

## Combination of Genomic and Proteomic Approaches to Characterize the Symbiotic Population of the Banana Aphid (Hemiptera: Aphididae)

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**ABSTRACT** Aphids are known to live in symbiosis with specific bacteria called endosymbionts that have positive or negative impacts on their hosts. In this study, six banana aphid (*Pentalonia nigronervosa* Coquerel) strains from various geographical origins (Gabon, Madagascar, and Burundi) were screened to determine their symbiotic content, using complementary genomic (16S rDNA sequencing and specific polymerase chain reaction) and proteomic (two-dimensional difference gel electrophoresis coupled with protein identification by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry) approaches. Despite the geographical heterogeneity, the combined methods allowed us to identify the same two symbionts in the six aphids strains tested: *Buchnera aphidicola* and *Wolbachia*. Although *B. aphidicola* is found in almost all aphid species, the systematic presence of *Wolbachia* in banana aphids is particularly interesting, as this bacterium usually has a low prevalence in aphid species. Phylogenetic analyses showed that the *Wolbachia* sp. strain found in *P. nigronervosa* was very similar to the strain present in aphids of the genus *Cinara*, known to have developed a strong and long-term symbiotic association with *Wolbachia*. The high level of asexual reproduction in *P. nigronervosa* could be linked to the presence of *Wolbachia*, but its prevalence also suggests that this symbiotic bacterium could play a more essential role in its aphid host.

**KEY WORDS** banana aphid, symbiosis, *Buchnera aphidicola*, *Wolbachia*

Many insects are known to live symbiotically with intracellular microorganisms that influence their development (Braendle et al. 2003), nutrition (Douglas 1998), reproduction and speciation (Thompson 1987, Simon et al. 2011), and protection and immunity (Teixeira et al. 2008). Aphids (Hemiptera: Aphidoidea), in particular, harbor several bacterial endosymbionts that are usually classified as obligate (primary) or facultative (secondary) symbionts. Primary symbionts are very similar, whereas secondary symbionts can vary in shape and structure (Abbot and Moran 2002).

*Buchnera aphidicola* is an obligate symbiont present in almost all aphid species (Buchner 1965, Baumann et al. 1995). Exceptions are species from the tribe *Ceratiophidini* that harbor extracellular yeast-like symbionts of the phylum *Ascomycotina* instead of *B. aphidicola*. This fact is sometimes interpreted as being evidence of symbiont replacement (Fukatsu and Ishikawa 1996).

*Buchnera* provides aphids with essential amino acids that are not available in the plant phloem. It is therefore essential for the insect to survive (Baumann et al. 1995, Douglas 1998). Reciprocally, the bacterium cannot survive more than a few hours outside its host (Baumann and Moran 1997). This co-evolution between aphids and *Buchnera* has apparently led to the deletion of several genes that were not essential for a symbiotic lifestyle (Gil et al. 2002).

These symbiotic bacteria are located in specialized cells called bacteriocytes or mycetocytes that are aggregated in organs called bacteriomes or mycetomes. They are maternally transmitted by colonization of the developing eggs and embryos at an early stage, which could explain this symbiont distribution (Gómez-Valero et al. 2004).

In addition to *Buchnera*, aphids can harbor secondary symbionts (Tsuchida et al. 2005). These bacteria are not required for survival but they have various effects, either positive or negative, on their insect hosts: protection against high temperatures and natural enemies (Oliver et al. 2003), loss of fecundity and cytoplasmic incompatibility (Clancy and Hoffmann 1996, Fukatsu et al. 2001), fitness effects (Chen et al. 2000), host plant utilization (Tsuchida et al. 2004), and body color changes (Tsuchida et al. 2010). Secondary symbionts can differ among host species, and also

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Table 1. *P. nigronervosa* strains used in this study

Strain no.	Country of origin	City of origin
1	Burundi	Bujumbura (Center)
2	Burundi	Bujumbura (Isale)
3	Burundi	Bujumbura (Cibitoke)
4	Cabon	Libreville
5	Madagascar	Arivonimamo
6	Madagascar	Ambodifontsy

among individual hosts within species (Oliver et al. 2010).

Until recently, eight secondary symbionts have been described in aphids: *Serratia symbiotica*, also referred to as PASS for pea aphid secondary symbiont, R-type symbiont or S- symbiont (Koga et al. 2003); *Hamiltonella defensa*, also named PABS for pea aphid Bemisia-type symbiont (Darby et al. 2001); *Rickettsia* or PAR for pea aphid *Rickettsia* (Chen et al. 1996); *Spiroplasma* sp (Fukatsu et al. 2001); *Wolbachia* (Gómez-Valero et al. 2004); *Arsenophonus*; *Regiella insecticola* (Chen and Purcell 1997); and, more recently, a facultative symbiont of the genus *Rickettsiella* (Tsuchida et al. 2010).

Among these, *Wolbachia* is particularly interesting, as it can distort the reproducing capabilities of its host. *Wolbachia* has rarely been found in aphids (Werren et al. 1995), even if it is commonly found in insects (at least in 60% of arthropod species). This alphaproteobacterium is mainly maternally transmitted, but cases of horizontal transfer have been reported (O'Neill et al. 1992).

The banana aphid *Pentalonia nigronervosa* Coquerel (Hemiptera: Aphididae) is the main vector of the banana bunchy top virus (BBTV; Yasmin et al. 2001), which is considered as the most serious viral banana disease worldwide (Thomas and Iskra-Caruana 2000). Despite the negative impact of *P. nigronervosa* on banana production, its symbiotic population has never been investigated. The objective of this study is to gain robust information about the symbiotic populations in several *P. nigronervosa* strains from various geographical origins by combining three independent protocols. This work could provide some interesting prospects about aphid-plant-virus interactions, especially as some symbiont proteins are believed to interact with virus particles in the case of persistent viruses transmitted by aphids. These data could prove useful for the development of an efficient control method against BBTV.

## Materials and Methods

**Insects.** Aphids were maintained at 25°C in a long-day photoperiod (16-h light periods) on tissue-cultured banana plantlets of the Williams variety. Plants were replaced when they outgrew the boxes or when they were covered with honeydew. All aphid strains were reared in isolated growth chambers. Table 1 shows the different *P. nigronervosa* strains used in this study.

**Genomic Approach. DNA Extraction.** Adult aphids were soaked in 100% ethanol for 1 min to eliminate confounding bacteria present on their surface before total DNA extraction using the Qiagen DNeasy blood and tissue kit (QIAGEN, Chatsworth, CA). DNA quality was evaluated using a NanoDrop spectrophotometer (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE). Two ratios of absorbance were measured: 260/280 nm and 260/230 nm. DNA was considered to be of good quality and purity when those ratios were around 1.8 for 260/280 and in the range of 2.0–2.2 for 260/230.

**16S rDNA Amplification and Sequencing.** This experiment was first conducted with *P. nigronervosa* strain 2 (Table 1), from Bujumbura (Isale), to screen symbiotic populations without any preconceived idea or bias linked to the use of specific primers. After DNA extraction from a single aphid, almost the entire length of bacterial 16S rDNA ( $\approx$ 1.5 kb) was amplified by polymerase chain reaction (PCR) using primers 16SA1 (5'-AGACITTTGATCMTGGCTCAG-3') and 16SB1 (5'-TACGGYTACCTTGTTACGACTT-3') following the temperature program described by Fukatsu and Nikoh (1998). PCR amplifications were performed in 50  $\mu$ l reactions containing 2  $\mu$ l of DNA, 5  $\mu$ l of 10 $\times$  reaction buffer (Bioline), 5  $\mu$ l deoxynucleotide triphosphate mixture (25 mM each), 1.5  $\mu$ l of MgCl<sub>2</sub> (50 mM), 1  $\mu$ l of each primer (25  $\mu$ M), 0.2  $\mu$ l of *Taq* polymerase (Hybripol, 1 U/ $\mu$ l), and 34.3  $\mu$ l of water.

To determine if several sequences from different microorganisms could be in the amplified DNA, PCR products with the correct size were cloned with the TOPO TA-cloning kit and the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA), using TOPI0 competent cells and following the manufacturer's instructions. The length of the inserted fragment was checked, and colonies of interest were cultured in 1.5 ml of Luria-Bertani medium supplemented with ampicillin and subjected to a classical plasmid extraction protocol (Sambrook and Russel 2006).

Products were then digested with the four-base-recognizing restriction endonuclease *Hae*III (New England BioLabs, Ipswich, MA) and electrophoresed in agarose gels to type the cloned 16S rDNA.

A dye terminator-labeled cycle sequencing reaction was performed on the purified plasmids with sequencing primers 16SA1 and EUB1405R (5'-GACGGGCGGTGTGTRCA-3') using the temperature program described by Kaiwa et al. (2010). The reaction products were analyzed with a 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA).

The resulting sequences were cleaned from plasmid and primer sequences. Contigs were made using ATGC sequence assembly software (GENETYX Corporation, Tokyo, Japan) and were further compared with databases using blastn. The complete 16S rDNA sequences generated in this study were recorded in GenBank under accession numbers KC522606 for *Wolbachia* and KC683897 for *B. aphidicola*.

The experiment was completed by the analysis of the V3 region of the 16S rDNA for all the aphid strains described in Table 1, using universal primers F 357

**Table 2.** Specific primers used in this study

Target symbiont	Target gene	Primer name	Primer sequence (5'-3')	Product size (kb)	References
<i>Buchnera</i>	16SrDNA	16SA1 ApisP1	AGAGTTTGATCMTGGCTCAG TTCCACTGTGGCTGGTTA	0.321	Fukatsu and Nikoh 1998 Tsuchida et al. 2002
PASS	16SrDNA	16SA1 PASScomp	AGAGTTTGATCMTGGCTCAG GCAATGTCTTATTAACACAT	0.48	Fukatsu and Nikoh 1998 Fukatsu et al. 2000
PAUS	16SrDNA	U99F 16SB4	ATCGGGGAGTAGCTTGCTAC CTAGAGATCGTCGCCTAGGTA	0.2	Sandström et al. 2001 Tsuchida et al. 2002
PABS	16SrDNA	PABSF 16SB1	AGCACAGTTTACTGAGTTCA TACGGYTACCTTGTTACGACTT	1.66	Darby et al. 2001 Fukatsu and Nikoh 1998
<i>Rickettsia</i>	16SrDNA	16SA1 Rick16SR	AGAGTTTGATCMTGGCTCAG CATCCATCAGCGATAAAATCTTTC	0.2	Fukatsu and Nikoh 1998 Fukatsu et al. 2001
<i>Rickettsiella</i>	16SrDNA	P136RIC211F P136RIC470R	GGGCCTTGGCCTCTAGGT TGGGATCCGTCACAGTAATCGA	0.26	Tsuchida et al. 2010 Tsuchida et al. 2010
<i>Spiroplasma</i>	16SrDNA	16SA1 TKSSspR	AGAGTTTGATCMTGGCTCAG TAGCCCTGGCTTCTTGCTAA	0.51	Fukatsu and Nikoh 1998 Fukatsu et al. 2000
<i>Wolbachia</i>	16SrDNA	16SA1 W2	AGAGTTTGATCMTGGCTCAG CTTCTGTGAGTACCGTCATTATC	0.45	Fukatsu and Nikoh 1998 Heddi et al. 1999
<i>Arsenophonus</i> sp	16SrDNA	16SA1 Ars16SR	AGAGTTTGATCMTGGCTCAG TTAGTCCCGGAGGCCACAGT	0.96	Fukatsu and Nikoh 1998 Tsuchida et al. 2002

(5'-CCTACGGGAGGCAGCAG-3') and R 538 (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al. 1993). The amplified PCR products were cloned using the Fermentas CloneJET PCR cloning kit (St. Leon-Rot, Germany). Plasmid DNA was then purified using the Fermentas GeneJET Plasmid Miniprep kit and sent to Macrogen (Seoul, Korea) for sequencing.

**Symbiont Detection by Targeted PCR.** To confirm the results obtained from 16S rDNA characterization, a targeted detection of symbionts was made for all the aphid strains listed in Table 1, using the specific primers listed in Table 2 and the thermal cycle described by Tsuchida et al. (2002).

To avoid false-positive and false-negative results, genomic DNA samples from several *Acyrtosiphon pisum* Harris (Hemiptera: Aphididae) strains known to harbor specific symbionts (obtained from T. Tsuchida, Toyama University, Japan) were used as positive controls in all PCR reactions. A negative control was also made, using water instead of DNA. Diagnostic PCRs were made on 20 individuals for each aphid strain.

**Phylogenetic Analyses.** Phylogenetic trees were constructed using MEGA 5 software (Tamura et al. 2011), and all the alignments were done using Muscle (Edgar 2004). Neighbor-joining trees were created using the Kimura two-parameter model and pairwise deletion. Bootstrap analyses were done with 1,000 replications. Maximum parsimony analysis yielded the same results altogether.

**Proteomic Approach. Total Protein Extraction.** For total protein extraction, 200 mg of strain 2 aphids (Table 1) collected in Bujumbura (Isale) was crushed in 35 ml of a 7 M urea, 2 M thiourea, 20 mM Tris buffer (pH 8.5) including 2% CHAPS and then centrifuged at 15,000 × g for 15 min at 4°C. Supernatant was collected and proteins were precipitated using the 2D Clean-Up Kit (Amersham Biosciences, Amersham, UK) according to the manufacturer's instructions.

**Symbiont Protein Extraction.** Symbiont protein extraction was carried out with 200 mg of aphids from the

same strain, using a Percoll gradient according to the protocol of Charles and Ishikawa (1999).

**Analytical Two-Dimensional Gel Electrophoresis.** We used the protocol described by Francis et al. (2010), with slight modifications. Protein quantification was carried out using the RCDC quantification kit from Bio-Rad (Hercules, CA). The protein extracts were then labeled with three different CyDyes (GE Healthcare Europe GmbH, Freiburg, Germany): Cy2 (blue, 488 nm), Cy3 (green, 532 nm), and Cy5 (red, 633 nm). The labeling was done by mixing 12.5 μg of each protein sample with the corresponding CyDye for 30 min. The labeling was then stopped by adding 10 mM lysine.

The samples corresponding to the different treatment groups were: a total protein extraction from *P. nigronervosa*, a symbiont protein extraction of *P. nigronervosa*, and a total protein extraction of an *A. pisum* clone with a unique endosymbiont (*B. aphidicola*). The three samples were analyzed per pair and all combinations were tested. For each gel, samples were labeled either with Cy3 or Cy5 and mixed with an internal reference standard protein mixture (pooled from equal aliquots from all the experimental samples) labeled with Cy2. Protein two-dimensional electrophoresis and picking and identification by mass spectrometry were performed as described by Francis et al. (2010).

## Results

**Genomic Approach.** Almost the entire eubacterial 16S rDNA from the symbionts of one Burundian strain of *P. nigronervosa* (strain 2, from Bujumbura [Isale]) was amplified, cloned, and sequenced.

Analysis of the cloned fragments revealed nine sequence types that could be associated to two different bacterial species.

The generated contig sequence associated to the first organism was 1,449-bp-long and showed 96% sequence similarity with the 16S rDNA sequences of

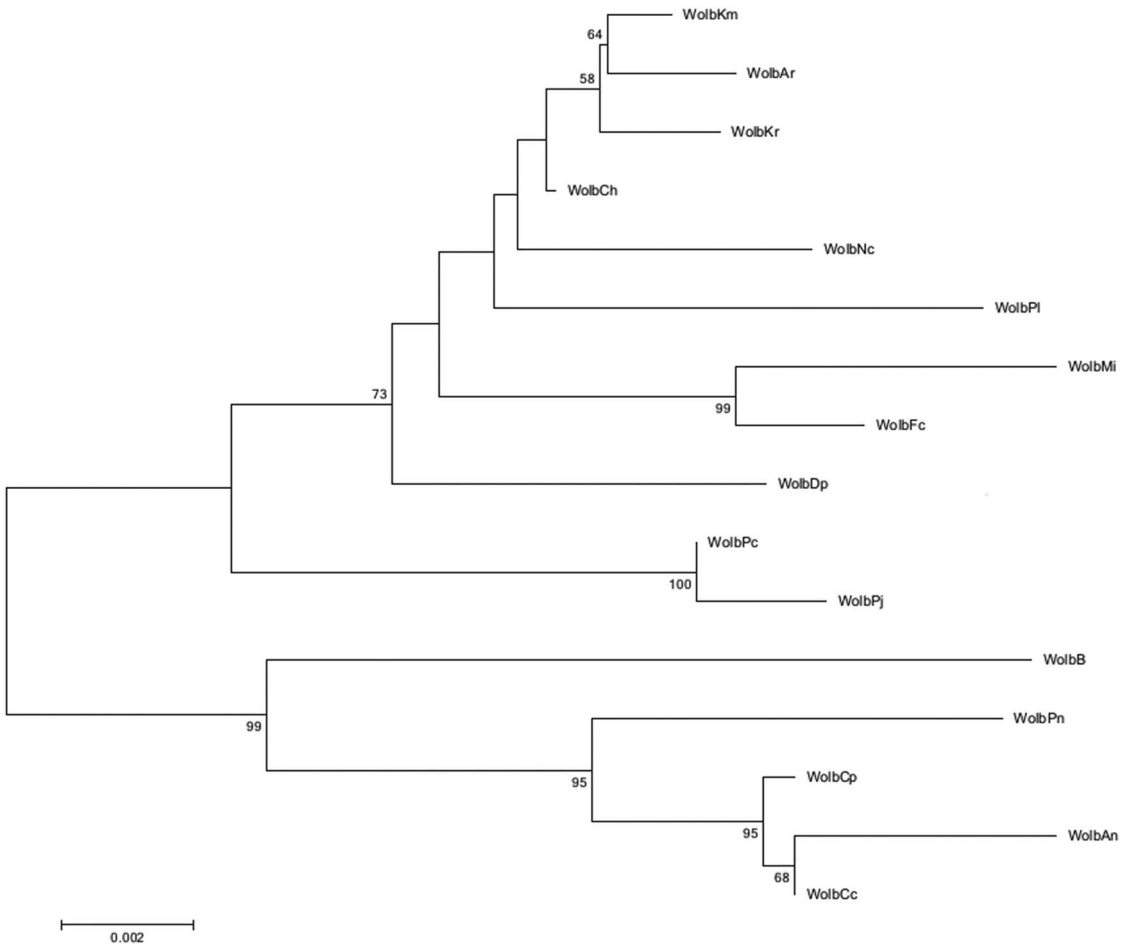


Fig. 1. Phylogenetic tree of *Wolbachia* based on 16S rDNA sequences. The tree was constructed using the neighbor-joining algorithm and the Kimura two-parameter model and pairwise deletion in MEGA 5. Bootstrap values obtained with 1,000 resamplings are shown at the nodes, although values less than 50% were not reported. The following strains and accession numbers were used: *Wolbachia* isolated from *Aphis nerii* (WolbAn, JN384083.1), *Archarius roelofsi* (WolbAr, AB604662.1), *Bryobia* sp (WolbB, EU499316.1), *Cinara cedri* (WolbCc, JN384079.1), *Curculio hachijoensis* (WolbCh, AB604661.1), *Cinara pinea* (WolbCp, JN384082.1), *Drosicha pinicola* (WolbDp, AB491204.1), *Folsomia candida* (WolbFc, EU831094.1), *Koreoculio minutissimus* (WolbKm, AB604663.1), *Kleidocerys resedae* (WolbKr, JQ726771.1), *Mesaphorura italica* (WolbMi, AJ575104.1), *Nephila clavata* (WolbNc, AF232234.1), *Pseudolynchia canariensis* (WolbPc, DQ115537.1), *Penicillidia jenyntii* (Wolb Pj, AB632591.1), *Pentastiridius leporinus* (WolbPl, FN428797.2), and *Pentalonia nigronervosa* (WolbPn, KC522606.1).

*B. aphidicola* of the aphids *Uroleucon ambrosiae* Thomas (Hemiptera: Aphididae), *A. pisum*, and *Uroleucon sonchi* L. (Hemiptera: Aphididae).

The sequence associated to the second organism (1,425 bp) was 97% similar to the 16S rDNA sequences of *Wolbachia* symbionts isolated from various insect species, including three aphid species. Phylogenetic analyses further confirmed these results and the identity of the detected symbiont (Fig. 1). The constructed tree showed that the 16S rDNA sequence corresponding to *Wolbachia* from *P. nigronervosa* clustered with *Wolbachia* sequences from other aphid species, including *Cinara cedri* Mimeur (Hemiptera: Aphididae), *Cinara pinea* Mordvilko (Hemiptera: Aphididae), and *Aphis nerii* Boyer de Fonscolombe (Hemiptera: Aphididae).

In addition, the V3 region of the bacterial 16S rDNA was amplified for five other *P. nigronervosa* strains. Amplification repeatedly yielded a heterogeneous amplification product of 200 bp. Ninety-six colonies were randomly selected for each aphid strain after cloning. Sequencing of the purified products revealed the presence of *B. aphidicola* and *Wolbachia* in all aphid strains.

Specific PCR tests made with the primer pairs 16SA1/ApisP2 and 16SA1/W2 yielded amplification products of 321 and 450 bp, respectively, confirming the presence of *B. aphidicola* and *Wolbachia* in all the collected *P. nigronervosa* strains.

None of the other primers listed in Table 2 produced PCR amplification products, suggesting the absence of other symbionts.

**Table 3.** Symbiont proteins identified in this study

MW	pI	Score	Coverage	Peptide no.	Protein identification	Accession	Organism
42,729	5.85	206	56%	16	OmpF-like porin	NP_240177	<i>Buchnera aphidicola</i>
57,988	5.14	225	59%	35	Chaperonin GroEL	YP_002468346	<i>Buchnera aphidicola</i>
48,470	6.23	50	15%	6	MesJ protein	YP_005619866	<i>Buchnera aphidicola</i>
59,662	9	58	20%	13	Anthranilate synthetase	AAR99727.1	<i>Buchnera aphidicola</i>
129,552	8.88	64	8%	9	RecC	YP_802836.1	<i>Buchnera aphidicola</i>
65,260	9.14	56	25%	14	Sulfite reductase subunit beta	YP_005619593.1	<i>Buchnera aphidicola</i>
78,765	8.81	72	15%	12	Ankyrin repeat domain	WP_010405507.1	<i>Wolbachia</i>
45,578	8.21	84	27%	10	Fic family protein	NP_967022	<i>Wolbachia</i>

MW, molecular weight; pI, isoelectric point; Score, Mowse score according to Mascot research (Pappin et al. 1993); Coverage, percentage of the protein sequence identified; Peptide no., number of peptide hits for each protein; Accession, accession number on NCBI, Organism: related organism for protein identification.

**Proteomic Approach.** We used the two-dimensional difference gel electrophoresis method, which only requires a single gel to reproducibly detect differences between fluorescently tagged protein samples (Unlü et al. 1997). This technique allowed us to identify six proteins belonging to *B. aphidicola* and two proteins belonging to *Wolbachia*, confirming their presence in the banana aphids we sampled (Table 3).

In particular, we detected a GroEL protein and an OmpF-like protein, both known to be abundant in *B. aphidicola*, and an ankyrin repeat domain protein that is specific to *Wolbachia*.

### Discussion

Symbiotic populations hosted by aphids can be very diverse and complex. New facultative symbionts are regularly described (Guay et al. 2009, Tsuchida et al. 2010) and novel effects of endosymbionts on host ecology and physiology are still unraveled (Hosokawa et al. 2010). Additional undiscovered associations between aphids and bacterial symbionts are therefore quite likely to exist (Russell and Moran 2006). The necessity to couple several methods for studying symbiosis and its relevance has already been highlighted (Fukatsu and Nikoh 1998). The endosymbiotic microbiota is indeed complex, with multiple symbionts co-existing inside one insect body, and this can sometimes lead to artifacts when using genomics-based methods only. It is therefore useful to analyze the microbial DNA sequences obtained in connection with other methods. That is why we chose to combine genomic and proteomic approaches in this study, to get a full view of the symbiotic population in the banana aphid.

Through the combination of those two methods, two bacterial symbionts were identified of the six banana aphid strains we screened: *B. aphidicola* and *Wolbachia*.

Surprisingly, and despite geographical heterogeneity, there was no difference in symbiont composition between strains. It is indeed usual to detect differences in secondary symbiont occurrences between aphids of the same species, especially when host plants and geographical origins vary (Oliver et al. 2010). However, examples of stable associations exist but remain rare (Gómez-Valero et al. 2004).

The coexistence of two symbionts in the banana aphid is not remarkable by itself, as multiple endo-

symbiosis is known to occur in aphids (Tsuchida et al. 2002, Moran and Dunbar 2006): up to half of aphid species may contain a secondary symbiont (Chen et al. 1996). However, co-infections are often unstable (Moran and Dunbar 2006), because the coexistence of multiple symbionts can be costly for the host (lower growth and fecundity rates).

*B. aphidicola* has been detected in almost all the aphid species tested so far in the literature, and found to be essential for their normal development and reproduction. We confirmed its presence in the banana aphid by 16S rDNA sequencing, targeted PCR, and a proteomic approach. Interestingly, a GroEL protein from *B. aphidicola* was identified. This molecular chaperone is considered as the most abundant protein in *Buchnera* in several studies (Charles et al. 2011, Poliakov et al. 2011). In particular, the GroEL protein of *Buchnera* was found to play a role in the transmission of persistent viruses of the Luteoviridae family. Such an impact of *Buchnera* proteins on BBTV transmission will require further investigation, as it could provide information about virus–host interactions and be a first step in the development of a potential biological control method against the disease.

The presence of the obligate symbiont was confirmed by the detection of an OmpF-like protein also known to be present in high concentrations in *Buchnera* (Maezawa et al. 2006), with a predicted capacity to transport small molecules across inner membranes. Finally, an anthranilate synthase protein involved in the biosynthesis of tryptophan, an essential amino acid known to be provided by *Buchnera* to the host, was also found (Charles et al. 2011).

The detection of *Wolbachia* in *P. nigronervosa* is more interesting. *Wolbachia* has indeed only been detected in 22 aphid species so far, with prevalence values ranging between 0.4 and 100% within species (Wang et al. 2009, Augustinos et al. 2011, Jones et al. 2011).

This secondary symbiont was moreover surprisingly detected in all six banana aphid strains tested in the current study. The strains were from various geographical origins, quite distant from one another. In particular, two of them were from Madagascar Island, without any contact with the African continent. This strongly suggests a vertical transmission of *Wolbachia* in *P. nigronervosa* and a well-established symbiotic association. This hypothesis is strengthened by the

fact that a nearly systematic occurrence of *Wolbachia* was recorded in another aphid species of the genus *Pentalonia* (*Pentalonia caladii* [Hemiptera: Aphididae]) in Hawaii (Jones et al. 2011).

The presence of *Wolbachia* in the banana aphid was confirmed by the detection of an ankyrin repeat domain protein. While these domains are relatively rare in bacteria, the *Wolbachia* genome is however characterized by an unusually large number of genes encoding for proteins containing ankyrin repeat domains (Iturbe-Ormaetxe et al. 2005) that are proposed to play a functional role in symbiotic biology and in host-symbiont interactions (Wu et al. 2004, Iturbe-Ormaetxe et al. 2005). In particular, genes encoding proteins with ankyrin repeat domains are believed to be linked to the cytoplasmic incompatibility phenotype (Tram et al. 2003, Iturbe-Ormaetxe et al. 2005).

The phylogenetic tree based on the 16S rDNA (Fig. 1) shows banana aphid *Wolbachia* among other *Wolbachia* strains hosted in various insect and nematode species. When constructing this tree, we were confronted with nodes with low bootstrap values. Classifying *Wolbachia* strains in invertebrates was already considered as a hard task by several authors (Fenn et al. 2006, Augustinos et al. 2011). Indeed, horizontal transfer events of *Wolbachia* genome fragments are known to occur and to make phylogenetic analysis more complicated, as nuclear gene copies would evolve in a different way than cytoplasmic copies of *Wolbachia* genes.

Regarding our tree, we can nevertheless see that the *Wolbachia* strain recorded in *P. nigronervosa* is very similar to the strains in aphid species of the genus *Cinara*. This is interesting because several studies have confirmed the prevalence of *Wolbachia* in *Cinara* species of different origins, also suggesting a strong and long-term symbiotic association with it (Gómez-Valero et al. 2004, Wang et al. 2009).

The *Wolbachia* sp. found in the banana aphid is also close to the one found in phytophagous spider mites of the genus *Bryobia* (Acari: Tetranychidae). Both these mites and aphids of the genus *Cinara* are characterized by a high level of asexual reproduction, and this phenomenon is believed to be directly linked to the presence of *Wolbachia* (Gómez-Valero et al. 2004, Ros et al. 2008). The capacity of *Wolbachia* to induce parthenogenesis, cytoplasmic incompatibility, and feminization has indeed been well studied in various insect species (Rousset et al. 1992, Stouthamer et al. 1993, Clancy and Hoffmann 1996) and is believed to promote bacterial spread within a species.

Similarly to *Bryobia* and *Cinara*, *P. nigronervosa* is characterized by parthenogenetic reproduction; hardly any sexual morphs are observed (Footitt et al. 2010). This could be the consequence of a stable infection by *Wolbachia*. Nevertheless, complete asexuality is rare in the animal kingdom (Burt 2000) and asexuals are considered to have limited adaptive potential (Ros et al. 2008). Bacterial infection could however play a role in the adaptive success of asexual species. An important presence and a stable infection of the banana aphid by *Wolbachia* could thus be ex-

plained if the bacterium has positive effects on the banana aphid. This hypothesis is strengthened by the fact that superinfections in insects are likely to be maintained when hosts receive more benefits than costs from symbiosis (Oliver et al. 2006).

Potential beneficial roles of *Wolbachia* have been broadly investigated these last years. Studies have been made on mosquitoes (Diptera: Culicidae) and *Drosophila* (Diptera: Drosophilidae) evidencing that *Wolbachia* could manipulate the host antioxidant system in a manner that is beneficial to host survival (Brennan et al. 2008, Brownlie et al. 2009). The bacterium was also shown to improve its host fitness in certain conditions (Dobson et al. 2002, Weeks et al. 2007).

*Wolbachia* was shown to play a nutritional role in *Drosophila*, but also in the bedbug *Cimex lectularius* L. (Hemiptera: Cimicidae), in which it was identified to be associated to bacteriocytes (Brownlie et al. 2009, Hosokawa et al. 2010). Regarding its prevalence, a possible role of *Wolbachia* in the banana aphid could be, similarly to *Buchnera*, to supply essential nutrients and vitamins to its host or to protect it from plant-produced defense molecules. *P. nigronervosa* effectively feed on banana phloem, which is rich in phenolic compounds (Pothavorn et al. 2010) known to have negative effects on aphids in various host plants (Goławska 2010). Arthropod *Wolbachia* are mostly known to be parasites, but the possibility of a more mutualistic role in insects could not be excluded (Fukatsu et al. 2001, Weeks et al. 2007).

This study identified *B. aphidicola* and *Wolbachia* in six strains of banana aphids from various origins. Combining proteomic and genomic methods allowed us to get a thorough view of the symbiont population of *P. nigronervosa* for the first time. Our results suggest that a strong link exists between *P. nigronervosa* and *Wolbachia*, and this long-term relationship could explain the anholocyclic status of the aphid species. A possible role of *Wolbachia* in banana aphid nutrient supply cannot be excluded and will deserve further investigation.

## References Cited

- Abbot, P., and N. A. Moran. 2002. Extremely low levels of genetic polymorphism in endosymbionts (*Buchnera*) of aphids (*Pemphigus*). *Mol. Ecol.* 11: 2649–2660.
- Augustinos, A. A., D. Santos-García, E. Dionyssopoulou, M. Moreira, A. Papapanagiotou, M. Scarvelakis, V. Doudoumis, S. Ramos, A. F. Aguiar, P.A.V. Borges, et al. 2011. Detection and characterization of *Wolbachia* infections in natural populations of Aphids: is the hidden diversity fully unraveled? *PLoS ONE* 6: e28695. doi:10.1371/journal.pone.0028695.
- Baumann, P., and N. A. Moran. 1997. Non-cultivable microorganisms from symbiotic associations of insects and other hosts. *Anton. Leeuw. Int. J. G.* 72: 39–48.
- Baumann, P., L. Baumann, C. Y. Lai, D. Rouhbachsh, N. A. Moran, and M. A. Clark. 1995. Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annu. Rev. Microbiol.* 49: 55–94.

- Braendle, C., T. Miura, R. Bickel, A. W. Shingleton, S. Kambhampati, and D. L. Stern. 2003. Developmental origin and evolution of bacteriocytes in the aphid-*Buchnera* symbiosis. *PLoS Biol.* 1: e21. doi:10.1371/journal.pbio.0000021.
- Brennan, L. J., B. A. Keddie, H. R. Braig, and H. L. Harris. 2008. The endosymbiont *Wolbachia pipientis* induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. *PLoS ONE* 3: e2083. doi:10.1371/journal.pone.0002083.
- Brownlie, J. C., B. N. Cass, M. Riegler, J. J. Witsenburg, I. Iturbe-Ormaetxe, E. A. McGraw, and S. L. O'Neill. 2009. Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. *PLoS Pathog.* 5: e1000368. doi:10.1371/journal.ppat.1000368.
- Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. *J. Basic Microbiol.* 7: 198.
- Burt, A. 2000. Perspective: sex, recombination, and the efficacy of selection—was Weismann right? *Evolution* 54: 337–351.
- Charles, H., and H. Ishikawa. 1999. Physical and genetic map of the genome of *Buchnera*, the primary endosymbiont of the pea aphid *Acyrtosiphon pisum*. *J. Mol. Evol.* 48: 142–150.
- Charles, H., S. Balmand, A. Lamelas, L. Cottret, V. Pérez-Brocal, B. Burdin, A. Latorre, G. Febvay, S. Colella, F. Calevro, et al. 2011. A genomic reappraisal of symbiotic function in the aphid *Buchnera* symbiosis: reduced transporter sets and variable membrane organisations. *PLoS ONE* 6: e29096. doi:10.1371/journal.pone.0029096.
- Chen, D. Q., and A. H. Purcell. 1997. Occurrence and transmission of facultative endosymbionts in aphids. *Curr. Microbiol.* 34: 220–225.
- Chen, D. Q., B. C. Campbell, and A. H. Purcell. 1996. A new *Rickettsia* from a herbivorous insect, the pea aphid *Acyrtosiphon pisum* (Harris). *Curr. Microbiol.* 33: 123–128.
- Chen, D. Q., C. B. Montllor, and A. H. Purcell. 2000. Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrtosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. *Entomol. Exp. Appl.* 95: 315–323.
- Clancy, D. J., and A. A. Hoffmann. 1996. Cytoplasmic incompatibility in *Drosophila simulans*: evolving complexity. *Trends Ecol. Evol.* 11: 145–146.
- Darby, A. C., L. M. Birkle, S. L. Turner, and A. E. Douglas. 2001. An aphid-borne bacterium allied to the secondary symbionts of whitefly. *FEMS Microbiol. Ecol.* 36: 43–50.
- Dobson, S. L., E. J. Marsland, and W. Rattanadachakul. 2002. Mutualistic *Wolbachia* infection in *Aedes albopictus*: accelerating cytoplasmic drive. *Genetics* 160: 1087–1094.
- Douglas, A. E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. *Annu. Rev. Entomol.* 43: 17–37.
- Edgar, R. C. 2004. Muscle: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32: 1792–1797.
- Fenn, K., C. Conlon, M. Jones, M. A. Quail, N. E. Holroyd, J. Parkhill, and M. Blaxter. 2006. Phylogenetic relationships of the *Wolbachia* of nematodes and arthropods. *PLoS Pathog.* 2: e94. doi:10.1371/journal.ppat.0020094.
- Footitt, R. G., H. E. L. Maw, K. S. Pike, and R. H. Miller. 2010. The identity of *Pentalonia nigronevosa* Coquerel and *P. caladii* van der Goot (Hemiptera: Aphididae) based on molecular and morphometric analysis. *Zootaxa* 2358: 25–38.
- Francis, F., F. Guillonneau, P. Leprince, E. De Pauw, E. Haubruge, L. Jia, and F. L. Goggin. 2010. Tritrophic interactions among *Macrosiphum euphorbiae* aphids, their host plants and endosymbionts: investigation by a proteomic approach. *J. Insect Physiol.* 56: 575–585.
- Fukatsu, T., and H. Ishikawa. 1996. Phylogenetic position of yeast-like symbiont of *Hamiltonaphis styraci* (Homoptera, Aphididae) based on 18S rDNA sequence. *Insect Biochem. Mol. Biol.* 26: 383–388.
- Fukatsu, T., and N. Nikoh. 1998. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). *Appl. Environ. Microbiol.* 64: 3599–3606.
- Fukatsu, T., N. Nikoh, R. Kawai, and R. Koga. 2000. The secondary endosymbiotic bacterium of the pea aphid *Acyrtosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* 66: 2748–2758.
- Fukatsu, T., T. Tsuchida, N. Nikoh, and R. Koga. 2001. *Spiroplasma* symbiont of the pea aphid, *Acyrtosiphon pisum* (Insecta: Homoptera). *Appl. Environ. Microbiol.* 67: 1284–1291.
- Gil, R., B. Sabater-Munoz, A. Latorre, F. J. Silva, and A. Moya. 2002. Extreme genome reduction in *Buchnera* spp.: toward the minimal genome needed for symbiotic life. *Proc. Natl. Acad. Sci. U.S.A.* 99: 4454–4458.
- Goławska, S. 2010. Effect of various host-plants on the population growth and development of the pea aphid. *J. Plant Prot. Res.* 50: 224–228.
- Gómez-Valero, L., M. Soriano-Navarro, V. Pérez-Brocal, A. Heddi, A. Moya, J. M. García-Verdugo, and A. Latorre. 2004. Coexistence of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. *J. Bacteriol.* 186: 6626–6633.
- Guay, J. F., S. Boudreault, D. Michaud, and C. Cloutier. 2009. Impact of environmental stress on aphid clonal resistance to parasitoids: role of *Hamiltonella defensa* bacterial symbiosis in association with a new facultative symbiont of the pea aphid. *J. Insect Physiol.* 55: 919–926.
- Heddi, A., A.-M. Grenier, C. Khachatourian, H. Charles, and P. Nardon. 1999. Four intracellular genomes direct weevil biology: nuclear, mitochondrial, principal endosymbiont, and *Wolbachia*. *Proc. Natl. Acad. Sci. U.S.A.* 96: 6814–6819.
- Hosokawa, T., R. Koga, Y. Kikuchi, X. Y. Meng, and T. Fukatsu. 2010. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl. Acad. Sci. U.S.A.* 107: 769–774.
- Iturbe-Ormaetxe, I., G. R. Burke, M. Riegler, and S. L. O'Neill. 2005. Distribution, expression, and motif variability of ankyrin domain genes in *Wolbachia pipientis*. *J. Bacteriol.* 187: 5136–5145.
- Jones, R. T., A. Bressan, A. M. Greenwell, and N. Fierer. 2011. Bacterial communities of two parthenogenetic aphid species cocolonizing two host plants across the Hawaiian islands. *Appl. Environ. Microbiol.* 77: 8345–8349.
- Kaiwa, N., T. Hosokawa, Y. Kikuchi, N. Nikoh, X. Y. Meng, N. Kimura, M. Ito, and T. Fukatsu. 2010. Primary gut symbiont and secondary, *Sodalis*-allied symbiont of the scutellerid stinkbug *Cantao ocellatus*. *Appl. Environ. Microbiol.* 76: 3486–3494.
- Koga, R., T. Tsuchida, and T. Fukatsu. 2003. Changing partners in an obligate symbiosis: a facultative endosymbiont can compensate for loss of the essential endosymbiont *Buchnera* in an aphid. *Proc. R. Soc. B Biol. Sci.* 270: 2543–2550.
- Maewaza, K., S. Shigenobu, H. Taniguchi, T. Kubo, S. Aizawa, and M. Morioka. 2006. Hundreds of flagellar basal bodies cover the cell surface of the endosymbiotic bacterium

- Buchnera aphidicola* sp. strain APS. *J. Bacteriol.* 188: 6539–6543.
- Moran, N. A., and H. E. Dunbar. 2006. Sexual acquisition of beneficial symbionts in aphids. *Proc. Natl. Acad. Sci. U.S.A.* 103: 12803–12806.
- Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59: 695–700.
- Oliver, K. M., J. A. Russell, N. A. Morant, and M. S. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* 100: 1803–1807.
- Oliver, K. M., N. A. Moran, and M. S. Hunter. 2006. Costs and benefits of a superinfection of facultative symbionts in aphids. *Proc. R. Soc. B Biol. Sci.* 273: 1273–1280.
- Oliver, K. M., P. H. Degnan, G. R. Burke, and N. A. Moran. 2010. Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. *Annu. Rev. Entomol.* 55: 247–266.
- O'Neill, S. L., R. Giordano, A. M. Colbert, T. L. Karr, and H. M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl. Acad. Sci. U.S.A.* 89: 2699–2702.
- Pappin, D. L., P. Hojrup, and A. J. Bleasby. 1993. Rapid identification of proteins by peptide-mass fingerprinting. *Curr. Biol.* 3: 327–332.
- Poliakov, A., C. W. Russell, L. Ponnala, H. J. Hoops, Q. Sun, A. E. Douglas, and K. J. Van Wijk. 2011. Large-scale label-free quantitative proteomics of the pea aphid-*Buchnera* symbiosis. *Mol. Cell. Proteomics* 10: M110.007039. doi:10.1074/mcp.M110.007039-1-17.
- Pothavorn, P., K. Kitdamrongsont, S. Swangpol, S. Wongniam, K. Atawongsa, J. Svasti, and J. Somana. 2010. Sap phytochemical compositions of some bananas in Thailand. *J. Agric. Food Chem.* 58: 8782–8787.
- Ros, V. I., J. A. Breeuwer, and S. B. Menken. 2008. Origins of asexuality in *Bryobia* mites (Acari: Tetranychidae). *BMC Evol. Biol.* 8: 153.
- Rousset, F., D. Bouchon, B. Pintureau, P. Juchault, and M. Solignac. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proc. R. Soc. B Biol. Sci.* 250: 91–98.
- Russell, J. A., and N. A. Moran. 2006. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc. R. Soc. B Biol. Sci.* 273: 603–610.
- Sambrook, J., and D. W. Russel. 2006. Preparation of plasmid DNA by alkaline lysis with SDS: minipreparation. *Cold Spring Harb. Protoc.* 1: 1657–1669.
- Sandström, J. P., J. A. Russell, J. P. White, and N. A. Moran. 2001. Independent origins and horizontal transfer of bacterial symbionts of aphids. *Mol. Ecol.* 10: 217–228.
- Simon, J. C., S. Boutin, T. Tsuchida, R. Koga, J. F. Gallic, A. Frantz, Y. Outreman, and T. Fukatsu. 2011. Facultative symbiont infections affect aphid reproduction. *PLoS ONE* 6: e21831. doi:10.1371/journal.pone.0021831.
- Stouthamer, R., J.A.J. Breeuwer, R. F. Luck, and J. H. Werren. 1993. Molecular identification of microorganisms associated with parthenogenesis. *Nature* 361: 66–68.
- Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. Mega5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739.
- Teixeira, L., Á. Ferreira, and M. Ashburner. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biol.* 6: 2753–2763.
- Thomas, J. E., and M. L. Iskra-Caruana. 2000. Bunchy top. In D. R. Jones (ed.), *Diseases of Banana, Abacá and Enset*. CABI Publishing, Wallingford, United Kingdom.
- Thompson, J. N. 1987. Symbiont-induced speciation. *Biol. J. Linn. Soc.* 32: 385–393.
- Tram, U., P. M. Ferree, and W. Sullivan. 2003. Identification of *Wolbachia*-host interacting factors through cytological analysis. *Microbes Infect.* 5: 999–1011.
- Tsuchida, T., R. Koga, H. Shibao, T. Matsumoto, and T. Fukatsu. 2002. Diversity and geographic distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid, *Acyrtosiphon pisum*. *Mol. Ecol.* 11: 2123–2135.
- Tsuchida, T., R. Koga, and T. Fukatsu. 2004. Host plant specialization governed by facultative symbiont. *Science* 303: 1989.
- Tsuchida, T., R. Koga, X. Y. Meng, T. Matsumoto, and T. Fukatsu. 2005. Characterization of a facultative endosymbiotic bacterium of the pea aphid *Acyrtosiphon pisum*. *Microbiol. Ecol.* 49: 126–133.
- Tsuchida, T., R. Koga, M. Horikawa, T. Tsunoda, T. Maoka, S. Matsumoto, J. C. Simon, and T. Fukatsu. 2010. Symbiotic bacterium modifies aphid body color. *Science* 330: 1102–1104.
- Unlü, M., M. E. Morgan, and J. S. Minden. 1997. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. *Electrophoresis* 18: 2071–2077.
- Wang, Z., Z. R. Shen, Y. Song, H. Y. Liu, and Z. X. Li. 2009. Distribution and diversity of *Wolbachia* in different populations of the wheat aphid *Sitobion miscanthi* (Hemiptera: Aphididae) in China. *Eur. J. Entomol.* 106: 49–55.
- Weeks, A. R., M. Turelli, W. R. Harcombe, K. T. Reynolds, and A. A. Hoffmann. 2007. From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS Biol.* 5: e114. doi:10.1371/journal.pbio.0050114.
- Werren, J. H., D. Windsor, and L. Guo. 1995. Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. B Biol. Sci.* 262: 197–204.
- Wu, M., L. V. Sun, J. Vamathevan, M. Riegler, R. Deboy, J. C. Brownlie, E. A. McGraw, W. Martin, C. Esser, N. Ahmadinejad, et al. 2004. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. *PLoS Biol.* 2: e69. doi:10.1371/journal.pbio.0020069.
- Yasmin, T., S. Khalid, M. H. Soomro, S. A. Malik, H. Shah, and I. Ahmad. 2001. Specificity of host-pathogen interaction of Banana bunchy top disease. *J. Biol. Sci.* 1: 212–213.

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