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Microbiomes of the "*Candidatus* Phytoplasma solani" vectors *Hyalesthes obsoletus* Signoret isolated from different host plants

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Summary

Because of the potential impact of bacterial symbionts on the adaptation of hemipteran insects to plant hosts, we characterized the entire microbial community present in various populations of *H. obsoletus*. Insects sampled from *Lavandula* and *Salvia* were significantly smaller than those sampled from *Urtica* and *Convolvulus*. As shown by the two mtDNA markers 16SrRNA-tRNALeu-ND1 and COI-tRNALeu-COII, *Hyalesthes* specimen from *Lavandula* were genetically different from that of *Salvia* as it was the case between specimen from *Urtica* compared to specimen from *Convolvulus*. Analysis of their microbiome by deep sequencing of the amplified V4V5 variable region of the 16Sr DNA showed that insects sampled on *Lavandula* and *Salvia* possessed a specific bacterium identified by a V4V5 16srDNA sequence 100% identical to the 16SrDNA of a Bacterium of *Irenimus aequalis*.

Introduction

Hyalesthes obsoletus is one of the Cixiidae able to transmit "Candidatus *Phytoplasma solani*" to cultivated and wild plants, the latter frequently serve as sources of inoculums for crops. In France, "*Ca*. P. solani" is the agent of Bois Noir in vineyard, stolbur in solanaceous plants, and lavender decline (Boudon-Padieu 2003, 2005). *H. obsoletus* spread different strain of the "*Ca*. P. solani" belonging to three *tuf* genotypes (Langer and Maixner, 2004). In France, *H. obsoletus* can fully complete its lifecycle on *Convolvulus arvensis*, *Lavandula angustifolia* and *Urtica dioica* (Sforza *et al.*, 1999; Johannesen *et al.*, 2008). All these populations correspond to a single species despite their specialization on different host plants (Maixner, 2007). However, *H. obsoletus* populations from lavender have not been characterized yet, and a novel plant host (*Salvia sclarea*) has been found in south eastern of France (Chuche *et al.*, 2013).

Many symbionts of insects have been described so far and are thought to contribute greatly to the evolutionary and ecological success of insects in broad ecosystems. By their metabolic potential, endosymbionts help insects to feed on imbalanced food resources such as phloem sap (Moya *et al.*, 2008). For the pea aphid, host plant specialization is related both to chromosomal loci of the aphid and facultative endosymbiotic bacterium (Simon *et al.*, 2003; Leonardo and Muiru, 2003; Tsuchida *et al.*, 2004). Symbiotic bacteria can also have positive effect on pathogen transmission, vector susceptibility to natural enemies and insecticide resistance, or negative effect such as sex-ratio disturbance. Some endosymbionts have been identified in the Cixiidae (Bressan and Mulligan, 2013), and *Hyalesthes* in particular (Gonella *et al.*, 2011). But if the plant specialization has been attributed in some cases to chromosomal loci of *H. obsoletus* (Johannesen *et al.*, 2008; Kosovac *et al.*, 2013) the role of their facultative endosymbiotic bacteria on this specialization is not known yet.

Therefore, the goal of this study is to better characterize *H. obsoletus* in relation to the host plant in the light of its genetic variability and symbiont composition. The genomic variability of insects was analyzed with mitochondrial genetic (mtDNA) markers to test for differentiation among host-plant populations. We used two mtDNA markers previously analysed by Johannasen et al. (2008): 16SrRNA-tRNALeu-ND1 and COI-tRNALeu-COII. The microbiome of sap feeding vectors can easily be characterized using next generation sequencing approaches (Hail *et al.*, 2012; Jing *et al.*, 2014). Our study also aimed to provide rDNA-targeted metagenomic study of *Hyalesthes* bacteria isolated from different plant host. Due to its reproducibility and broad range, we chose sequencing the variable region V4-V5 of 16S rRNA gene (Claesson *et al.*, 2010).

Materials and Methods

1- *Hyalesthes obsoletus* sampling and observation

Insects were collected in France on *Urtica dioica*, *Salvia sclarea* and *Lavandula angustifolia*, and, from Germany on *Convolvulus arvensis* (M. Maixner).

Insect individuals (20 insects per population) were measured under a stereo microscope.

2- DNA extraction, amplification and pyrosequencing

Insect DNA was extracted as described in Maixner et al. (1995). For each plant host, DNA from twenty insects per genus was extracted, quantified using an Epoch spectrophotometer (BioTek Instruments, Inc), pooled and stored at -20°C until been used for amplification. The V4-V5 variable region of bacterial 16S rDNA was amplified using the primers V4-forward (5'-AYTGGGYDTAAAGNG) and V5-reverse (5'-CCGTCAATTYYTTTRAGTTT) (Claesson *et al.*, 2010), and sequenced using the NGS technology (Genomic and Transcriptomic Facility of Bordeaux).

3- Data processing of sequencing reads

In total, between 723190 and 1060765 paired end reads were generated for insect samples. Taxonomic identifications were performed according to the pipeline establish by USEARCH and Qiime. First, reads were preprocessed, thus sequences were quality trimmed and cleaned from remaining sequencing adapters. Overlapping pairs were assembled to create longer sequences and exactly duplicated sequences and singletons have been filtered out (USEARCH). Remaining sequences have been clustered into OTUs (Operational Taxonomy Unit) with the Bayesian classifier CROP, at a 5% dissimilarity. Chimeras were excluded by comparison with the "Gold" database from the Broad Institute. Using Qiime scripts, remaining sequences were then Blast against the GreenGenes 16S database to produce a taxonomic assignation and a global OTU table. The OTU table has been manually curated to remove chimera, duplicated OTU, and low coverage clusters.

4- Mitochondrial variability studies of *H. obsoletus*

Genomic DNA of individual insects was analyzed after sequencing the two mtDNA markers CO I-tRNA(Leu)-CO II (CO I) and 16S-tRNA(Leu)-ND1 (16S) as described by Johannesen et al. (2008).

Results

1- Morphological diversity of *H. obsoletus*

Whatever the plant host, females were systematically taller than males (Fig. 1). Females sampled on *L. angustifolia* and *S. sclarea* were about 20% smaller than the ones from *C. arvensis* and *U. dioica* and males were about 15% smaller. This clearly demonstrates that phenotypes of *H. obsoletus* vary according to of the host plant.

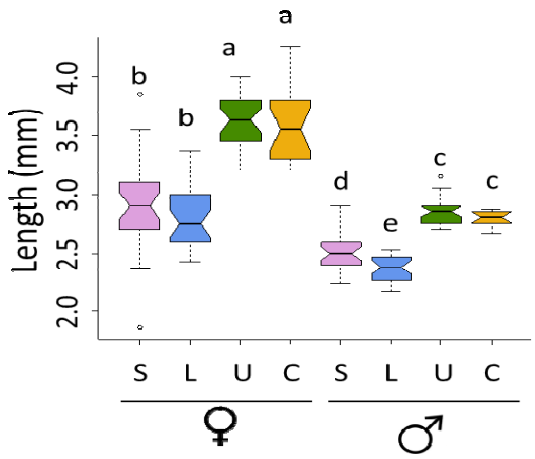

Figure 1. Length of *H. obsoletus* females and males sampled on *Salvia sclarea* (S), *Lavandula angustifolia* (L), *Urtica dioica* (U) and *Convolvulus arvensis* (C). Statistical differences were measured using the Kruskal-Wallis test (p<1%).

2- Genetic differentiation of *H. obsoletus* revealed by mtDNA polymorphism

To correlate morphological observations with genotypes of *H. obsoletus*, we analyzed genetic diversity of the four populations using mitochondrial makers. The mitochondrial haplotypes are shown in the table 1. All insects sampled from *Salvia* or *Lavandula* showed the same mtDNA haplotype per host plant. Haplotype diversity was the highest for insects sampled from *Convolvulus*.

Table 1. Genetic polymorphism of *H. obsoletus* using mtDNA markers.

Locality	Country	Host plant	Year	Number of insects	Number of mtDNA haplotypes (CO I - 16S)*			
					aa	ab	bb	new
Haut-Rhin	France	*Urtica*		10	8		1	1 (nd$^{\$}$-p)
Rhineland-Palatinate	Germany	*Convolvulus*		10			5	2 (δ-b) 2 (ε-b) 1 (ζ-b)
Alpes Hautes Provence	France	*Salvia*		14			14	
Vaucluse	France	*Lavandula*		9		9		

* CO I: CO I-tRNA(Leu)-CO II DNA region, ; 16S: ribosomal RNA (16S)-tRNA(Leu)-ND1 DNA region

$ nd: not determined

3- Identification of *H. obsoletus* associated bacteria

On average, a total of 431,815 sequences were generated from the different *H. obsoletus* populations in relation with host plants and 0.7 % of the sequences did not found homology in the sequence databanks (Greengene and BLASTn). The overall bacterial diversity was estimated between 8 and 18 genera (Figure 2).

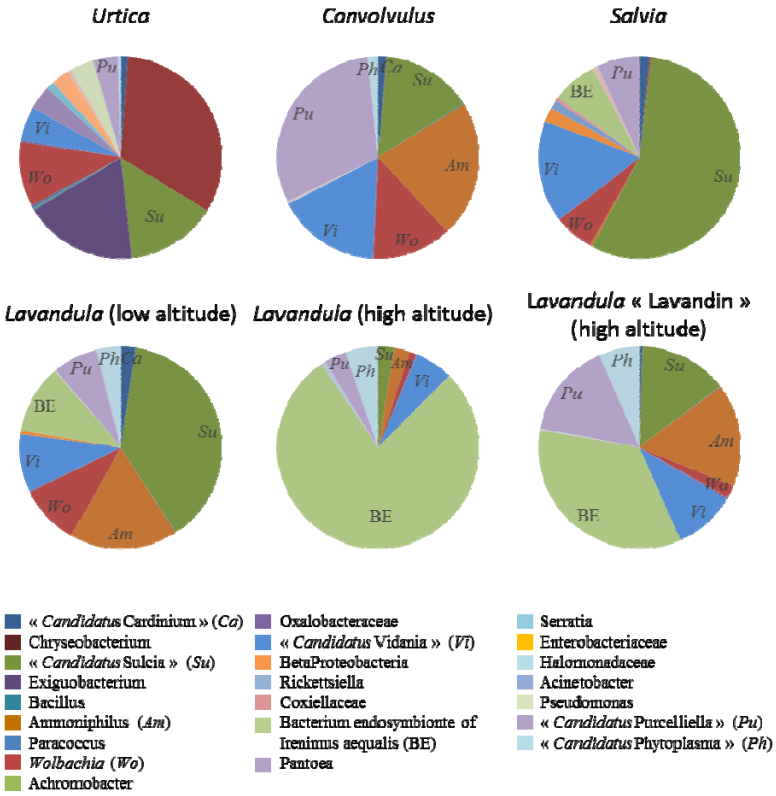

Figure 2. Relative abundance of OTUs found in *Hyalesthes obsoletus* collected on *Urtica dioica*, *Convolvulus arvensis*, *Salvia sclarea* and *Lavandula. Angustifolia* (Genera corresponding to more than 0.1% are shown).

Gamma Proteobaceria contain the higher diversity of bacterial genera among the genera associated to the OTUs. "*Ca*. Purcelliella", "*Ca*. Sulcia", "*Ca*. Vidania" and *Wolbachia* were the most dominant genus associated with *H. obsoletus* and were found in all populations studied. Figure 2 also shows that differences can be observed between host plants. In insects sampled on *Lavandula* cultivated at a high altitude the most abundant OUT was identical to the 16SrDNA of an *Irenimus aequalis* bacterium (GenBank: KJ494864.2). OTUs corresponding to this bacterium were also found in *H. obsoletus* from *Salvia*, but not in insects collected on *Urtica* and *Convolvulus*. The data also

show that we were able to detect "*Ca*. Phytoplasma" in all *H. obsoletus* populations expect in insects from *Salvia*.

Discussion

The relationships between insect phenotype/haplotype and their host plants is complex. Mitochondrial DNA analysis showed different profiles depending on the host plant and the localization. Our study confirms that *Urtica Hyalesthes* population differs slightly between years. Indeed, the population of *Urtica H. obsoletus* used in this study was sampled in 2013 from Alsace, a place closed to Turckheim from which the population studied by Johannesen was sampled in 2006 (Johannesen *et al.*, 2008). In both cases the mtDNA haplotypes were the same (aa) except for one new haplotype in our case. In the study of Johannesen and collaborators, the population of *Hyalesthes* sampled from *Urtica* in Germany showed the same haplotypes during two consecutive years. By the same time this haplotype was different from those found from *Urtica H. obsoletus* sampled in the South East of France in 2006 (Johannesen *et al.*, 2008). It seems that geographical localization rather than the host plant is responsible for their difference in mitochondrial markers. However, insects sampled in the same area but from *Salvia* or *Lavandula* showed different haplotypes.

To complete the description of *Hyalesthes* and because of the importance of symbionts during insect life, we wanted to characterize the entire microbial community present in *H. obsoletus* sampled from different host plants. The primers used to perform the DNA bank before metagenomic might not amplify all 16S rRNA gene as it is known that Chlamydia-related bacteria have a divergent 16S sequence that could be missed with consensus primer (Lienard *et al.*, 2011). However one goal of this study was to compare the bacteria associated with insects in relation with their host plant. The number of abundant OTU in these vectors vary between 7 and 11, figures in frame with the composition of the microbiome of phloem feeding insects previously estimated (<10 bacteria OTUs) (Jing *et al.*, 2014).

The conserved OTUs between all the *Hyalesthes* populations represented the bacterial genus "*Ca*. Sulcia", "*Ca*. Purcelliella", "*Ca*. Vidania". They were also found by Gonella et al. using sequencing of 16SrDNA amplified fragments that were separated by denaturing gradient gel electrophoresis (Gonella *et al.*, 2011). These bacteria are supposed to be obligate symbionts. "*Ca*. Sulcia" is one of the two symbionts associated with the Auchenorrhyncha (Hemiptera), the other been "*Ca*. Nasuia" (Bennett et al., 2014). These two symbionts kept their ability to synthetize essential amino acids that are not found in the sap of plants hosts (McCutcheon and Moran, 2010).

Wolbachia and "*Ca*. Cardinium" were found in all populations of *Hyalesthes* tested here, and were present in half of tested insects of the Gonella's study (Gonella *et al.*, 2011). *Wolbachia* and "*Ca*. Cardinium" are interesting symbionts as it has been shown that they manipulate the reproduction of their insect host (Ma *et al.*, 2014). Further studies are needed to characterize these two symbiont populations at subspecies level.

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Acknowledgement

We thank Mickael Maixner for providing gDNA of *Hyalesthes* sampled from *Convolvulus*. Part of the experiments was performed at the Genomic and Transcriptomic Facility of Bordeaux (grants from the Conseil Regional d'Aquitaine n°20030304002FA and 20040305003FA and from the European Union, FEDER n°2003227 and from Investissements d'avenir, Convention attributive d'aide N°ANR-10-EQPX-16-01). This work was funded by INRA grant from the department of Plant Health and Environment (SPE 2014) and the Structure Fédérative de Recherche 'Biologie Intégrative et Ecologie' (University of Bordeaux).