-mix targeting most known *Bacteria* readily visualized cysts containing bacteria, but did not result in hybridisation of the '*Ca.* P. salmonis' specific probe and bacterial cells within inclusions (Figure 3). Only occasionally, faint signals with the '*Ca.* P. salmonis' specific probe were apparent, diffusely distributed throughout the tissues, which might represent single bacterial cells or small cell clusters of '*Ca.* P. salmonis' external to cysts. This confirmed our PCR-based results and demonstrated clearly that the cysts in these gill samples contained bacteria other than '*Ca.* P. salmonis'.

Novel betaproteobacterium associated with epitheliocystis

To identify the bacteria associated with epitheliocystis in these fish, we used a broad PCR assay targeting the 16 S rRNA genes of most *Bacteria* (primers 616V/1492R; Table S1). RFLP analysis of the cloned PCR products from one fish revealed three different patterns. The clone representing the most abundant RFLP pattern (comprising 7 of 15 clones) showed only low sequence similarity to known *Betaproteobacteria* (around 88%), but was nearly identical (99.2%) to a partial 16 S rRNA gene sequence identified by denaturing gradient gel electrophoresis (DGGE) during a recent survey of gill-associated microbiota in Atlantic salmon [36].

To test whether this abundant phylotype was associated with cysts in the investigated fish population, we developed an oligonucleotide probe specific for this phylotype and used FISH on both sections and gill tissue squashes. The bacteria located within cysts could be readily visualized with the oligonucleotide probe BraCy-129. The simultaneous application of a bacterial probe mix and a probe targeting many *Betaproteobacteria* confirmed the absence of '*Ca.* P. salmonis' and demonstrated that the novel betaproteobacterial phylotype is the only cyst-associated microbe in these samples (Figure 4).

Phylogenetic analysis showed that the abundant phylotype established a novel, deep branching lineage within the *Betaproteobacteria* (Figure 5). Depending on the phylogenetic analysis method used, the novel lineage clusters together with other bacteria found in diverse environments, albeit with low bootstrap values, which

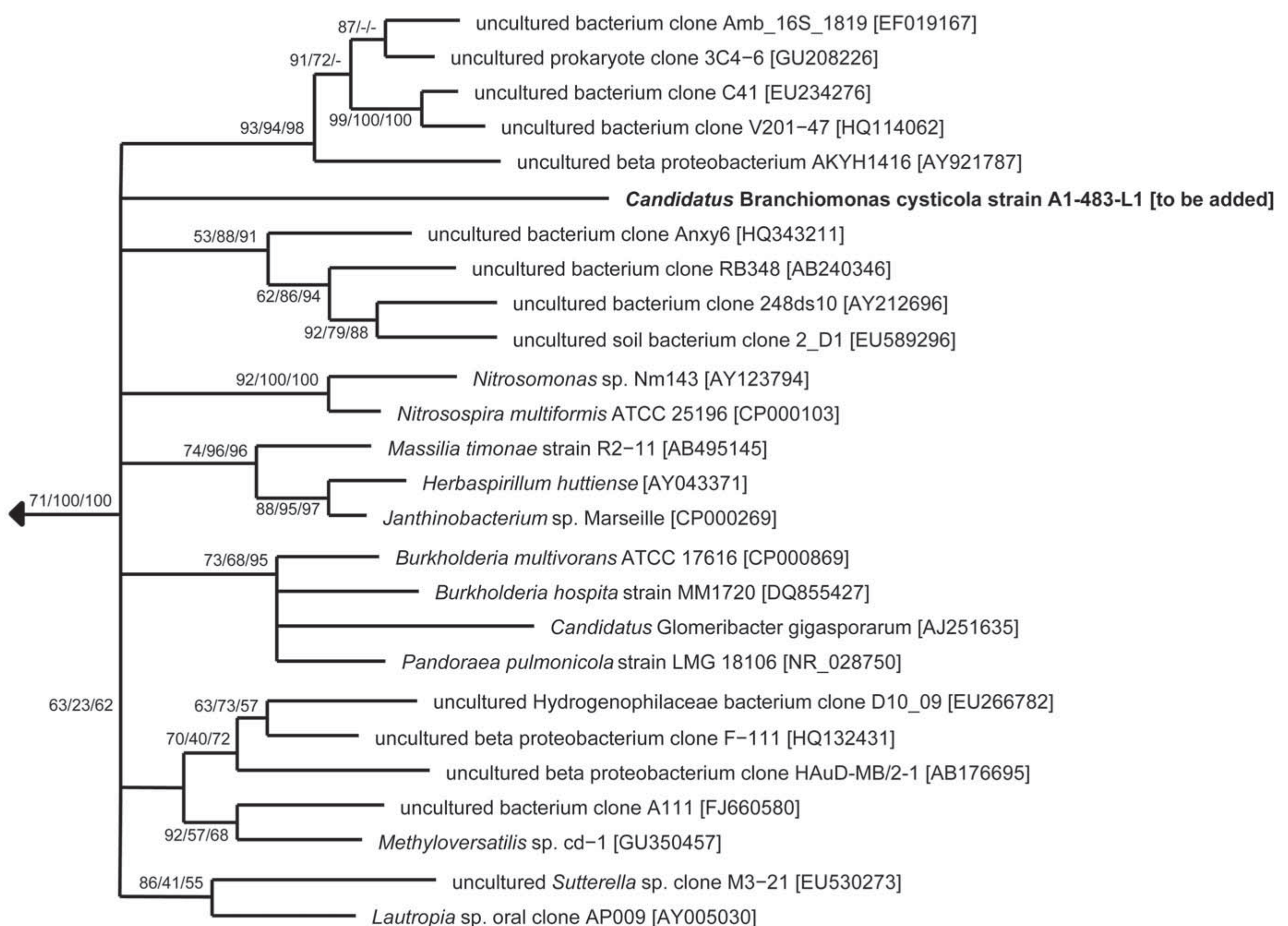

Figure 5. Phylogenetic relationship of '*Ca.* Branchiomonas cysticola' with the *Betaproteobacteria*. A 16 S rRNA-based TREEPUZZLE tree is shown. The branching order near the root of the tree varies between different treeing methods and can thus not be reliably resolved. TREEPUZZLE support and bootstrap values for maximum parsimony, maximum likelihood and Neighbor-Joining (1000 resamplings) are indicated at the inner nodes. GenBank accession numbers are given in the square brackets. Bar, 10% estimated evolutionary distance.
doi:10.1371/journal.pone.0032696.g005

does not allow reliable determination of its' position within the *Betaproteobacteria*. The low degree of phylogenetic relationship of the novel gill-associated bacterium to other *Betaproteobacteria* (less than 95% 16 S rRNA sequence similarity) justifies its' classification into a new genus. According to the recommendations of Murray and Stackebrandt [37] we propose, therefore, the provisional name '*Candidatus* Branchiomonas cysticola', for the novel, epitheliocystis-associated bacteria identified in this study. The proposed genus name is the combining form of gr. noun branchia (meaning gills) with gr. noun monas (meaning a unit, monad) while the species name is derived from new lat. cystis (meaning membranous sac, pouch (from gr. Kystis)) and lat. verb colere/lat. noun incola (meaning to inhabit/inhabitant or dweller).

Discussion

Until the present study, epitheliocystis in fish gills has generally been assumed to be caused by members of the *Chlamydiae*. This assumption has been based on ultrastructural studies corroborated more recently by molecular methodology. The identification of '*Ca*. B. cysticola' in this study represents the first demonstration of epitheliocystis-associated bacteria related to the *Betaproteobacteria* in any marine or freshwater fish species. This suggests that epitheliocystis is a condition that can be caused by different, evolutionary distinct bacteria.

The overall cyst morphology and the very pleomorphic bacterial cell morphotypes observed in the present study are highly similar to the intermediate and reticulate bodies reported previously [16,19]. The electron-dense elementary bodies reported by Nylund and co-workers [16], which are not documented outside the *Chlamydiae*, were not observed. Interestingly, the intermediate body-like morphotypes are also very similar to those observed in some beta-proteobacteria causing intracellular respiratory infections in mammals [38]. Thus, while histological and ultrastructural similarities exist, the overall cell morphologies observed in the present study do not appear to be consistent with a chlamydial life cycle.

Although fluorescence *in situ* hybridization unambiguously identified '*Ca*. B. cysticola' as the cyst-forming agent in the fish analysed in the present study (Figure 4), we also detected '*Ca*. P. salmonis' (but no other *Chlamydiae*) by PCR. Fluorescence *in situ* hybridization demonstrated that '*Ca*. P. salmonis' is in fact a rare member of the gill-associated microbiota and not responsible for the cysts in these samples (Figure 3). *Chlamydiae* have been detected and sequenced in association with epitheliocystis in a number of fish species [17,19,20,21,22,39,40]. While *in situ* evidence based on riboprobing (using polynucleotide probes) has been presented in some cases [17,19,20,21] the link between retrieved DNA sequences and the bacterial cells within the respective cysts has not always been conclusively confirmed [22,39,40]. The possibility exists therefore that some of the chlamydial sequences detected by PCR and associated with epitheliocystis may in fact represent organisms external to the cysts.

In conclusion, we have identified a novel agent of epitheliocystis in sea-farmed Atlantic salmon for which we propose the name '*Candidatus* Branchiomonas cysticola'. The diversity in bacteria now known to be associated with epitheliocystis in seawater farmed Atlantic salmon probably explains the lack of association between 'Ca. P. salmonis' and observed cyst number in a previous study [12]. Future analysis should thus ideally not rely on PCR-based detection methods alone, but should include evaluation of PCR results by an *in situ* technique such as FISH. Interestingly there is molecular evidence that '*Ca*. B. cysticola' is also a member of the normal gill microbiota of apparently healthy sea farmed Atlantic salmon [36]. It remains to be determined how widespread these bacteria are, and what their contribution is to the aetiology of epitheliocystis and PGI.

Supporting Information

Figure S1 Evaluation of the newly designed' *Candidatus* Piscichlamydia salmonis' specific probe Psc-523 using Clone-FISH.
(PPTX)

Table S1 PCR primers and fluorescently labelled probes used in this study.
(DOCX)

Acknowledgments

A special thanks to Marine Harvest Norway AS and staff members. Preparation for electron microscopy by Lene Hermansen, Department for Basic Sciences and Aquatic Medicine, Norwegian School of Veterinary Science. Histological sections were made by Randi Terland and colleagues at the Norwegian Veterinary Institute.

Author Contributions

Conceived and designed the experiments: ET AK SOM TS KF DJC MH. Performed the experiments: ET AK TS SOM. Analyzed the data: ET AK MH SOM. Contributed reagents/materials/analysis tools: ET AK SOM TS KF DJC MH. Wrote the paper: ET AK SOM TS KF DJC MH.

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A novel betaproteobacterial agent of gill epitheliocystis in seawater farmed Atlantic salmon (*Salmo salar*)

Elena R. Toenshoff *et al*.

Supporting Information

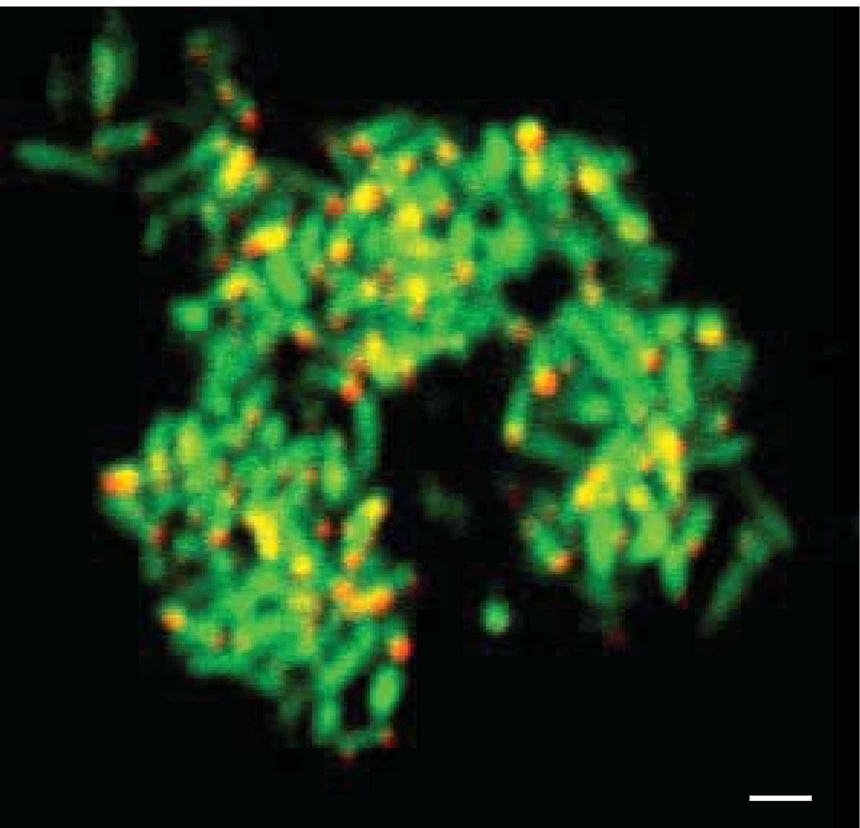

Figure S1. Evaluation of the newly designed ‚*Candidatus* Piscichlamydia salmonis‘ specific probe Psc-523 using Clone-FISH. Paraformaldehyde-fixed *E. coli* JM109 (DE3) cells containing the in vivo transcribed ‘*Candidatus* Piscichlamydia salmonis‘ 16S rRNA were hybridized. An oligonucleotide probe mix targeting most *Bacteria* labelled with Fluos (green) and the ‚*Candidatus* Piscichlamydia salmonis‘ specific probe Psc-523 labelled in Cy3 (red) was used. The combined fluorescence signal of both probes appears yellow, demonstrating that probe Psc-523 is suitable to detect ‘*Candidatus* Piscichlamydia salmonis‘. Scale bar represents 2 µm.

Table S1. PCR primers and fluorescently labelled probes used in this study.

Short name	Sequence (5´-3´)	Specificity	Target site[b]	Annealing temperature/ Formamide concentration	Reference
PCR primer					
616V	AGA GTT TGA TYM TGG CTC	16S rRNA gene, most *Bacteria*	8-25	52°C	[1]
1492R	GGY TAC CTT GTT ACG ACT T	16S rRNA gene, most *Bacteria* and *Archaea*	1492-1510		[2]
Univ1390R	GAC GGG CGG TGT GTA CAA	16S rRNA gene, most *Bacteria*	1391-1407	56°C	[3]
SigF2	CRG CGT GGA TGA GGC AT	16S rRNA gene, *Chlamydiales*	40-56	56-60°C	[4]
SigR2	TCA GTC CCA RTG TTG GC	16S rRNA gene, *Chlamydiales*	309-325	60°C	[4]
Pisci211F	GAG CCT TGT GGT TTG AGA GC	16S rRNA gene, '*Candidatus* Piscichlamydia salmonis'	211-230	70°C	This study
Pisci1363R	GAA CGT ATT CAC GGC GCT AT	16S rRNA gene, mainly '*Candidatus* Piscichlamydia salmonis'	1363-1382		This study
Oligonucleotide probes					
EUB338-I [a]	GCT GCC TCC CGT AGG AGT	16S rRNA, most *Bacteria*	338-355	10-50%	[5]
EUB338-II [a]	GCA GCC ACC CGT AGG TGT	16S rRNA, bacteria not covered by probe EUB338-I, e.g. many *Planctomycetes*	338-355	10-60%	[6]
EUB338-III [a]	GCT GCC ACC CGT AGG TGT	16S rRNA, bacteria not covered by probe EUB338-I, e.g. many *Verrucomicrobia*	338-355	10-60%	[6]
Psc-523	CCC ACG TAT TAC CGC AGC	16S rRNA, '*Candidatus* Piscichlamydia salmonis'	524-541	35%	This study

BraCy-129	CCC ACC ACT AGA CAC GTT	16S rRNA, '*Candidatus* Branchiomonas cysticola'	129-146	35%	This study
BTWO23A	GAA TTC CAC CCC CCT CT	16S rRNA, many *Betaproteobacteria*	663-679	35%	[7]
BONE23A (competitor for BTWO23A)	GAA TTC CAT CCC CCT CT	16S rRNA, beta1-group of *Betaproteobacteria*	663-679	35%	[7]
NONEUB	ACT CCT ACG GGA GGC AGC	Control probe complementary to EUB338-I	338-355	10-60%	[8]

[a] EUB338-I, EUB338-II, and EUB338-III were applied simultaneously to target most *Bacteria*.

[b] Target site according to *E. coli* 16S rRNA gene numbering.

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Appendix

Scientific experience

Acknowledgements

Curriculum vitae

Publications in peer reviewed journals

([#] corresponding author)

1. Collingro A., **Toenshoff E. R.**, Taylor M. W., Fritsche T. R., Wagner M., Horn M[#]. (2005) *'Candidatus Protochlamydia amoebophila', an endosymbiont of Acanthamoeba spp.* Int J Syst Evol Microbiol 55: 1863-1866.

2. Stoecker K., Bendinger B., Schöning B., Nielsen P. H., Nielsen J. L., Baranyi C., **Toenshoff E. R.**, Daims H., Wagner M[#]. (2006) *Cohn's Crenothrix is a filamentous methane oxidizer with an unusual methane monooxygenase*. Proc Natl Acad Sci USA 7: 2363-2367 (Track II).

3. Heinz E., Kolarov I., Kästner C., **Toenshoff E. R.**, Wagner M., Horn M[#]. (2007) *An Acanthamoeba sp. containing two phylogenetically different bacterial endosymbionts*. Environ Microbiol 9: 1604-1609.

4. Schmitz-Esser S., **Toenshoff E. R.**, Haider S., Heinz E., Hoenninger V. M., Wagner M., Horn M[#]. (2008) *Diversity of bacterial endosymbionts of environmental Acanthamoeba isolates*. Appl Environ Microbiol 74: 5822-5831.

5. Heinz E., Pichler P., Heinz C., op den Camp H. J. M., **Toenshoff E. R.**, Ammerer G., Mechtler K., Wagner M., Horn M.[#]. (2010) *Proteomic analysis of the outer membrane of Protochlamydia amoebophila elementary bodies*. Proteomics 10: 4363-4376.

6. Haider S., Wagner M.[#], Schmid M. C., Sixt B. S., Christian J. G., Häcker G., Pichler P., Mechtler K., Müller A., Baranyi C., **Toenshoff E. R.**, Montanaro J., Horn M. (2010) *Raman microspectroscopy reveals long-term extracellular activity of chlamydiae*. Mol Microbiol 77: 687-700.

7. Schimak M. P.[#], **Toenshoff E. R.**, Bright M. (2011) *Simultaneous 16S and 18S rRNA fluorescence in situ hybridization (FISH) on LR White sections demonstrated in Vestimentifera (Siboglinidae) tubeworms*. Acta Histochem, 114: 122-30.

8. **Toenshoff E. R.**, Penz T., Narzt T., Collingro A., Schmitz-Esser S., Pfeiffer S., Klepal W., Wagner M., Weinmaier T., Rattei T., Horn M[#]. (2011) *Bacteriocyte-associated gammaproteobacterial symbionts of the Adelges nordmannianae/piceae complex (Hemiptera: Adelgidae)*. ISME J, 6: 384-396.

9. **Toenshoff E. R.**, Kvellestad A., Mitchell S. O., Steinum T., Falk K., Colquhoun D.[#], Horn M. (2012) *A novel betaproteobacterial agent of gill epitheliocystis in seawater farmed Atlantic salmon (Salmo salar)*. PLoS ONE 7: e32696.

10. **Toenshoff E. R.**, Gruber D., Horn M#. (2012) *Co-evolution and symbiont replacement shaped the symbiosis between adelgids (Hemiptera: Adelgidae) and their bacterial symbionts*. Environ Microbiol, Doi: 10.1111/j.1462-2920.2012.02712.x.

Manuscripts in preparation

(* joint first authors, # corresponding author)

1. **Toenshoff E R**, Gruber D, Horn M#. *Pineus strobi (Hemiptera: Adelgidae) contains novel gammaproteobacterial symbionts*. Manuscript in preparation for Environ Microbiol.

2. Mitchell S O*, Steinum T*, **Toenshoff E R**, Kvellestad A, Falk K, Horn M, Colquhoun D#. Prevalence of the epitheliocystis agent *'Candidatus Branchiomonas cysticola' in seawater farmed Atlantic salmon (Salmo salar) from Norway and Ireland*. Manuscript in preparation.

3. Siegl A., **Toenshoff E. R.**, Mitchell S. O., Tischler P., Weinmaier T., Rattei T., Horn M#. *Metagenomic reconstruction of the genome of 'Candidatus Clavochlamydia salmonicola', a member of the Chlamydiae associated with the gills of salmonid fish*. Manuscript in preparation.

Oral presentations

Talks at national and international scientific meetings

1. **Toenshoff E. R.,** Narzt T., Collingro A., Schmitz-Esser S., Klepal W., Wagner M., Horn M. (2005) *The silver fir woolly aphid Dreyfusia nordmannianae (Adelgidae) contains novel gammaproteobacterial endosymbionts*. 5th International Workshop on new Techniques in Microbial Ecology (INTIME), Lunz am See, Austria, June 2005.

2. **Toenshoff E. R.,** Narzt T., Collingro A., Schmitz-Esser S., Klepal W., Wagner M., Horn M. (2006) *The silver fir woolly aphid Dreyfusia nordmannianae (Adelgidae) contains novel gammaproteobacterial endosymbionts*. 5th International Symbiosis Society Congress (ISS), Vienna, Austria, August 2006.

3. **Toenshoff E. R.,** Narzt T., Pfeiffer S., Schmitz-Esser S., Collingro A., Klepal W., Wagner M., Horn M. (2007) *Adelgids (Hemiptera: Sternorrhyncha: Adelgidae) harbour novel proteobacterial endosymbionts*. 10th International Colloquium on Endocytobiology and Symbiosis, Gmunden, Austria, September 2007.

4. **Toenshoff E. R.**, Penz T., Narzt T., Pfeiffer S., Tischler P., Rattei T., Klepal W., Wagner M., Horn M. (2009) *Molecular ecology of bacteriocytes-associated endosymbionts in adelgids*. 7th International Workshop on new Techniques in Microbial Ecology (INTIME), Lackenhof, Austria, August 2009.

5. **Toenshoff E. R.**, Penz T., Narzt T., Pfeiffer S., Tischler P., Rattei T., Klepal W., Wagner M., Horn M. (2009) *Molecular ecology of bacteriocytes-associated endosymbionts in adelgids*. 6th International Symbiosis Society Congress (ISS), Madison, Wisconsin, USA, August 2009.

Invited seminar

1. **Toenshoff E. R.,** Narzt T., Pfeiffer S., Schmitz-Esser S., Collingro A., Klepal W., Wagner M., Horn M. (2009) *Molecular ecology of symbionts in adelgids*. Seminar at the Department of Forest- and Soil Science, University of Natural Resources and Applied Life Science, Vienna, Austria, March 2009.

Meetings/Poster

(* presenting author)

1. Stoecker K.*, Schöning B., Bendinger B., Nielsen P. H., **Toenshoff E. R.**, Daims H., Wagner M. (2005) *Single cell tools identified the yet uncultured Crenothrix polyspora Cohn as novel filamentous methanotroph with an unusual methane monooxygenase*. 3rd International Conference on Analysis of Microbial Cells at the Single Cell Level, Semmering, Austria, May 2005. (**talk**)

2. Stoecker K.*, Schöning B., Bendinger B., Nielsen P. H., Nielsen J. L., Baranyi C., **Toenshoff E. R.**, Daims H., Wagner M. **(**2005) *Single cell tools identified the yet uncultured Crenothrix polyspora Cohn as novel filamentous methanotroph with an unusual methane monooxygenase*. 5th International meeting on Novel Techniques in Microbial Ecology (INTIME), Lunz am See, Austria, June 2005. (**talk**)

3. Stoecker K.*, Schöning B., Bendinger B., Nielsen P. H., Nielsen J. L., Baranyi C., **Toenshoff E. R.**, Daims H., Wagner M. (2005) *Crenothrix polyspora Cohn identified as novel filamentous methanotroph with an unusual methane monooxygenase*. VAAM Jahrestagung, Göttingen, Germany. September 2005. (**talk**)

4. Schmitz-Esser S.*, Haider S., Heinz E., **Toenshoff E. R.**, Hoenninger V. M., Stoecker K., Wagner M., Horn M. (2006) *Bacterial endosymbiont diversity within novel environmental Acanthamoeba isolates*. 5th International Symbiosis Society Congress (ISS), Vienna, Austria, August 2006. (**poster**)

5. Stoecker K.*, Schöning B., Bendinger B., Nielsen P. H., Nielsen J. L., Baranyi C., **Toenshoff E. R.**, Daims H., Wagner M. (2006) *Crenothrix polyspora Cohn identified as novel filamentous methanotroph with an unusual methane monooxygenase*. Internal Seminar at the group of Prof. Dr. Collin Murrell, University of Warwick, United Kingdom. (**talk**)

6. Kolarov I., **Toenshoff E. R.**, Schmitz-Esser S.*, Wagner M., Horn M. (2007) *Identification and localisation of two secreted proteins of the amoeba symbiont Protochlamydia amoebophila*. 10th International Colloquium on Endocytobiology and Symbiosis, Gmunden, Austria, September 2007. (**poster**)

7. Schmitz-Esser S.*, Haider S., Hoenninger V. M., Heinz E., **Toenshoff E. R.**, Stoecker K., Wagner M., Horn M. (2007) *Diversity of bacterial endosymbionts of novel environmental Acanthamoeba isolates*. 10th International Colloquium on Endocytobiology and Symbiosis, Gmunden, Austria, September 2007. (**poster**)

8. **Toenshoff E. R.***, Narzt T., Pfeiffer S., Schmitz-Esser S., Collingro A., Klepal W., Wagner M., Horn M. (2008) *Adelgids (Hemiptera: Sternorrhyncha: Adelgidae) harbour novel proteobacterial endosymbionts*. VAAM-/GBM Jahrestagung 2008, Frankfurt, Germany, March 2008. (**poster**)

9. **Toenshoff E. R.***, Narzt T., Pfeiffer S., Klepal W., Wagner M., Horn M. (2008) *Novel proteobacterial endosymbionts of adelgids*. 5th International Wolbachia Conference, Kolymbari, Crete, Greece, June 2008. (**poster**)

10. **Toenshoff E. R.**, Mitchell S. O., Siegl A.*, Tischler P., Rattei T., Horn M. (2010) *Environmental genome sequencing of `Candidatus Clavochlamydia salmonicola´, a member of the Chlamydiae associated with the gills of salmonid fish*. 8. Deutscher Chlamydienworkshop, Herrsching, Germany, February 2010. (**talk**)

11. Siegl A.*, **Toenshoff E. R.**, Mitchell S. O., Tischler P., Weinmaier T., Rattei T., Horn M. (2011) *Metagenomic reconstruction of the genome of ‘Candidatus Clavochlamydia salmonicola’, a member of the Chlamydiae associated with the gills of salmonid fish*. 5th Biennial Meeting of the Chlamydia Basic Research Society (CBRS), Redondo Beach, USA, March 2011. (**talk**)

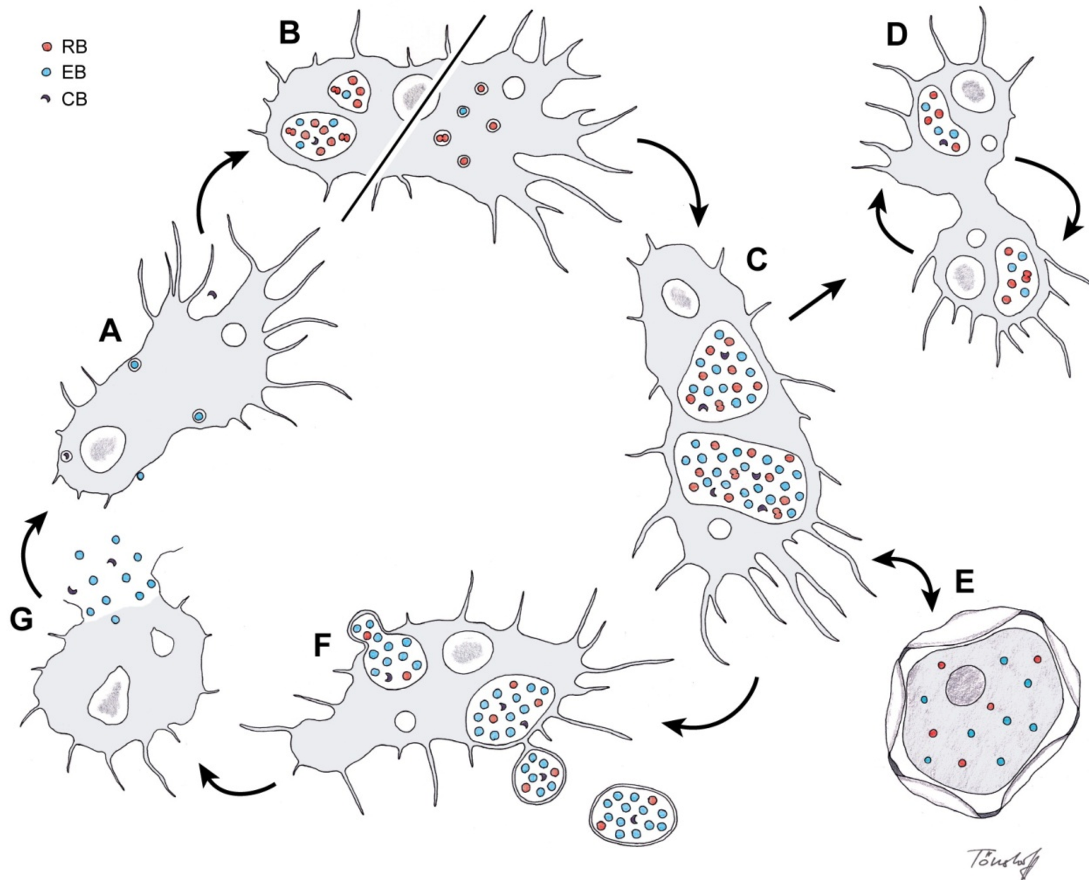
12. Mitchell S. O.*, **Toenshoff E. R.**, Kvellestad A., Steinum T., Falk K., Colquhoun D., Horn M. (2011) *Evidence for a non-chlaymdial agent of ephitheliocystis in Atlantic salmon*. European Association of Fish Pathologists (EAFP) Conference 2011. (**talk**)

Teaching experience

2006 - 2011	Advisor of guest scientists at the Department of Microbial Ecology (DoME), University of Vienna (Austria): Terje Steinum (two weeks in 2006), Rok Kostanjšek (one week in 2007, one month in 2011), Susie Mitchell (two weeks in 2009), Omri Finkel (two weeks in 2010)
2005, 2006, 2010	Supervisor of the two-week undergraduate student course *Fluorescence in situ hybridisation (FISH) – Identification of uncultivated microorganisms*, DOME
2007, 2008, 2009	Supervision of diploma student at DoME: Christian Kästner, Irina Kolarov, Stefan Pfeiffer, Thomas Penz

Contributed figure

Horn M. (2008) *Chlamydiae as symbionts in eukaryotes*. Ann. Rev. Microbiol. 62: 113-131



The developmental cycle of chlaymdia-like bacteria in free-living amoebae is illustrated by a drawing. The amoebal biphasic life-cycle is pictured showing the trophozoite (A-D, F, G) and cyst (E) stage. The different chlamydial developmental forms are displayed in different colours. Reticulate bodies (RBs) are shown in red; elementary bodies (EBs) in blue, the proposed crescent bodies (CBs) are shown in dark purple.

Acknowledgements

Special thanks to my supervisor Matthias Horn for support and funding, for interesting scientific group meetings and fruitful discussions during my time at DoME.

I thank Michael Wagner, the head of DoME, for interesting discussions during the seminars.

I am thankful to Celine Lesaulnier, Sebastian Lücker, Kilian Stoecker and Dagmar Woebken for critical reading of my thesis.

Special thanks to Astrid Horn and Stefan Schmitz-Esser (Schmitzi) for your great support and especially for interesting discussions. You both brought microbiology and science into my heart.

Thanks to my diploma students Irina Kolarov, Christian Kästner, and Stefan Pfeiffer for interesting discussions during your theses.

I am grateful to my diploma student Thomas Penz for the help with screening of the clone library. Thomas, you did a great job! I will never forget our time during sampling of the adelgids.

Thanks to all DoME members past and present for a good time at DoME and especially a great time in the lab. Special thanks to the 'Chlamydia-Group' for great group meetings.

I am thankful to Sebastian Lücker and Roland Hatzenpichler for having a good time.

Many thanks to my Viennese friends Basti, Bettina, Birgit, Christoph, Ingrid, Linus, Michi, Sigrid and Thomas for funny beer nights with Chinese food and pizza. Without you, my Viennese world would have been pretty lonely.

In my heart of hearts, special thanks to my parents (Annett and Heiko) and sisters (Lara, Sophia-Selina) for your support. This thesis is as much your merit as it is mine. Thanks a million!

Last, I want to thank Inger, Orna, Basti, Angela, Wai, Dave, Anna, Dieter and many more, which helped me to keep on track in the last two years. Thanks a lot!

Curriculum vitae

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Date of birth	March 20, 1978
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Education

Date	2005-2012
Affiliation	Department of Microbial Ecology, University of Vienna, Austria
PhD thesis	*Diversity and ecology of bacteriocyte-associated symbionts in adelgids (Hemiptera: Adelgidae)* under the supervision of Prof. Dr. Matthias Horn
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Affiliation	Department of Microbial Ecology, University of Vienna, Austria
Master thesis	*Identification of endosymbionts of Adelges nordmannianae & Ultrastructure Research of endosymbionts of acanthamoebae* under the supervision of Prof. Dr. Matthias Horn
Date	1998 - December 2004
Affiliation	Technische Universität München, Germany
Studies	Biology, to major in Microbiology
Final degree	Dipl.-Biol. Univ. (Master of Science), grade achieved: 1,7; “gut bestanden”
Date	1984-1998
Affiliations	Secondary schools (Gymnasium Tutzing, Germany; Kleines Privates Lehrinstitut Derksen, Munich, Germany) Primary schools (Martin-Kneidl-Volksschule, Grünwald, Germany; Otto-Bernheimer Grundschule Feldafing, Germany)
Title of qualification	high school diploma