# Microbiota associated with field-collected populations of the pine false webworm, *Acantholyda erythrocephala* (Hymenoptera: Symphyta: Pamphiliidae).

**SERG International Project 2006/10 - 2008/10**

# Microbiota associated with field-collected populations of the pine false webworm, *Acantholyda erythrocephala* (Hymenoptera: Symphyta: Pamphiliidae).

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**Abstract**

Little is known about the microbial interactions that occur within sawfly hosts and an increased knowledge might contribute to a better understanding of host population dynamics and ecology. In this study, pine false webworms, *Acantholyda erythrocephala* (Hymenoptera, Symphyta, Pamphiliidae) were collected from locations near Barrie, Ontario over several years and a survey of their associated microbiota undertaken. Total DNA was extracted from individual insects and polymerase chain reaction (PCR) used to amplify the conserved 16S ribosomal RNA gene from microbiota. Denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP) were undertaken to separate bacterial clones associated with the host insect. Sequencing of the PCR products revealed a dominance of α- and γ-Proteobacteria, with most sequences showing high similarity to bacteria previously identified from other insect species. No known symbiotic endobacteria, such as *Wolbachia*, were identified in any of pine false webworms collected.

**Résumé**

On en connaît peu sur les interactions microbiennes qui se produisent dans les hôtes de la tenthrède, et une meilleure connaissance à cet égard pourrait contribuer à une meilleure compréhension de la dynamique et de l'écologie des populations hôtes. Dans la présente étude, des pamphiles introduits du pin, *Acantholyda erythrocephala* (Hymenoptera, Symphyta, Pamphiliidae), ont été ramassés près de Barrie, en Ontario, durant plusieurs années, et un relevé de leur microbiote a été réalisé. De l'ADN total a été extrait d'insectes individuels, et la réaction en chaîne de la polymérase (PCR) a permis d'amplifier le gène d'ARN ribosomique 16s du microbiote. Une électrophorèse en gel de gradient dénaturant (DGGE) et un polymorphisme de restriction (RFLP) ont été réalisés sur des clones bactériens associés à l'insecte hôte. Le séquençage des produits de la PCR a révélé une dominance de protéobactéries α et γ, la plupart des séquences montrant une forte similarité avec des bactéries identifiées précédemment sur d'autres espèces d'insectes. Aucune endobactérie symbiotique connue, dont *Wolbachia*, n'a été repérée dans aucun des pamphiles introduits du pin recueilli.

## Introduction

The pine false webworm (PFW) (*Acantholyda erythrocephala*, Pamphiliidae) is an Eurasian sawfly that was introduced into eastern North America from Europe sometime prior to 1925 (Lyons 1995). Since the 1970s, PFW has been known to cause moderate to severe defoliation in saw-timber pine stands in Bruce, Grey and Simcoe counties in Ontario (Lyons 1995). Adult sex ratios are even or tend toward being male biased (Asaro and Allen 1999). There is little natural mortality in these PFW populations and survival of each life stage can be high. However, populations do fluctuate from year to year but the cause of these fluctuations is not known.

Culture-independent analysis using 16S ribosomal RNA (16S rRNA) gene-based methodologies have been widely used to investigate microbe-host interactions (Egert et al. 2003, Reeson et al. 2003, Benson et al. 2004, Dunn and Stabb 2005, Moreno et al. 2006). Using broad-range polymerase chain reaction (PCR) primers that target highly conserved regions of bacterial 16S rRNA genes, many species of bacteria can be identified without isolation and *in vitro* culture (Head et al. 1998, Reeson et al. 2003). PCR fragments can then be separated by cloning, screened using restriction fragment length polymorphism (RFLP) and sequenced for identification purposes. Alternatively, denaturing gradient gel electrophoresis (DGGE) allows sequence-specific separation of a mixture of partial 16S rRNA amplicons of the same length, facilitating the profiling of bacterial communities (Muyzer et al. 1993). Phylogenetic identification can then be achieved by DNA band excision and direct sequencing. We used 16S rRNA gene fragment PCR, DGGE, fragment cloning and sequencing to survey populations of PFW in Ontario for associated bacteria. Sequencing of the PCR products revealed a dominance of $\alpha$- and $\gamma$-Proteobacteria with most sequences showing high similarity to bacteria previously identified from other insect species or environmental samples. No known symbiotic endobacteria, such as *Wolbachia*, were identified in any of PFW collected.

## Materials and methods

*1. Pine false webworms collected in 2003-2004*

Larvae, pronymphs, eonymphs, pupae and adult PFW were collected in Bruce, Grey, and Simcoe Counties in Ontario, in spring and summers 2003 and 2004, and were transported live to the Canadian Forest Service - Atlantic Forestry Centre (CFS-AFC), Fredericton, New Brunswick (Zahner et al. 2008). Eonymphs, pronymphs, and pupae were maintained in clean sand at 4°C and were processed within 48 h of arrival. Field-caught adults were provided with potted, 3-year-old red pine trees (*Pinus resinosa*) for oviposition and larvae were allowed to feed on the same trees. PFW rearing was performed in the laboratory at 20°C under a 16-h light:8-h dark cycle. To process specimens, all life stages were first surface cleansed with quick rinses (1 min each) in 0.3% aqueous NaOH, then 70% ethanol, followed by a rinse in sterile distilled water. Internal tissues were then carefullly dissected from different PFW life stages: larvae (n = 25), pronymphs (n = 24), eonymphs (n = 20), pupae (n = 30), and adults (n = 4). Eggs, in groups of 20 (n = 20 groups), were also processed. Specimens were placed in FastRNA Tubes (Savant Bio101 Inc.) containing 1 mL of phosphate buffered saline (PBS), pH 7.2. Homogenization was conducted in a Fast Prep 120 (Bio101 Inc.) at a setting of 4 for 30 s. For bacteriological analysis duplicate 100-μL volumes were removed, serially diluted in PBS, and used as inocula for nutrient agar (NA) plates with aerobic incubation at 25°C. Plates were examined daily over 14 days and samples of the dominant colony types were subcultured into pure culture. PCR assays were performed using DNA extracted from duplicate 200-μL aliquots of the tissue homogenates which had also been examined for culturable bacteria. The broadly conserved bacterial 16S rDNA gene primers p515F and p806R (Table 1) were used. For denaturing gradient gel electrophoresis (DGGE), the p515F primer was modified at the 5' end with a 40-bp GC rich clamp sequence that terminated gel migration of products within a concentration gradient of urea/formamide. Amplicons were examined by electrophoresis using 1.5% agarose gels and ethidium bromide staining. For DGGE analysis of the 16S rRNA gene amplicons, amplicons were separated in a 40–70% gradient using the DCode system (Bio-Rad, Hercules, CA) for 14 h at 80 V in 1X Tris/Borate/EDTA (TBE) buffer at a constant temperature of 60°C. Relative mobility standards consisted of p515F (no GC clamp)/p806R PCR amplicons produced from laboratory subcultures of the dominant bacterial species cultured from the insect samples and identified by nucleotide sequence similarity to known 16S rDNA sequences using BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990). Gels were stained with ethidium bromide and documented with an Imagemaster digital camera and associated annotation software (Amersham Pharmacia Biotech). DGGE bands of interest were extracted and sequenced.

*2. Pine false webworms collected in 2006*

Pine false webworms from near Craighurst, Simcoe County, Ontario were collected as second- to fourth-instar larvae and, for consistency, we focused the research on fourth- and fifth-instar larvae (Graham et al. 2008). All individuals sampled were shipped with and fed foliage (red or white (*P. strobus*) pine) from the locations where they were collected, and all were alive at the time of processing for DNA. DNA was individually isolated from 24 PFW larvae which were surface cleaned prior to processing by submerging in 0.3% aqueous NaOCl for 1 min and rinsed in two changes of distilled water. Total DNA was purified from whole larvae using DNeasy Tissue Kit (Qiagen, Valencia, CA) (Haynes et al. 2003, Behar et al. 2005).

Bacteria were surveyed using two techniques: DGGE and PCR-RFLP. For DGGE, specific 16S rRNA gene primers U984-GC and L1401 were used to amplify regions V6 to V8 and p515F-GC and p806R were used to amplify region V4 (Table 1). Reaction mixtures (50 µL) contained PCR buffer (10 mM Tris-HCl pH 8.3 at 25 °C, 50 mM KCl, 1.5 mM MgCl, 0.001% gelatin), 10 µM each of dATP, dTTP, dCTP, and dGTP, 0.1 µM of each primer, 1 unit *Taq* polymerase (Qiagen) and approximately 10 ng insect genomic DNA template. PCR reactions were done using a Mastercycler EP thermal cycler (Eppendorf, Westbury, NY) with the settings: (i) 94 °C for 5 min, 1 cycle; (ii) 94 °C for 20 sec, 52 °C for 20 sec, 72 °C for 45 sec, 40 cycles; (iii) 72 °C for 5 min, 1 cycle. PCR products were separated by DGGE using the DCode system (BioRad, Hercules, CA) according to the manufacturer's instructions. Gels consisted of 1-mm thick 6% polyacrylamide with a denaturing gradient of 30%–70% (100% denaturant corresponds to 7 M urea and 40% vol/vol deionized formamide) and 1 x TAE buffer (40 mM Tris-acetate, pH 8.0, 2 mM EDTA). Electrophoresis was performed at 60 °C and 80 V in 1 x TAE running buffer for 16 h, and gels were stained with SYBR Gold nucleic acid stain (Invitrogen). The DNA bands were excised with a sterile razor blade and placed in 100 µL of sterile distilled $H_2O$ at 94 °C for 5 min to elute DNA for sequencing.

For PCR-RFLP, DNA from individual PFW were pooled and used as a template for PCR amplification of bacterial 16S rRNA genes using universal primers 27F and 1492R (Table 1). PCR was carried out as above using the settings: (i) 94°C for 5 min, 1 cycle; (ii) 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 min, 35 cycles; (iii) 72°C for 5 min, 1 cycle. PCR products were gel purified and extracted (QIAquick Gel Extraction Kit, Qiagen), cloned into pGEMT-Easy vector (Promega, Madison, WI), and DH5α *E. coli* cells (Invitrogen) transformed. The region containing the insert was amplified using M13 universal primers (Invitrogen). Additional PCR reactions were similarly carried out in 25 µL volumes. Ninety-six positive clones were amplified and screened. The PCR products from clones with an approximate 1500 bp insert were digested with the restriction enzymes *Msp*I and *Hae*III (*sensu* Hogg and Lehane 2001). Clones were grouped according to their novel RFLP profile. A representative of each group was cultured and the plasmid purified using QIAprep Spin Miniprep Kit (Qiagen).

Plasmid inserts and PCR products were sequenced at the Ontario Genomics Innovation Centre (Ottawa, ON). Sequences were viewed using BioEdit (Hall 1999) and edited to remove vector and primer regions. Preliminary identification against previously published sequences were provided by BLAST (Altschul et al. 1990) and Ribosomal Database Project II (RDP) (East Lansing, MI) similarity rank program (Cole et al. 2005). Sequences were checked for possible chimerical nature using RDP Chimera Check program (Cole et al. 2005).

*3. Pine false webworms collected in 2008-2009*

Nymphs and pupae were collected from soil near Craighurst, Simcoe County, Ontario (the same location as in 2006) in late-May and early-June in both 2008 and 2009. Eggs were collected on white pine needles in May 2009. Samples were transported to CFS-AFC as described above. Where necessary, PFW larvae were reared on red pine as was done in 2003-2004, above. Prior to DNA extraction, specimens were individually rinsed three times with sterile distilled water followed by washing with 0.3% aqueous NaOH for 3 min, then 70% ethanol, followed by rinses in sterile distilled water. Hemolymph was first collected into sterile 1.5-mL microcentrifuge tubes by poking a hole in the insect cuticle using a flame-sterile dissecting needle. Fat bodies were dissected into sterile 1.5-mL microcentrifuge tubes and rinsed with sterile PBS. The remaining tissues (e.g. cuticle, gut, muscle) were similarly placed in 1.5-mL microcentrifuge tubes and rinsed with sterile PBS.

Each specimen sample was then homogenized using a sterile pestil and DNA was extracted with DNeasy tissue kit (Qiagen, ON) following the manufacture's instructions. DNA concentration was measured and stored in -20°C.

The 16S rRNA gene universal primers 27F, 1492R, 515F, 565F, 881R, and 1545R were initially used in different combinations between the (Table 1) for PCR. Following this specific primers were designed to amplify 16S rDNA from certain bacterial groups. Additionally, primers specific to certain known insect symbionts, (i.e. *Cardinium*, *Flavobacterium*, *Rickettsia* and *Wolbachia*) were also used in PCR reactions (Bourtzis and Miller 2003) (Table 1). GoTaq DNA polymerase (Promega, ON) was used for the PCR. The 25-µL reaction mix contains 50 ng DNA. The PCR was conducted at 94°C for 2 min, 30 cycles of 94°C for 20s, 50-61°C for 1 min, and 72°C for 1.5 min. The end of each cycle was followed by a 10 min extension at 72°C. One tenth of the PCR product was electrophoresed on a 1% agarose gel in 0.5X TBE buffer. Following electrophoresis, the gel was ethidium bromide stained before being photographed. The remaining PCR product was purified and cloned with pGEM-T Easy System II and sent to McGill University and Genome Québec Innovation Centre (Montréal, QC) for sequencing from both ends with M13F and M13R primers. The vector sequence was removed and the sequence ends were trimmed with high stringency with the computer software DNASTAR lasergene 7 (DNASTAR, Inc. WI. USA). The sequences were finally hand edited and used as queries to search the GenBank nr database with BLAST (Altschul et al. 1990). Query nucleotide sequence showing 97% identity or more to the subject under 100% coverage was considered from the same bacterial species.

Bacterial 16S rRNA gene sequences from PFW collected in 2006 and 2008-2009 and subject sequences with high sequence identities from the GenBank nr database were aligned using ClustalX 2.09 (Larkin et al. 2007). Two overlapping regions were recognized at 700-935 bp (Fig. 1) and 1075-1340 bp (Fig. 2). Neighbor-joining phylogenetic analysis for each overlapping region using Mega3.1 (Kumar et al. 2004). The 16S rRNA gene sequence of *Haladaptatus* sp. (Archaea) (GenBank Acc. No. FJ773394) was used as the out-group and the trees were rooted to the out-group.

**Table 1.** PCR primers for detection of bacteria in pine false webworm.

| Primer<br><br>Years used | Type | Sequence | References, otherwise from congeneric 16S rDNA sequences in GenBank |
|---|---|---|---|
| **2003-2004** | | | |
| 515F | Universal | 5'-GCCAGCAGCCGCGGTAA-3' | Relman 1993 |
| P806R | E. coli nt 806-787 | 5'-GGACTACCAGGGTATCTAAT-3' | Relman 1993 |
| **2006** | | | |
| 515F | Universal | 5'-GCCAGCAGCCGCGGTAA-3' | Relman 1993 |
| 27F | Universal | 5'-AGAGTTTGATCCTGGCTCAC-3' | Hogg and Lelane 2001 |
| U984-GC | E. coli V6 | 5'-CGCCCGGGGCGCGCCCCGGGCGGGGCG GGGGCACGGGGGGAACGCGCCGAACCTTAC-3' | Frederick and Caesar 2000 |
| **2008-2009** | | | |
| 27F | Universal | 5'-AGAGTTTGATCCTGGCTCAC-3' | Hogg and Lelane 2001 |
| 1492R | Universal | 5'-GGTTACCTTGTTACGACTT-3' | Hogg and Lelane 2001 |
| 515F | Universal | 5'-GCCAGCAGCCGCGGTAA-3' | Relman 1993 |
| P806R | E. coli nt 806-787 | 5'-GGACTACCAGGGTATCTAAT-3' | Relman 1993 |
| U984-GC | E. coli V6 | 5'-CGCCCGGGGCGCGCCCCGGGCGGGGCG GGGGCACGGGGGGAACGCGCCGAACCTTAC-3' | Frederick and Caesar 2000 |
| R-1401 | E. coli V8 | 5'GCGTGTGTACAAGACCC-3' | Nübel et al. 1996 |
| 881R | Degenerate universal | 5'- GGA CTA CYM GGG TAT CTA ATC C -3' | |
| 565F | Degenerate universal | 5'-GTGCCAGCAGCCGCGGTAAIAC-3' | |
| 1545R | Degenerate universal | 5'- TGACGGGCRGTGTGTACAAG-3' | |
| A437F | Alphaproteobacteria | 5'-AAGCCCCGGCTAACT TCGTGCCAGCAG -3' | |
| A907R | Alphaproteobacteria | 5'- GGTAAGGTTCTGCGCGTTGCTTCG -3' | |
| R340F | Rickettsia | 5'-GGAGGCAGCAGTGGGGAATATTGG-3' | |
| R1025R | Rickettsia | 5'-GCTGACGACAGCCATGCAACACCTG-3' | |
| R200F | Rickettsia | 5'- CGGAGGAAAGATTTATCGCTGATGG -3' | |
| R550F | Rickettsia | 5'- GTGCGTAGGCGGTTTAGTAAGTTGG -3' | |
| R840R | Rickettsia | 5'- CTCCCCAGGCGGAGTGCTTAATGCG -3' | |
| R1501R | Rickettsia | 5'- CAGTCGCTAATTTTACCGTGGTTGG -3' | |
| R872R | Rickettsia | 5'- AGTGCTTAATGCGTTAGCTGCGAAACC -3' | |
| W80F | Wolbachia | 5'- CGGAGTTATATTGTAGCTTGCTATGG -3' | |
| W458F | Wolbachia | 5'- AGTGAGGAAGATAATGACGGTACTC -3' | |
| W492F | Wolbachia | 5'- TGACGGTACTCACAGAAGAAGTCCT -3' | |
| W950R | Wolbachia | 5'- ACATGCTCCACCGCTTGTGCGGGT -3' | |
| W1030R | Wolbachia | 5'- AACCGACCCTATCCCTTCGAATAGG -3' | |
| W1498R | Wolbachia | 5'- CACTGATCCCACTTTAAATAACTCC -3' | |
| Ch225F | Chryseobacterium | 5'- CTCCGGTGGATAGAGATGGGCACG -3' | |
| Ch450F | Chryseobacterium | 5'- TGTATAGGGATAAACCTACTCTCGTG -3' | |
| Ch1010R | Chryseobacterium | 5'- ACCTGTCATTTCCCATTTAAGCCT -3' | |
| Ch1470R | Chryseobacterium | 5'- CCCTAGGCAGCTCCTATTACGGTC -3' | |
| FL330F | Flavobacterium | 5'- CTACGGGAGGCAGCAGTGAGGAAT -3' | |
| FL930R | Flavobacterium | 5'- GCGTATCATCGAATTAAACCACA -3' | |
| FL1385R | Flavobacterium | 5'- CTTCCATGGCTTGACGGGCGGTGTG -3' | |
| FL300F | Flavobacterium | 5'- CCACACTGGTACTGAGACACGGACC -3' | |
| FL1030R | Flavobacterium | 5'- ACGAGCTGACGACAACCATGCAGCACC -3' | |
| Ch-F | Cardinium | 5'-TACTGTAAGAATAAGCACCGGC-3' | De Luna et al. 2009 |
| Ch-R | Cardinium | 5'-GTGGATCACTTAACGCTTTCG-3' | De Luna et al. 2009 |
| EN60F | Cardinium | 5'- GCGTCCTATTAGCTAGTTGGTGAGGC -3' | |
| EN940R | Cardinium | 5'- TAAAGTTCCCACCATTATGTGCTGGCA -3' | |
| EN100F | Cardinium | 5'- GCTACGATGGGTAGGGGTTCTTAGTGG -3' | |
| EN908R | Cardinium | 5'- GTAAAAGGGTTTCGCTCGTTATAGGAC -3' | |
| ARC856F | Archaea | 5'-TAAAGGAATTGGCGGGGGA-3' | |
| ARC1345R | Archaea | 5'-TGAGGGGCGGTGTGTGCAAG-3' | |
| ARC35F | Archaea | 5'-CTGGTTGATCCTGCCAG-3' | |
| TP1374R | Archaea | 5'-TTCACCGCGAGTTGTTGATT-3' | |

## Results

Table 2 lists the bacteria identified from PFW collected from sites near Barrie, Ontario, from years that collections were made. Sixteen different bacteria were identified in 2003-2004, 27 in 2006 and 26 in 2008-2009. The bacteria identified included Gram-negative Flavobacteria, α-, β- and γ-Proteobacteria and Gram-positive Actinobacteria and Firmicutes (Figs. 1 and 2). However, no bacterium common to all years was identified judging by BLAST matches. Additional, detailed information for 2003-2004 and 2006 can be found in Zahner et al. (2008) and Graham et al. (2008), respectively.

**Table 2.** Bacteria identified from pine false webworm.

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| Bacterium | Clade | BLASTN match | RPD % identity |
|---|---|---|---|
| **2003-2004** Zahner et al. 2008 | | | |
| *Nocardia inordata* | Actinobacteria | AY191254 | 100 |
| Microbacteriaceae | Actinobacteria | AF408987 | 97 |
| *Bacillus sphaericus* | Firmicutes | AY304997 | 100 |
| *Eubacterium* sp. | Firmicutes | AY230774 | 94 |
| *Paenibacillus xyanilyticum* | Firmicutes | AY427832 | 100 |
| *Paenibacillus* sp. | Firmicutes | AY337581 | 100 |
| *Chryseobacterium* sp. | Flavobacteria | AY439233 | 99 |
| *Flavobacterium* sp. | Flavobacteria | AY162137 | 92 |
| *Leptotricia* sp. | Fusobacteria | AF189244 | 99 |
| *Pantoea agglomerans* | γ-Proteobacteria | AY315453 | 99 |
| *Photorhabdus luminescens* | γ-Proteobacteria | AY444555 | 99 |
| *Photorhabdus temperata* | γ-Proteobacteria | AY296252 | 99 |
| *Pseudomonas* sp. | γ-Proteobacteria | AY439233 | 99 |
| *Pseudomonas* sp. | γ-Proteobacteria | AY315457 | 100 |
| *Pseudomonas* sp. | γ-Proteobacteria | AY308054 | 99 |
| *Stenotrophomonas maltophilia* | γ-Proteobacteria | AY472115 | 98 |
| **2006** Graham et al. 2008 (isolate number, see Figs. 1 and 2) | | | |
| uncultured bacterium (PFW1) | Actinobacteria | AB288658 | 99 |
| *Propionibacterium acnes* (PFW2) | Actinobacteria | AY642054 | 100 |
| *Rhodococcus erythropolis* (PFW3) | Actinobacteria | AJ576250 | 97 |
| *Corynebacterium* sp. (PFW4) | Actinobacteria | AY677186 | 89 |
| *Bradyrhizobium* sp. (PFW5) | α-Proteobacteria | AF408969 | 98 |
| *Agrobacterium* sp. (PFW6) | α-Proteobacteria | DQ193597 | 99 |
| *Phyllobacterium* sp. (PFW7) | α-Proteobacteria | AF290483 | 98 |
| uncultured bacterium (PFW8) | α-Proteobacteria | AB186822 | 94 |
| *Sphingobium herbicidovorans* (PFW9) | α-Proteobacteria | AB022428 | 99 |
| *Methylobacterium* sp. (PFW10) | α-Proteobacteria | AY369236 | 96 |
| uncultured bacterium (PFW11) | α-Proteobacteria | AY162827 | 98 |
| *Novosphingobium* sp. (PFW12) | α-Proteobacteria | AB177883 | 97 |
| uncultured bacterium (PFW13) | α-Proteobacteria | AJ459874 | 98 |
| uncultured soil bacterium (PFW14) | α-Proteobacteria | DQ378222 | 94 |
| *Caulobacter* sp. (PFW15) | α-Proteobacteria | DQ163946 | 93 |
| Uncultured bacterium (PFW16) | α-Proteobacteria | AB074649 | 92 |
| *Staphylococcus* sp. (PFW17) | Bacilli | DQ170801 | 95 |
| *Leuconostoc* sp. (PFW18) | Firmicutes | AB008901 | 93 |
| *Rahnella* sp. (PFW19) | γ-Proteobacteria | DQ822730 | 95 |
| *Enterobacter* sp. (PFW20) | γ-Proteobacteria | DQ822723 | 94 |
| *Klebsiella* sp. (PFW21) | γ-Proteobacteria | DQ533885 | 90 |
| *Yersinia enterocolitica* (PFW22) | γ-Proteobacteria | Z75316 | 98 |
| *Stenotrophomonas* sp. (PFW23) | γ-Proteobacteria | AM421782 | 98 |
| *Pseudomonas* sp. (PFW24) | γ-Proteobacteria | AF290479 | 90 |
| *Stenotrophomonas* sp. (PFW25) | γ-Proteobacteria | DQ530137 | 98 |
| *Erwinia* sp. (PFW26) | γ-Proteobacteria | AJ971890 | 93 |
| *Serratia* sp. (PFW27) | γ-Proteobacteria | DQ321555 | 94 |

**Table 2** (concluded).

| Bacterium | Clade | BLASTN match | RPD % identity |
|---|---|---|---|
| **2008-2009** (isolate number, see Figs. 1 and 2) | | | |
| *Actinomyces* sp. (PFWB55) | Actinobacteria | AY008315 | 99 |
| *Nesterenkonia flava* (PFWB44) | Actinobacteria | EF680886 | 99 |
| *Segniliparus rotundus* (PFWB59) | Actinobacteria | FJ468343 | 99 |
| *Segniliparus rugosus* (PFWB37) | Actinobacteria | FJ593188 | 99 |
| Bacterium Ellin5129 (PFWB46) | Actinobacteria | AY234546 | 95 |
| *Pseudochrobactrum* sp. (PFWB39) | α-Proteobacteria | AB478897 | 96 |
| *Sphingomonas echinoides* (PFWB51) | α-Proteobacteria | EU730918 | 99 |
| *Bacteroides gracilis* (PFWB41) | Bacteroidetes | L37787 | 99 |
| *Burkholderia sediminicola* (PFWB33) | β-Proteobacteria | EU035613 | 94 |
| *Burkholderia* sp. (PFWB34) | β-Proteobacteria | EF075718 | 100 |
| *Delftia* sp. (PFWB57) | β-Proteobacteria | AB461757 | 99 |
| uncultured bacterium (PFWB50) | β-Proteobacteria | AB252903 | 95 |
| *Bacillus halodurans* (PFWB43) | Firmicutes | AB359904 | 99 |
| *Geobacillus pallidus* (PFWB48) | Firmicutes | AB198976 | 96 |
| *Chryseobacterium* sp. (PFWB60) | Flavobacteria | DQ301786 | 98 |
| *Aeromonas salmonicida* (PFWB56) | γ-Proteobacteria | FJ233861 | 99 |
| *Escherchia coli* (PFWB40) | γ-Proteobacteria | CU928164 | 99 |
| *Halomonas* sp. (PFWB36) | γ-Proteobacteria | AY687525 | 98 |
| *Halomonas* sp. (PFWB53) | γ-Proteobacteria | EF554886 | 95 |
| *Halomonas* sp. (PFWB52) | γ-Proteobacteria | DQ642806 | 93 |
| *Pseudomonas putida* (PFWB30) | γ-Proteobacteria | AF307869 | 95 |
| *Pseudomonas* sp. (PFWB29) | γ-Proteobacteria | AM419019 | 94 |
| *Pseudomonas* sp. (PFWB28) | γ-Proteobacteria | DQ778036 | 99 |
| *Stenotrophomonas* sp. (PFWB58) | γ-Proteobacteria | FJ626655 | 99 |
| *Chitinophaga* sp. (PFWB35) | Shingobacteria | FJ750951 | 99 |
| uncultured compost bacterium (PFWB42) | not known | DQ346527 | 96 |

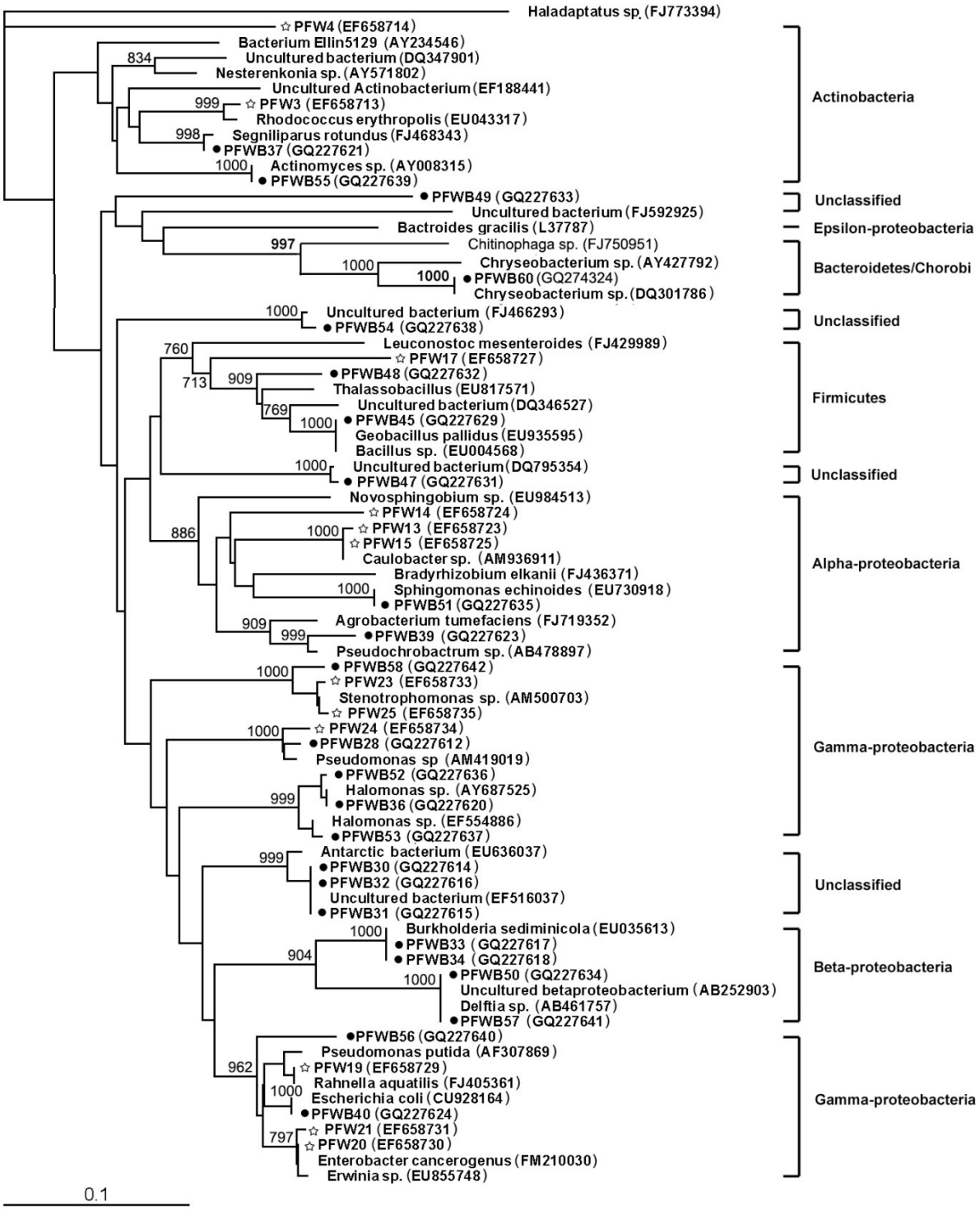


**Figure 1.** Neighbour-joining phylogenetic tree showing the relationship between bacteria from pine false webworm collected in 2006 (☆) and 2008-2009 (●) and other bacteria listed in GenBank based on partial (700-935 bp) 16S rRNA PCR-amplified gene sequences. GenBank accession numbers are shown in brackets. Pine false webworm bacteria identities are given in Table 2. *Haladaptatus* sp. (FJ773394) was used as the outgroup. The scale bar represents a 10% estimated difference in nucleotide sequence. Bootstrap values larger than 700 are shown at the nodes of the tree branches.

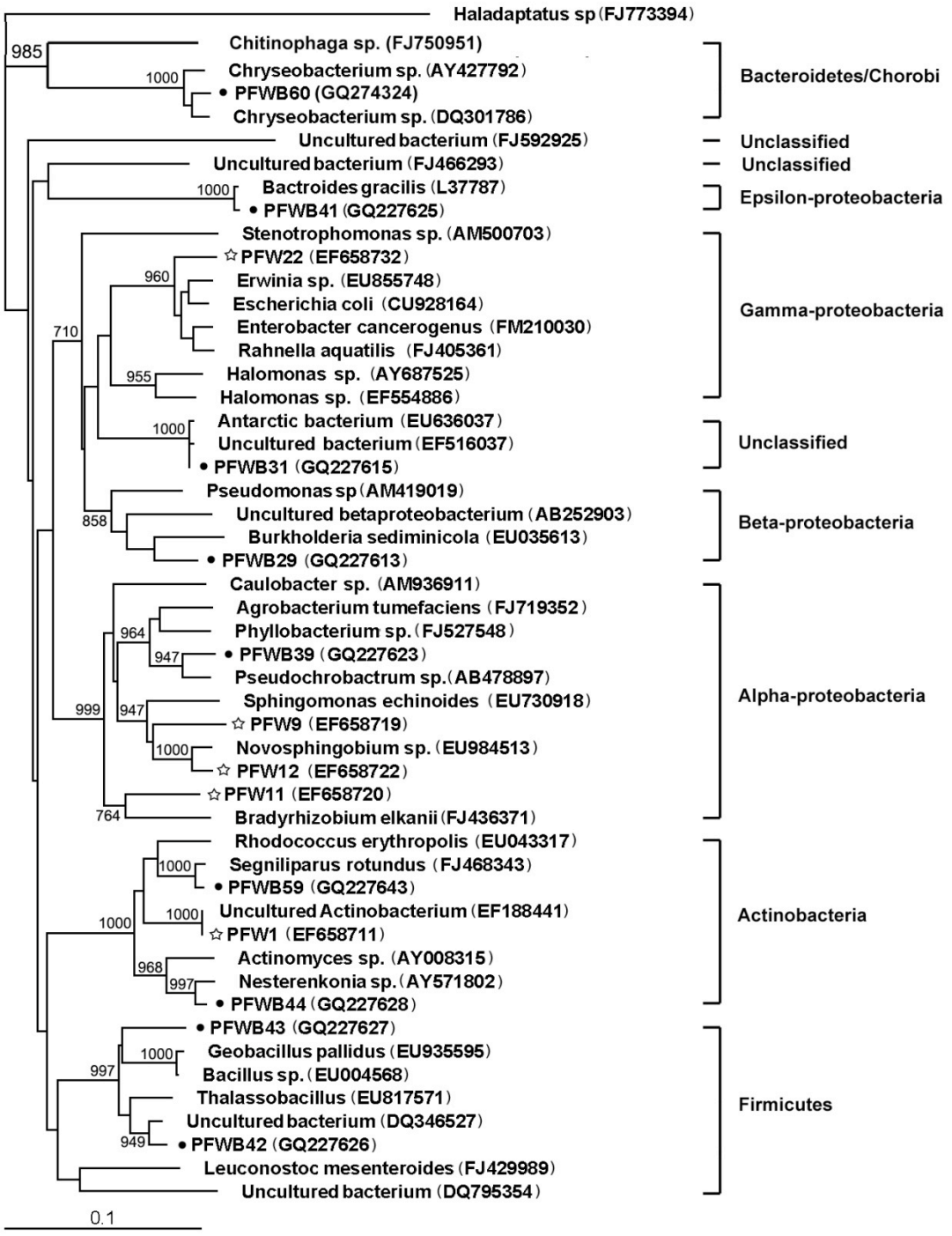


**Figure 2.** Neighbour-joining phylogenetic tree showing the relationship between bacteria from pine false webworm collected in 2006 (☆) and 2008-2009 (•) and other bacteria listed in GenBank based on partial (1075-1340 bp)16S rRNA PCR-amplified gene sequences. GenBank accession numbers are shown in brackets. Pine false webworm bacteria identities are given in Table 2. *Haladaptatus* sp. (FJ773394) was used as the outgroup. The scale bar represents a 10% estimated difference in nucleotide sequence. Bootstrap values larger than 700 are shown at the nodes of the tree branches.

**Discussion**

Insect origins date back to around the Silurian-Ordovician boundary some 430 million years ago (mya) (Gaunt and Miles 2002). Over evolutionary time, many symbiotic relationships between microbes and insects have been established. In certain cases specialized insect host cells (bacteriocytes) have evolved to house specific symbiotic bacteria (i.e. mealybugs and β-Proteobacteria; aphids, whiteflies and γ-Proteobacteria; cockroaches, termites and *Bacteroidetes*). Many "primary" or P-endosymbionts have been shown to provide a nutritional benefit to the host insect. P-endosymbionts have greatly reduced genomes compared to their wild-type ancestors. For example, *Buchnera* symbiosis in aphids originated ≈200 mya with an enterobacterial-like genome of 1,800-2,400 genes. This genome was reduced in size by 65-74% and achieved stasis relatively soon after the symbiosis was established (van Ham et al. 2003). The small size of P-endosymbiont genomes and their isolation can lead to degenerate genome evolution and loss of fitness to the point where their nutritional functions may be taken over by other unique symbiotic bacteria (von Dohlen et al. 2001). Other obligate bacterial associates of insects are not restricted to bacteriocytes and may be found in a number of different host cells and tissues. Certain of these bacteria are often referred to as "guest" microbes and are not necessarily mutualistic. For example, *Wolbachia* (Rickettsiaceae) are intracellular guest bacteria that have been found to infect a number of invertebrates including mites, crustaceans and insects. *Wolbachia*, and other guest bacteria, are transmitted maternally via the cytoplasm of the egg and *Wolbachia* is known to modify host reproduction in a number of ways (e.g. cytoplasmic incompatibility, male killing, feminization of genetic males). The effects on host reproduction are to the advantage of *Wolbachia*.

The Hymenoptera date back to the Lower Triassic (248 mya) (Labandeira and Sepkoski 1993) and are divided into two suborders, the Apocrita (ants, bees and wasps) and the Symphyta (wood wasps and sawflies). The Symphyta are considered to be the more primitive and make up only about 5% (6,000+ species) of described Hymenoptera of which the majority (≈5,300 species) are tenthredinoid sawflies. Symphyta also fill fewer ecological niches than members of the Apocrita (Kristensen 1999). Considering the ancient lineage of the Symphyta, we wondered whether there was an endosymbiont that was as common to sawflies as *Buchnera* is to aphids or *Blattabacterium* to cockroaches. In an earlier study (Graham et al. 2008), we used 16S rRNA gene fragment PCR - DGGE, fragment cloning and sequencing to survey bacteria associated with several sawfly species including PFW, mountain ash sawfly (*Pristophora geniculata*, Tenthredinidae), yellowheaded spruce sawfly (*Pikonema alaskensis*, Tenthredinidae), birch sawfly (*Arge pectoralis*, Agridae) and balsam fir sawfly (*Neodiprion abietis*, Diprionidae). As in the current study, most of the bacteria identified by Graham et al. (2008) were either α or γ-Proteobacteria, many of which had been identified in other insects, mostly from insect guts. All the sawflies examined had a low apparent diversity of bacteria (Graham et al. 2008). However, PFW and other sawflies may harbour additional bacteria taxa that yield poor or no 16S rRNA gene PCR amplification products with the methods used, as a result of PCR primer bias, low template abundance (Wintzingerode et al. 1997), or other factors (Janda and Abbott 2007, Luo et al. 2007).

The microbial habitat of the insect gut is influenced by food quality, which represents the major carbon source for gut-dwelling bacteria (Mohr and Tebbe 2006), and diet appears to be an important factor affecting the richness of the gut microbiota in insects. In addition, gut structure and physiology can also affect the microbial habitat. Insects possessing simple and straight alimentary canals, such as

Symphyta, Lepidoptera, and many Diptera, will generally have a lower diversity of gut microbes (Dillon and Dillon 2004) compared to insects, such as termites and cockroaches, that have evolved complex and convoluted guts to facilitate the retention of bacteria in specialized fermentation structures (Wigglesworth 1972, Brune and Friedrich 2000). Due to the selective diets of sawflies and the relatively simple gut morphology (Maxwell 1955), the low level of bacterial diversity observed in PFW would be expected. The fact that no bacterial taxon was identified in more than one of the collections of PFW might be indicative of the transient nature of many of these gut bacteria.

The only known obligate intracellular bacterium detected in a sawfly to date was *Wolbachia* sp. in mountain ash sawflies (Graham et al. 2008). A *Wolbachia* bacteriophage locus,WO orf7, that is thought to encode a capsid protein, was also detected in mountain ash sawfly larvae (Graham et al. 2008). Graham et al. (2008) carried out RT-PCR transcription analysis and *Wolbachia* surface protein (WSP) was detected but WO orf7 was not suggesting that WO orf7 is integrated in the bacterial genome as inactive prophages rather than as active virions as is the case, for example, with the bacteriophage found in the mosquito, *Culex pipiens* (Sanogo and Dobson 2006).

Results to date do not support the hypothesis that sawflies share a common obligate intracellular bacterium.

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