An intracellular symbiont and other microbiota associated with field-collected populations of sawflies (Hymenoptera: Symphyta)

Robert I. Graham, Viviane Zahner, and Christopher J. Lucarotti

Abstract: Six species of sawfly (Hymenoptera: Symphyta) from four taxonomic families (Agridae, Diprionidae, Pamphiliidae, and Tenthredinidae) were collected from locations across Canada and surveyed for their associated microbiota. Total DNA was extracted from individual insects, and polymerase chain reaction (PCR) was used to amplify the conserved 16S rRNA 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–DGGE and PCR–RFLP products revealed a dominance of α- and γ-Proteobacteria, with most sequences showing high similarity to bacteria previously identified from other insect species and environmental samples. Additionally, a strain of the bacterial endosymbiont *Wolbachia* and a *Wolbachia* bacteriophage were identified from the mountain ash sawfly (*Pristiphora geniculata*).

Key words: bacteria, endosymbiont, Hymenoptera, sawflies, *Wolbachia*.

Résumé : Six espèces de mouches à scie (Hyménoptères : Symphytes) appartenant à quatre familles taxonomiques (Agridae, Diprionidae, Pamphiliidae et Tenthredinidae) ont été recueillies dans différentes localités à travers le Canada et ont été examinées quant à la présence de microbiotes associés. L'ADN total a été extrait des insectes individuellement et une réaction en chaine par polymérase (PCR) a été utilisée pour amplifier la région conservée du gène de l'ARNr 16S des microbiotes. Des électrophorèses sur gel en gradient dénaturant (DGGE) et des analyses de polymorphisme de longueur des fragments de restriction (RFLP) ont été réalisées pour séparer les clones bactériens associés aux insectes hôtes. Le séquençage des produits obtenus en PCR–DGGE et en PCR–RFLP a révélé une dominance des protéobactéries α et γ, la plupart des séquences montrant un haut degré de similarité avec les bactéries identifiées précédemment chez d'autres insectes et chez d'autres échantillons environnementaux. De plus, une souche de l'endosymbiote bactérien *Wolbachia* et un bactériophage de *Wolbachia* ont été identifiés du Tenthrède du sorbier (*Pristiphora geniculata*).

Mots-clés : bactérie, endosymbiote, Hyménoptère, mouches à scie, *Wolbachia*.

[Traduit par la Rédaction]

Introduction

Sawflies (Hymenoptera: Symphyta) are common in temperate regions of the world and a number are known to cause significant defoliation in North American forests. Outbreaks of the balsam fir sawfly *Neodiprion abietis* (Diprionidae) have been well documented in eastern Canada on balsam fir (*Abies balsamea*) (Moreau et al. 2005; Moreau 2006). The yellowheaded spruce sawfly *Pikonema alaskensis* (Tenthredinidae) is a growing concern because of its defoliation of managed plantations of black spruce (*Picea mariana*) and white spruce (*Picea glauca*) (Johns et al. 2006). Pine false webworm (*Acantholyda erythrocephala* (Pamphiliidae)), introduced into North America about 80 years ago (Lyons 1995), has been reported causing substantial damage to eastern white pine (*Pinus strobus*) plantations in Ontario, Canada (Lyons 1995), and New York State (Asaro and Allen 1999). The introduced pine sawfly *Diprion similis* (Diprionidae), similarly imported into eastern North America from Europe, preferentially feeds on eastern white pine (Wallace and Cunningham 1995). The mountain ash sawfly *Pristiphora geniculata* (Tenthridinidae) is mainly a pest of ornamental trees, causing severe defoliation of mountain ash (*Sorbus americana*) (Rose and Lindquist 1997). The birch sawfly *Arge pectoralis* (Agridae) is a pest of yellow and white birch (*Betula alleghaniensis* and *Betula papyrifera*, respectively). Birch sawflies rarely defoliate widespread areas but can impact mixed woodlands in eastern North America (Rose and Lindquist 1997).

Bacteria are universally found in animal guts, with the host–microbe relationship ranging from pathogenic to mu-

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tualistic (Dillon and Dillon 2004). The significance of microbial populations associated with arthropods is poorly understood, chiefly because their effects on the host are so variable. Bacteria within the arthropod gut are known to be important in the breakdown, mineralization, and cycling of a range of organic compounds and nitrogen fixation (Cruden and Markovetz 1987; Breznak and Brune 1994; Nardi et al. 2002). As well as gut microbiota, intracellular bacteria have also been found in a number of arthropod tissues. Endosymbiotic bacteria have been found to supply their hosts with nutrients (Baumann et al. 1995; Blattner et al. 1997) and provide protection against infection by other microbes (Dillon and Charnley 1995; Dillon and Dillon 2004), whereas others, most notably *Wolbachia*, have been found to generate reproductive defects (O'Neill et al. 1997).

Culture-independent analysis using 16S rRNA based methodologies has 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 genes encoding 16S rRNA, sequences can be amplified without prior cultivation of the bacterium (Head et al. 1998). PCR fragments can then be separated by cloning, screened using restriction fragment length polymorphism (RFLP), and sequenced. Alternatively, denaturing gradient gel electrophoresis (DGGE) allows the 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). Identification can then be achieved by DNA band excision and direct sequencing.

Little information is available concerning the array of bacteria associated with sawflies. We surveyed the microbial communities associated with the six species of sawflies listed above as part of a broader investigation into sawfly population dynamics and identification of potential biological control agents. Culture-independent analysis using 16S rRNA methodologies and sequencing revealed a dominance of α- and γ-Proteobacteria, with most showing high similarity to bacteria previously identified from other insect species. Numerous gut-associated bacteria, believed to be involved in facilitating nutrient uptake by host insects, were discovered in all six sawfly species. Additionally, a strain of the bacterial endosymbiont *Wolbachia* and a *Wolbachia* bacteriophage were identified from the mountain ash sawfly.

Materials and methods

Insect collection

Sawflies were collected as second- to fourth-instar larvae from a number of locations throughout Canada. Balsam fir sawflies were collected from Deer Lake, Newfoundland, pine false webworms from Simcoe County, Ontario, yellow-headed spruce sawflies from Lethbridge, Alberta, and Clearwater Lake, Saskatchewan, mountain ash sawflies and introduced pine sawflies from York County, New Brunswick, and birch sawflies from Northumberland County, New Brunswick. For consistency, we focused the research on fourth- and fifth-instar larvae, and all individuals sampled were shipped with and fed foliage from the locations where they were collected, and all were alive at the time of processing for DNA and RNA.

DNA and RNA extraction

DNA was individually isolated from 24 *N. abietis*, 24 *A. erythrocephala*, 24 *P. alaskensis*, 24 *P. geniculata*, 12 *D. similis*, and 12 *A. pectoralis*. Insects were submerged in 0.3% aqueous NaOCl for 1 min and rinsed in two changes of distilled water. Total DNA was purified from whole larvae using a DNeasy tissue kit (Qiagen, Valencia, California) (Haynes et al. 2003; Behar et al. 2005). For RNA analysis, simultaneous RNA and DNA extraction from the same individual insect was achieved using TriZol (Invitrogen, Carlsbad, California) following the manufacturer's protocol.

PCR amplification and DGGE analysis of bacterial communities

Specific 16S rRNA gene primers U984-GC and L1401 (Nübel et al. 1996; Frederick and Caesar 2000) were used to amplify regions V6 to V8 and p515F-GC and p806R (Relman 1993) were used to amplify region V4. Reaction mixtures (50 μL) contained PCR buffer (10 mmol/L Tris–HCl (pH 8.3) at 25 °C, 50 mmol/L KCl, 1.5 mmol/L MgCl, and 0.001% gelatin), 10 μmol/L each of dATP, dTTP, dCTP, and dGTP, 0.1 μmol/L of each primer, 1 unit *Taq* polymerase (Qiagen), and approximately 10 ng of insect genomic DNA template. PCRs were done using a Mastercycler EP thermal cycler (Eppendorf, Westbury, New York) with the settings (*i*) 94 °C for 5 min, 1 cycle, (*ii*) 94 °C for 20 s, 52 °C for 20 s, 72 °C for 45 s, 40 cycles, and (*iii*) 72 °C for 5 min, 1 cycle. PCR products were separated by DGGE using the DCode system (BioRad, Hercules, California) 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 mol/L urea and 40% *v/v* deionized formamide) and 1× TAE buffer (40 mmol/L Tris–acetate (pH 8.0) and 2 mmol/L EDTA). Electrophoresis was performed at 60 °C and 80 V in 1× 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 razorblade and placed in 100 μL of sterile distilled water at 94 °C for 5 min to elute DNA for sequencing.

PCR amplification and PCR–RFLP of bacterial communities

The DNA from individuals of the same species was pooled and used as a template for PCR amplification of bacterial 16S rRNA genes using universal primers 27F and 1492R (Hogg and Lehane 2001). PCR was carried out as above using the settings (*i*) 94 °C for 5 min, 1 cycle, (*ii*) 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, 35 cycles, and (*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, Wisconsin), and DH5α *Escherichia coli* cells (Invitrogen) transformed. The region containing the insert was amplified using M13 universal primers (Invitrogen). Additional PCRs were similarly carried out in 25 μL volumes. Ninety-six positive clones per sawfly species tested were amplified and screened. The PCR products from clones

Fig. 1. Example of DGGE profiles of PCR-amplified partial 16S rRNA bacterial genes cloned from larval (*a*) *Arge pectoralis* and (*b*) *Pristiphora geniculata*. Profiles collected from the same sawfly species show high similarity between individuals (lanes 1–6). Bands were excised from the gels, purified, and sequenced.

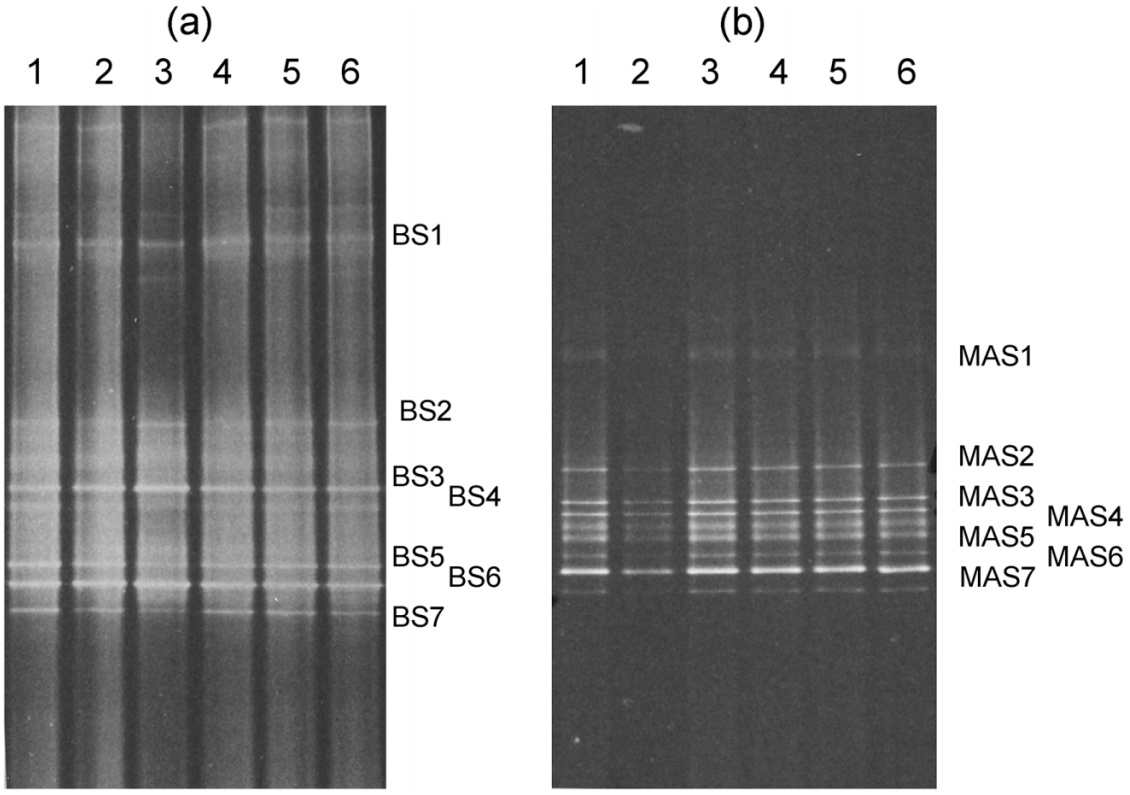
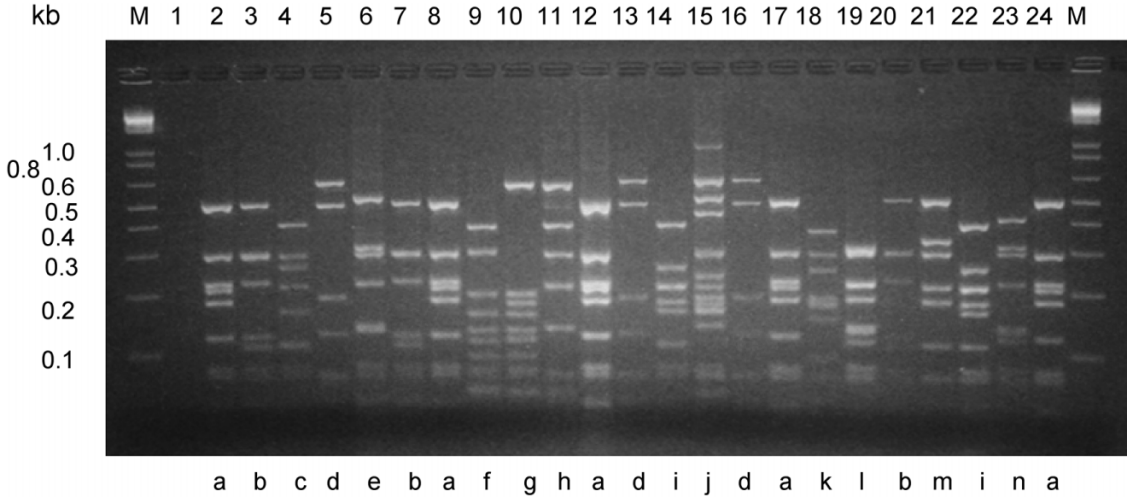


Fig. 2. Twenty-four PCR–RFLP profiles of 16S rRNA gene PCR-amplified clones from the host *Pristiphora geniculata* following cloning, enzyme digestion, gel electrophoresis, staining, and UV illumination. Letters below lanes indicate identical RFLP patterns. Clones showing differing RFLP profiles were purified and sequenced for further analysis. 1KbPlus ladder (Invitrogen) was used as a size standard (lane M).



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 a QIAprep spin miniprep kit (Qiagen).

Nucleotide sequencing, alignment, and phylogeny

Plasmid inserts and PCR products were sequenced at the Ontario Genomics Innovation Centre (Ottawa, Ontario). 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 the Ribosomal Database Project II (RDP) (East Lansing, Michigan) similarity rank program (Cole et al. 2005). Sequences were checked for possible chimerical nature using the RDP Chimera Check program (Cole et al. 2005). Sequence alignment was

Table 1. Diversity and phylotype of partial 16S rRNA bacterial sequences isolated from six species of Symphyta according to their homology to gene sequences in GenBank.

No.	Isolate	Primer pairs used[a]	Clade	Genus	BLASTN match	RDP % identity
Mountain ash sawfly (*Pristiphora geniculata*)						
1	MAS1	p515F and 27F	α-Proteobacteria	*Wolbachia* sp.	AY007547	97
2	MAS2	p515F and 27F	γ-Proteobacteria	*Yersinia pestis*	AE013632	91
3	MAS3	p515F and 27F	γ-Proteobacteria	*Yersinia* sp.	X89574	94
4	MAS4	p515F	γ-Proteobacteria	*Serratia* sp.	AB288309	99
5	MAS5	p515F and 27F	γ-Proteobacteria	*Pseudomonas* sp.	AM421162	98
6	MAS6	p515F and 27F	γ-Proteobacteria	Uncultured bacterium	EF127599	100
7	MAS7	p515F and 27F	γ-Proteobacteria	*Yersinia frederiksenii*	AY332821	93
Yellowheaded spruce sawfly (*Pikonema alaskensis*)						
8	YHSS1	p515F and 27F	Actinobacteria	*Corynebacterium* sp.	AJ438051	99
9	YHSS2	p515F and 27F	Actinobacteria	Uncultured bacterium	EF220354	99
10	YHSS3	p515F and 27F	α-Proteobacteria	Uncultured bacterium	AY394615	100
11	YHSS4	p515F and 27F	α-Proteobacteria	Uncultured bacterium	DQ163946	98
12	YHSS5	p515F and 27F	α-Proteobacteria	*Bradyrhizobium elkanii*	AY904749	98
13	YHSS6	p515F and 27F	α-Proteobacteria	*Bradyrhizobium* sp.	AY624135	99
14	YHSS7	p515F and 27F	α-Proteobacteria	*Sphingomonas* sp.	AJ001051	96
15	YHSS8	p515F and 27F	α-Proteobacteria	*Sphingomonas* sp.	Z23157	87
16	YHSS9	p515F and 27F	α-Proteobacteria	Uncultured bacterium	DQ163946	94
17	YHSS10	p515F	α-Proteobacteria	Uncultured bacterium	AJ459874	99
18	YHSS11	p515F and 27F	Bacilli	*Streptococcus* sp.	AY005042	99
19	YHSS12	p515F and 27F	Bacilli	*Staphylococcus* sp.	AE015929	100
20	YHSS13	27F	δ-Proteobacteria	Uncultured bacterium	AB286285	97
21	YHSS14	p515F	γ-Proteobacteria	*Yersinia* sp.	X89574	89
22	YHSS15	p515F	γ-Proteobacteria	Uncultured bacterium	AJ504511	98
23	YHSS16	p515F	γ-Proteobacteria	*Enterobacter* sp.	AF024609	88
24	YHSS17	p515F	γ-Proteobacteria	*Stenotrophomonas* sp.	DQ424872	90
25	YHSS18	p515F	γ-Proteobacteria	*Cellvibrio* sp.	AF452103	86
Balsam fir sawfly (*Neodiprion abietis*)						
26	BFS1	p515F	α-Proteobacteria	Uncultured bacterium	AJ459874	100
27	BFS2	p515F	α-Proteobacteria	*Sphingomonas* sp.	DQ207361	99
28	BFS3	p515F	α-Proteobacteria	*Sphingomonas* sp.	AY764287	98
29	BFS4	p515F and U984	α-Proteobacteria	*Caulobacter* sp.	DQ163946	93
30	BFS5	p515F and U984	γ-Proteobacteria	*Rahnella* sp.	U90758	99
31	BFS6	U984	γ-Proteobacteria	*Yersinia* sp.	AJ627599	99
32	BFS7	U984	γ-Proteobacteria	*Yersinia* sp.	AJ627599	99
33	BFS8	U984	γ-Proteobacteria	*Enterobacter* sp.	AY859722	97
34	BFS9	p515F	γ-Proteobacteria	*Enterobacter* sp.	DQ822723	99
Birch sawfly (*Arge pectoralis*)						
35	BS1	p515F	α-Proteobacteria	*Sphingomonas* sp.	DQ447783	99
36	BS2	p515F	γ-Proteobacteria	*Enterobacter* sp.	DQ884929	94
37	BS3	p515F	γ-Proteobacteria	Uncultured bacterium	AY226212	97
38	BS4	p515F	γ-Proteobacteria	*Yersinia* sp.	DQ917929	98
39	BS5	p515F	γ-Proteobacteria	*Enterobacter* sp.	DQ163940	93
40	BS6	p515F	γ-Proteobacteria	Uncultured bacterium	AY171318	95
41	BS7	p515F	γ-Proteobacteria	Uncultured bacterium	AY154518	93
Introduced pine sawfly (*Diprion similis*)						
42	IPS1	p515F	γ-Proteobacteria	*Rahnella* sp.	AM403660	89
43	IPS2	p515F	γ-Proteobacteria	*Rahnella* sp.	AM403661	95
Pine false webworm (*Acantholyda erythrocephala*)						
44	PFW1	27F	Actinobacteria	Uncultured bacterium	AB288658	99
45	PFW2	U984	Actinobacteria	*Propionibacterium acnes*	AY642054	100
46	PFW3	p515F	Actinobacteria	*Rhodococcus erythropolis*	AJ576250	97
47	PFW4	p515F	Actinobacteria	*Corynebacterium* sp.	AY677186	89

Table 1 (*concluded*).

No.	Isolate	Primer pairs used[a]	Clade	Genus	BLASTN match	RDP % identity
48	PFW5	U984	α-Proteobacteria	*Bradyrhizobium* sp.	AF408969	98
49	PFW6	U984	α-Proteobacteria	*Agrobacterium* sp	DQ193597	99
50	PFW7	U984	α-Proteobacteria	*Phyllobacterium* sp.	AF290483	98
51	PFW8	U984	α-Proteobacteria	Uncultured bacterium	AB186822	94
52	PFW9	27F	α-Proteobacteria	*Sphingobium herbicidovorans*	AB022428	99
53	PFW10	U984	α-Proteobacteria	*Methylobacterium* sp.	AY369236	96
54	PFW11	27F	α-Proteobacteria	Uncultured bacterium	AY162827	98
55	PFW12	27F	α-Proteobacteria	*Novosphingobium* sp.	AB177883	97
56	PFW13	27F	α-Proteobacteria	Uncultured bacterium	AJ459874	98
57	PFW14	27F	α-Proteobacteria	Uncultured soil bacterium	DQ378222	94
58	PFW15	p515F and U984	α-Proteobacteria	*Caulobacter* sp.	DQ163946	93
59	PFW16	U984	α-Proteobacteria	Uncultured bacterium	AB074649	92
60	PFW17	p515F	Bacilli	*Staphylococcus* sp.	DQ170801	95
61	PFW18	p515F and U984	Firmicutes	*Leuconostoc* sp.	AB008901	93
62	PFW19	p515F	γ-Proteobacteria	*Rahnella* sp.	DQ822730	95
63	PFW20	p515F	γ-Proteobacteria	*Enterobacter* sp.	DQ822723	94
64	PFW21	p515F	γ-Proteobacteria	*Klebsiella* sp.	DQ533885	90
65	PFW22	27F	γ-Proteobacteria	*Yersinia enterocolitica*	Z75316	98
66	PFW23	p515F	γ-Proteobacteria	*Stenotrophomonas* sp.	AM421782	98
67	PFW24	p515F	γ-Proteobacteria	*Pseudomonas* sp.	AF290479	90
68	PFW25	p515F	γ-Proteobacteria	*Stenotrophomonas* sp	DQ530137	98
69	PFW26	p515F and U984	γ-Proteobacteria	*Erwinia* sp.	AJ971890	93
70	PFW27	U984	γ-Proteobacteria	*Serratia* sp.	DQ321555	94

[a]The forward primer of the primer pair was used to sequence the isolate.

performed with Clustal W 1.8 using default parameters (Thompson et al. 1994) and phylogenetic analysis performed by neighbor-joining using Mega3.1 (Kumar et al. 2004). Sequences obtained in this study were compared with sequences available in GenBank.

Wolbachia and *Wolbachia* bacteriophage characterization

Wolbachia-specific primers Wsp81F and Wsp691R (Braig et al. 1998) were used to amplify a region of the *Wolbachia* surface protein gene (*wsp*). Primers ftsZf1 and ftsZr1 (Werren et al. 1995) were used to amplify a region of the *ftsZ* gene. To investigate the presence of a *Wolbachia* bacteriophage within the system, primers phgWOF and phgWOR (Masui et al. 2000) were used to amplify a region of the *Wolbachia* bacteriophage *ORF7* gene. The PCRs, sequencing, and phylogenetic analysis were undertaken as described for RFLP above. A duplex PCR protocol was designed to simultaneously determine the presence of *wsp* and *ORF7* loci within 22 second-instar and 20 fifth-instar *P. geniculata*. The same 42 *P. geniculata* larvae were tested for the presence of *wsp* and *ORF-7* gene transcription using host RNA extracts and duplex RT–PCR.

Accession numbers

All 70 unique 16S rRNA gene sequences are available in the GenBank database under accession Nos. EF658668 to EF658737. *Wolbachia ftsZ*, *wsp*, and *Wolbachia* bacteriophage *ORF7* sequences isolated from *P. geniculata* are available under accession Nos. EF658738, EF658739, and EF658740, respectively.

Results

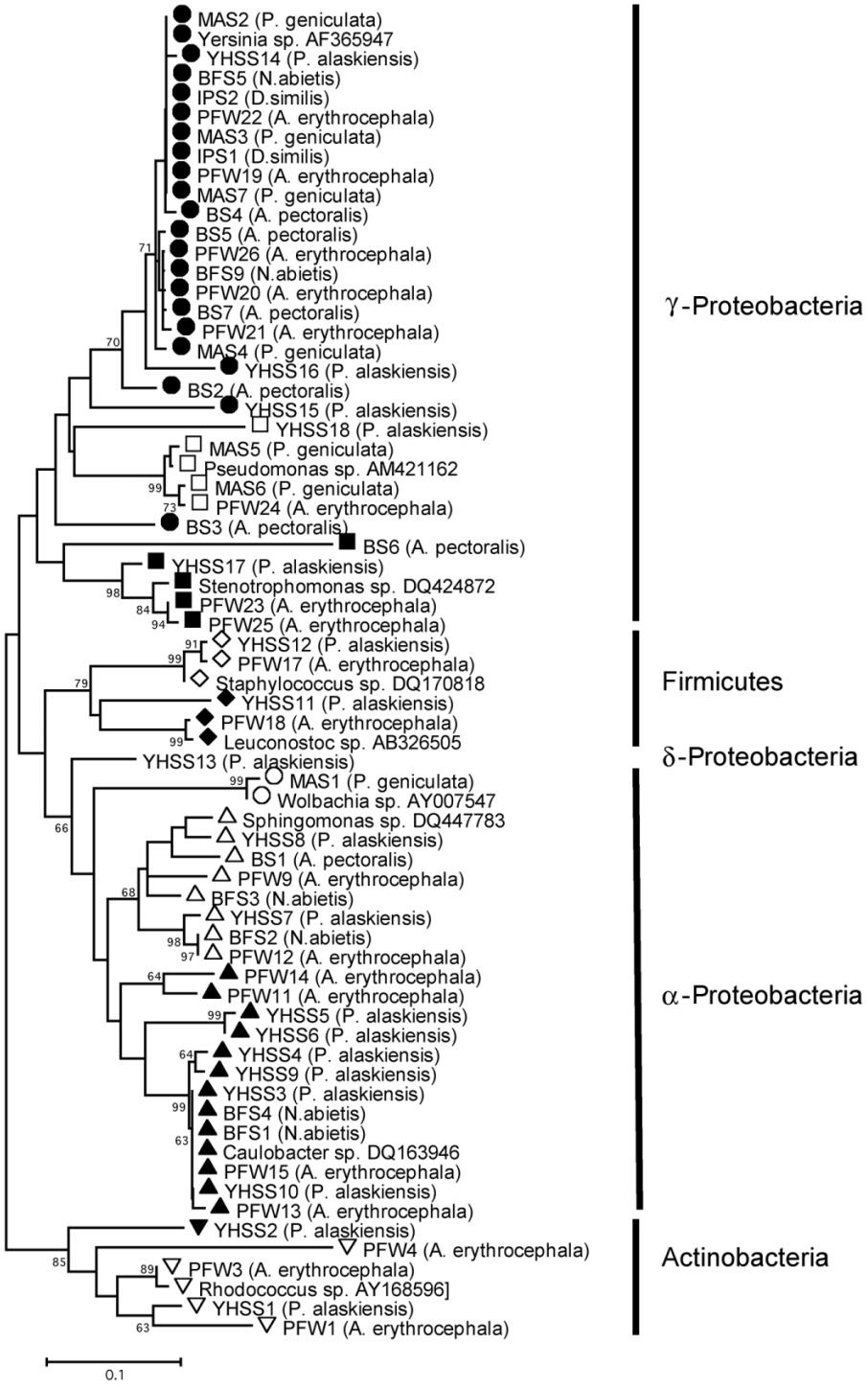
Identification of bacterial sequences

Variation in the associated bacterial communities between the different sawfly species was observed in PCR–DGGE profiles, but profiles from individuals of the same species were often identical. Sequencing of heterogenic clones and gel bands identified from PCR–DGGE (e.g., Fig. 1) and PCR–RFLP (e.g., Fig. 2) confirmed that each clone or band excised represented an individual DNA sequence corresponding to a different microbe. Sequence lengths of approximately 300 bases were achieved using primer p515F, 400 bases using primer U984, and 700 bases using primer 27F. Table 1 presents the bacteria identified from the six sawfly species tested. The results revealed a dominance of Gram-negative α- and γ-Proteobacteria, but members of the δ-Proteobacteria and Gram-positive Actinobacteria and Firmicutes were also detected (Fig. 3). *Acantholyda erythrocephala* and *P. alaskensis* contained the most bacterial entities, with 27 and 18 sequences detected, respectively. Only two bacterial sequences were identified from *D. similis*, both most closely related to sequences from the genus *Rahnella*.

Characterization of *P. geniculata – Wolbachia*

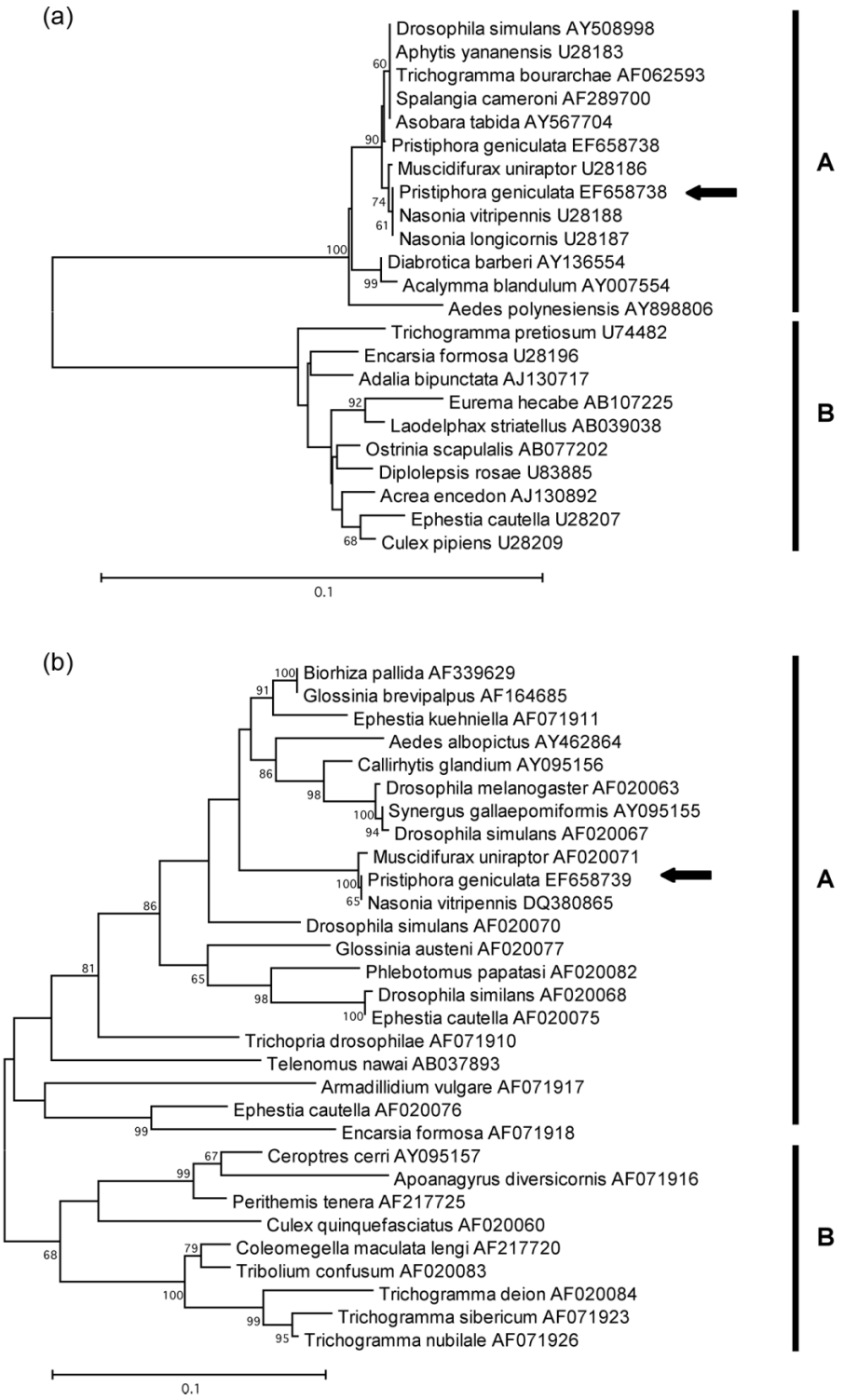
A strain of the bacterium *Wolbachia* was detected in *P. geniculata* using 16S rRNA gene amplification (sequence MAS1) (Table 1; Fig. 3). Fragments of *wsp* (586 bp) and *ftsZ* (929 bp) were further amplified and sequenced. Phylogenetic analysis of both the *ftsZ* (Fig. 4*a*) and *wsp* (Fig. 4*b*) gene fragments placed *P. geniculata – Wolbachia* in *Wolbachia* supergroup A. Primer pair phgWO amplified a 403 bp

Fig. 3. Neighbor-joining phylogenetic tree including the partial 16S rRNA PCR-amplified gene sequences from all six species of sawfly. The overlapping sequences obtained using primers 27F and p515 were used in the analysis. The scale bar represents a 10% estimated difference in nucleotide sequence. Numbers given at each node correspond to the percentage bootstrap values (for 1000 repetitions). Replicate numbers <60% were not included in the figure. Symbols indicate sequences belonging to the same bacterial taxonomic family: open squares, Pseudomonadaceae; solid circles, Enterobacteriaceae; solid squares, Xanthomonadaceae; solid diamonds, Leuconostrocaceae/ Streptococcaceae; solid triangles, Caulobacteraceae; open triangles, Sphingomonadaceae; open circles, Wolbachieae; solid inverted triangles, Propionibacteriaceae; open diamonds, Staphylococcaceae; open inverted triangles, Nocardiaceae.



fragment of *Wolbachia* bacteriophage *ORF-7*, indicating the presence of a bacteriophage. Both *wsp* and *ORF7* genes were detected in all 42 *P. geniculata* larvae using duplex PCR (Fig. 5, lanes 1–6). Duplex RT–PCR detected expression of *wsp* in all *P. geniculata* larvae but not *ORF7* (Fig. 5, lanes 8–13).

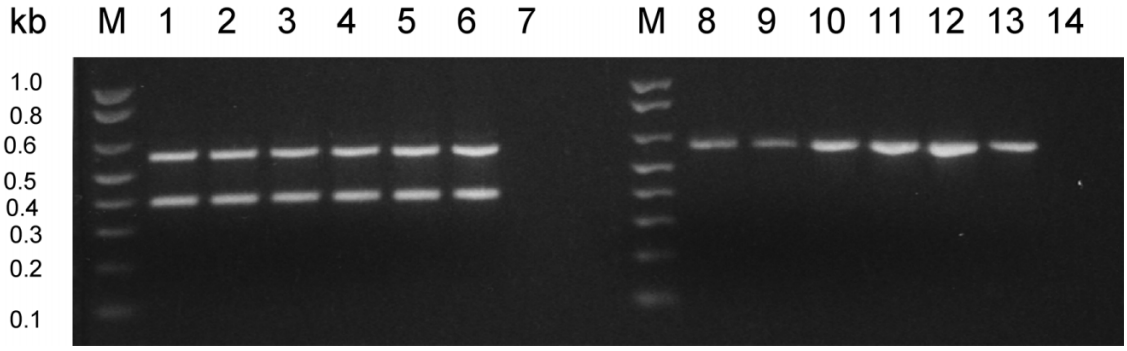
Fig. 4. Neighbor-joining phylogenetic tree showing the relationship between *Pristiphora geniculata – Wolbachia* and other identified *Wolbachia* spp. using (*a*) *ftsZ* and (*b*) *wsp* genes. Both genes show *P. geniculata – Wolbachia* strongly aligned to *Wolbachia* supergroup A (supergroups A and B are labeled on the right). The scale bars represent a 10% estimated difference in nucleotide sequence. Numbers given at each node correspond to the percentage bootstrap values (for 1000 repetitions). Replicate numbers <60% were not included in the figure.



Discussion

One hundred and twenty individuals representing six species from four taxonomic families of Symphyta were screened for the presence of associated microbiota using universal 16S rRNA gene primer sets. To our knowledge, a comprehensive description of microbiota associated with sawflies has not been previously performed. A related study (Whittome et al. 2007) specifically investigated only the midgut microbiota of *N. abietis* and found five bacterial se-

Fig. 5. Results of duplex PCR and duplex RT–PCR. Gel stained with ethidium bromide following electrophoresis at 80 V for 2 h. Lanes 1–6, duplex PCR using *Pristiphora geniculata* DNA showing both *wsp* (approximately 600 nt in size) and *ORF7* (approximately 400 nt in size) genes to be present; lane 7, distilled water negative control; lanes 8–13, duplex RT–PCR using *P. geniculata* RNA showing the presence of *wsp* expression but no evidence of *ORF7* expression; lane 14, distilled water negative control; lanes M, DNA size standards (1KbPlus ladder (Invitrogen)). A negative control for the presence of residual DNA in the RNA preparation was performed during all RT analyses (not shown in this figure).



quences identical to those from *N. abietis* detected in this current study. Our study focused on the microbiota associated with fourth- and fifth-instar larvae. However, limited 16S rRNA gene sequence analysis of early instar larvae and pupae suggested that microbiota were qualitatively similar throughout all of those life stages (data not shown). Further detailed characterization of the adult life stage would be necessary to determine whether microbial diversity changes over the sawfly life cycle. All sawflies were confirmed to have a low apparent diversity of bacteria (Table 1). The possibility should not be excluded, however, that sawflies may harbor additional bacterial 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).

We deliberately extracted total DNA from whole insects to allow for the identification of microbiota from all host tissues (Fukatsu and Nikoh 1998; Haynes et al. 2003; Schabereiter-Gurtner et al. 2003; Scoles 2004). The vast majority of bacterial sequences detected, however, were typically associated with insect gut habitats. The microbial habitat of the insect gut is greatly influenced by gut structure and physiology, in addition to food quality, which represents the major carbon source for gut-dwelling bacteria (Mohr and Tebbe 2006). Many termites and cockroaches have complex and convoluted guts (Wigglesworth 1972; Brune and Friedrich 2000) that have evolved to facilitate the retention of bacteria in specialized fermentation structures. Insects possessing simple and straight alimentary canals, such as the Symphyta, Lepidoptera, and many Diptera, will generally have a lower diversity of gut microbes (Dillon and Dillon 2004). In their larval stages, sawflies are phytophagous. Diet appears to be an important factor affecting the richness of the gut microbiota in insects. For example, depending on the source of diet tested, the gypsy moth *Lymantria dispar* (Lepidoptera: Lymantriidae) was found to have a microbial diversity ranging from 15 phylotypes at its most complex to seven phylotypes at its simplest (Broderick et al. 2004). Because of the selective diet of sawflies and the relatively simple gut morphology (Maxwell 1955), the low level of bacterial diversity observed in this study would be expected.

Many of the bacteria identified in this study are likely to have originated from host foodplants. *Rahnella* spp. have been isolated from foliage (Hashidoko et al. 2002; Izumi et al. 2006), indicating that the sequences detected may have originally been acquired through host diet. Members of this bacterial genus have been shown to ferment several polysaccharides (Brenner et al. 1998) and to have a role in nitrogen fixation (Brenner et al. 1998; Izumi et al. 2006). This latter feature would be particularly important for nitrogen recycling in nutrient-poor habitats and may promote the retention of this species within the sawfly gut as a beneficial symbiont. *Yersinia* spp. are ubiquitous in insect guts (Ulrich et al. 1981) and were found in four species of sawfly tested here. No beneficial characteristics have been attributed to *Yersinia* spp., and their widespread presence in soils and detritus suggests that this bacterium is more likely to be a transient microbe ingested with food matter rather than a permanent flora of sawfly guts. Many of the other α- and γ-Proteobacteria identified in this study are also likely to have originated from foliage contamination. We identified a sequence from *A. erythrocephala* similar to that of a Gram-positive *Leuconostoc* sp. detected in the termite *Cryptotermes domesticus* (Ohkuma and Kudo 1998). Species belonging to the *Leuconostoc* and *Streptococcus* (identified from *P. alaskensis*) are lactic acid bacteria, a group of bacteria abundant in termite guts (Tholen et al. 1997) and other insect intestines (Hunt and Charnley 1981; Ulrich et al. 1981). These bacteria are most likely involved in carbohydrate fermentation and nutrient uptake, producing lactic acid as the end product.

Isolating DNA from whole insects provided a greater possibility of detecting endosymbiotic bacteria residing in tissues other than only the gut lumen. To our knowledge, this is the first study to report the identification of *Wolbachia* (Alphaproteobacteria: Rickettsiales) (Lo et al. 2007) in the Symphyta. Detailed surveys of *Wolbachia* distributions in Britain (West et al. 1998), Panama (Werren et al. 1995), and North America (Werren and Windsor 2000; Floate et al. 2006) established that 16%–46% of insects sampled con-

tained *Wolbachia*, indicating that 1–5 million insect species could be infected in total (Werren et al. 1995). Hilgenboecker et al. (2008) suggested that previous estimates of *Wolbachia* infection may be low because, in many cases, only one or a few individuals of any given arthropod species were sampled. They estimated that 66% of arthropods may be infected by *Wolbachia* and that infection in any one species would involve either most or only a few individuals (Hilgenboecker et al. 2008). In addition to other tissues, *Wolbachia* infect the reproductive tissues of arthropods and are, therefore, primarily transmitted vertically from parent to offspring. Infections can quickly spread through populations, predominantly by causing postzygotic reproductive incompatibility (termed cytoplasmic incompatibility or CI), and are known to cause parthenogenesis in parasitic wasps and feminization of genetic males in isopods (Breeuwer and Werren 1990; O'Neill et al. 1992; Rousset et al. 1992; Stouthamer et al. 1993). In this current study, all *P. geniculata* tested were found to harbor *Wolbachia*, and preliminary results from different tissue types showed that *Wolbachia* was present in the gut, the head capsule, and the fat body of *P. geniculata* (data not shown).

Phylogenetic analysis using *wsp* and *ftsZ* genes placed *P. geniculata* – *Wolbachia* in *Wolbachia* supergroup A (Lo et al. 2002), most closely aligned with *Wolbachia* in *Nasonia vitripennis* (Hymenoptera: Pteromalidae) (West et al. 1998) (Fig. 4). A *Wolbachia* bacteriophage gene was also amplified from *P. geniculata*. Bacteriophages are frequently found in association with free-living bacteria, both as exogenic phages (virions) and as prophages integrated into the bacterial genome. *Wolbachia* endosymbionts have also been found to harbor a number of phage elements (Masui et al. 2000, 2001; Sanogo and Dobson 2004, 2006) that are believed to represent sources of genetic diversity between strains of *Wolbachia* (Sanogo and Dobson 2004). In the mosquito *Culex pipiens*, expressed bacteriophage *ORF7* genes were detected by RT–PCR, suggesting the presence of active virions (Sanogo and Dobson 2006). In the current study, experiments examining the expression of bacteriophage genes produced negative results, suggesting that bacteriophages from the *P. geniculata* – *Wolbachia* are only present as inactive *Wolbachia*-integrated prophages.

In summary, PCR–DGGE and PCR–RFLP methodologies have been used to profile bacterial communities associated with six species of Symphyta. Culture-independent analysis revealed a relatively simple community, particularly from *N. abietis*, *P. geniculata*, *D. similis*, and *A. pectoralis*, an attribute that may be related to the food source of these insects. Further analysis of the interactions between sawflies and their microbial communities, specifically those related to known endosymbionts, will help interpret the role that these bacteria play in the population dynamics and biology of these insects.

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