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## Endophytic Bacteria Associated to Sharpshooters (Hemiptera: Cicadellidae), Insect Vectors of *Xylella fastidiosa* subsp. *pauca*

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### Abstract

*Xylella fastidiosa* subsp. *pauca* causes citrus variegated chlorosis (CVC) disease in Brazil, resulting in significant production losses in the citrus industry. *X. fastidiosa* subsp. *pauca* is mainly transmitted by three species of sharpshooters (Hemiptera: Cicadellidae) in Brazil; *Dilobopterus costalimai* (Young), *Acrogonia citrina* Marucci & Cavichioli and *Oncometopia facialis* (Signoret). We identified bacterial communities associated with the heads of surface-sterilized insect vectors of *X. fastidiosa* subsp. *pauca* that were collected from CVC affected citrus groves in Brazil. Bacteria were isolated and analyzed by amplified ribosomal DNA restriction analysis (ARDRA) and sequencing, revealing the presence, among the most abundant genera, of the well-known citrus endophytes *Methylobacterium* spp. and *Curtobacterium* spp. Specific PCR systems for the detection of these genera indicated high frequencies of presence of these bacteria in sharpshooters. The remaining bacterial community was compared in distinct vector species and at different period of the year by denaturing gradient gel electrophoresis (DGGE), showing its responsiveness to the climate change over the year. These results represent a new basis for the knowledge about the interaction symbiotic-pathogenic bacteria inside insect vectors and provide a basis for further work on the biocontrol of plant bacteria like *X. fastidiosa*.

**Keywords:** citrus variegated chlorosis (CVC); *Curtobacterium* sp.; *Methylobacterium* sp.

### Introduction

Citrus variegated chlorosis (CVC) was first reported in Brazil in 1987 [1,2] and by 2000, the disease affected already 34% of the 200 million sweet orange trees in state of São Paulo. By 2005, the percentage had increased to 43%, and CVC was present in all citrus growing regions of Brazil [3,4]. The disease is caused by the xylem-limited Gram-negative bacterial pathogen, *Xylella fastidiosa* subsp. *pauca* [5,6].

Endophytic microorganisms live within plants without causing apparent harm to the host. We have studied the possible use of endophytes as vehicles to control both phytopathogens and insects [7-10]. Endophytes colonize ecological niches similar to that of phytopathogens [11,12]. The co-localization enables the interaction between both, pathogens and endophytes, creating the possibility to endophytes to act as biocontrol agents [13,14,15]. Biocontrol activity of the endophytic bacterial community may be through the synthesis of structural compounds, such as siderophores and extracellular enzymes [16,17], and the induction and expression of general molecular-based plant immunity [16]. Alternatively, they may act by niche competition, preventing pathogens from becoming established in a host [18].

In citrus, several endophytic bacteria were isolated and *Methylobacterium* spp. and *Curtobacterium flaccumfaciens* were further determined as the main endophytic species interacting with *X. fastidiosa* subsp. *pauca* [19,20]. These species vary in population density when CVC-affected and asymptomatic plants are compared. Later, Lacava et al. [21] reported that the growth of *X. fastidiosa* subsp. *pauca* was inhibited by endophytic *C. flaccumfaciens* and stimulated by *Methylobacterium* sp. A similar effect was demonstrated by the reduced severity of the *X. fastidiosa* subsp. *pauca* colonization in plants priorly colonized by *C. flaccumfaciens* [22].

Cicadellinae leafhoppers, commonly named sharpshooters, are xylem-feeder insects [23]. An association has been observed between

the xylem-feeding habit of sharpshooters and their ability to transmit *X. fastidiosa* [24-27]. In Brazilian citrus groves, *Dilobopterus costalimai*, *Oncometopia facialis* and *Acrogonia citrina* are the most common sharpshooters found [25-27]. After acquisition of *X. fastidiosa* by the insects, colonies of bacterial cells were visible in the cibarium and pre-cibarium of transmitting insects attached to the foregut walls [21,28] and also in insect honeydew [29]. The transmission efficiency of bacteria is a measure of the ability to successfully acquire bacteria from an infected plant and transmit to healthy ones. The transmission rates for the main species associated with CVC vary from 1% to 5% [26,30,31].

Many aspects can influence the transmission of a pathogen by an insect vector such as the low concentration of *X. fastidiosa* subsp. *pauca* cells in the citrus plants [22] and the low number of colonized vessels in infected plants [32]. The interaction between different bacteria inside the insect foregut may also influence the transmission, as once inside the foregut, bacterial interaction, such as competition for nutrients, space and other complex interactions could occur. In this context, the aims of this work were (i) to access the bacterial population associated with the main sharpshooters responsible for the transmission of *X. fastidiosa* subsp. *pauca* in citrus; (ii) to evaluate the diversity of heterotrophic bacteria by amplified ribosomal DNA restriction analysis (ARDRA);

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(iii) to compare the bacterial community presents in the insect heads from distinct species and collected from citrus at distinct period of the year by denaturing gradient gel electrophoresis (DGGE).

## Materials and Methods

### Sampled vectors of *Xylella fastidiosa* subsp. *pauca*

Insects affiliated to the species *Oncometopia facialis*, *Acrogonia citrina* and *Dilobopterus costalimai* were collected from plants of sweet orange (*Citrus sinensis* L. Osbeck), cultivated in an orange groves affected by *X. fastidiosa* subsp. *pauca* located in the city of Bebedouro (State of São Paulo, Brazil/20°56'58"S48°28'45"W). Three distinct sampling were made, at dates of March 23<sup>th</sup>, May 05<sup>th</sup> and June 14<sup>th</sup>. Insect sweep nets were used to capture the vectors, which were further transported to the laboratory [24].

### Isolation of heterotrophic bacteria from insects

At each sampling date, ten insects of each species (*O. facialis*, *A. citrina* and *D. costalimai*) were selected for bacterial isolation (total of 90 analyzed insects: 3 species x 3 dates x 10 insects). Vectors were surface sterilized by immersion in 75% ethanol for two min, transferring them to a container with sodium hypochlorite solution (2% available Cl<sup>-</sup>) for two min, and rinsing them twice in sterile double-distilled water [33]. After sterilization their heads were cut off, and triturated separately in 1 ml of saline solution (NaCl 0.85 %). An aliquot of 100 µl of this suspension and 1:10 dilutions were plated on non-selective TSB 5% medium (Tryptone Soy Broth, DIFCO) amended with agar (1.5%). Plates were incubated at 28°C for 10 days, colonies were counted, classified based on their morphology and the number of colony forming unities per insect head (CFU/insect head) was determined.

### DNA extraction from bacterial isolates

DNA was extracted from fresh bacteria cultures of 120 isolates (Insect Associated Bacterium - IAB) with the following protocol according [24]. A 1.5 ml sample of an overnight bacterial culture was centrifuged for 2 min at 12.000 X g and resuspended in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), recentrifuged, and resuspended in 500 µl of TE buffer plus 0.5 g of 0.1 mm diameter glass beads and 30 µl of 10% sodium dodecyl sulfate. The cells were homogenized for 30 seg in a bead beater (Braun cell homogenizer; B. Braun, Melsungen, Germany). A 500 µl volume of Tris-buffered phenol was then added; the solution was mixed and centrifuged for 10 min at 12.000 X g. The aqueous phase was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform-isoamyl alcohol (24:1), and the DNA was precipitated with isopropanol (1/6 actual volume) incubated for 5 min at room temperature and collected by centrifugation (10 min at 12.000 X g). The pellet was washed with 70% ethanol, air dried, and resuspended in 30 µl of double-distilled water.

### PCR for 16S rDNA amplification

The amplification of the 16S rDNA from the isolates was carried out in a 50µl reaction volume containing 1µl bacterial DNA (approx.

20 ng), 0.4 µmol<sup>-1</sup> of each primer (R1378 and PO27F, details in Table 1), 200 µmol<sup>-1</sup> of each dNTPs, 3.75 mmol<sup>-1</sup> of MgCl<sub>2</sub>, 2.5 U Taq DNA polymerase (Invitrogen, São Paulo, Brazil) in 10 mmol<sup>-1</sup> Tris-HCl (pH 8.3) and 10 mmol<sup>-1</sup> KCl. The amplification protocol consisted of an initial step at 94°C for 4 min, followed by 30 amplification cycles of 94°C for 1 min, 62°C for 1 min and 72°C for 1 min with a final extension at 72°C for 10 min. Amplification products were separated by electrophoresis by spotting 5µl of the PCR reaction mixture onto 1% agarose gel and visualized under ultra-violet light after staining with ethidium bromide (0.5 µg ml<sup>-1</sup>).

### ARDRA and ribotype selection

After the amplification of bacterial 16S rDNA from 120 bacterial isolates (40 isolates from each insect species) the amplified fragments (1360bp) were digested with the restriction enzyme *AluI* (AG/CT) (Fermentas, Vilnius, Lithuania) for 5 hours at 37°C and visualized in a 1.4% agarose gel stained with ethidium bromide (0.5 µg ml<sup>-1</sup>) under UV light. The resulting patterns were analyzed, and distinct cleavage patterns were considered as ribotypes [34].

### Sequencing of the 16S rDNA gene from the selected isolates

Later, isolate representatives of main ribotypes had their 16S rDNA genes partially sequenced and blasted against the nr/nt database at the GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>) and classified at Ribosomal Data Project (<http://rdp.cme.msu.edu/>). Also, the phylogeny of obtained sequences was reconstructed in a tree composed by Insect Associated Bacteria 16S rDNA sequences, sequences from type strains of the genera *Methylobacterium* and *Curtobacterium* (obtained from Ribosomal Data Project database), and sequences from previously isolated citrus endophytic bacteria from these genera [17]. Alignment and clustering were performed by Kimura-2 parameter and Neighbour-joining methodology. The phylogenetic and molecular analyses were conducted using MEGA version 4.1 [35], where 1000 replication of bootstrap analyses were performed.

### DNA extraction from insect heads

After surface sterilization the heads of insects were cut off, frozen with liquid nitrogen and triturated, separately, in 1 ml of extraction buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0, SDS 10%). The total DNA extraction was carried out according to the bacterial DNA extraction protocol [33]. From each insect species, DNA from 15 heads was extracted at each sampling date.

### Detection of *Methylobacterium mesophilicum* and *Curtobacterium flaccumfaciens* by specific PCR

DNA extracted from insect heads was tested for the presence of *M. mesophilicum* and *C. flaccumfaciens*, the most important endophytes associated with *X. fastidiosa* subsp. *pauca*, by using specific primers [36,37]. Due to a limitation in the amount of bacterial DNA from one single insect head, a nested approach was applied. First, universal bacterial 16S rDNA PCR amplification, using primers PO27F and

Primer	Target DNA	Sequence (5'- 3')	Reference
MMV6	<i>Methylobacterium mesophilicum</i> 16SrDNA	ACGTGGAGAGATTACGGG	Rossetto et al., 2000
CF16S	<i>Curtobacterium flaccumfaciens</i> 16SrDNA	ATCAGGAGCTTGCTCCTGTGA	Rossetto et al., 2000
530R	16S rDNA	CCGCGGCKGCTGGCAC	Lane, 1985
R1378	Bacterial 16S rDNA	CGGTGTGTACAAGCCCCGGGAACG	Heuer et al., 1997
U968-GC	Bacterial 16S rDNA	CGCCCCGGGCGCGCCCCGGGCGGGGGCACGGGGGAACCGAAGAACCTTAC	Heuer et al., 1997
PO27F	Bacterial 16S rDNA	GAGAGTTTGTATCTGGCTCAG	Lane, 1985

Table 1: Primers used in this study, target organism and sequence.

Sharpshooter species	Ribotype	Isolate	GenBank accession numbers	Best Blast match (similarity)	RDP Classification (taxonomic level)	
<i>O. facialis</i>	I	IAB68	GU573523	<i>Methylobacterium radiotolerans</i> strain P (99%)	<i>Methylobacterium radiotolerans</i> AY616142 (97%)	
		IAB47	GU573507	<i>Methylobacterium</i> sp. P11E (94%)	<i>Methylobacterium</i> sp. D32231 (86%)	
		IAB73	GU573526	<i>Methylobacterium</i> sp. PB183 (97%)	<i>Methylobacterium</i> sp. D32231 (96%)	
		IAB35	GU573498	<i>Methylobacterium</i> sp. C1FA3 (98%)	Uncultured <i>Methylobacteriaceae</i> AY360526 (95%)	
		IAB36	GU573499	<i>Methylobacterium</i> sp. C1FA3 (98%)	<i>Methylobacterium</i> sp. D32231 (97%)	
		IAB76	GU573529	<i>Methylobacterium</i> sp. C1FA3 (99%)	<i>Methylobacterium</i> sp. D32231 (98%)	
		IAB77	GU573530	<i>Methylobacterium</i> sp. C1FA3 (99%)	<i>Methylobacterium</i> sp. D32231 (100%)	
		IAB90	GU573538	<i>M. radiotolerans</i> strain P (98%)	<i>M. radiotolerans</i> AY616142 (97%)	
		II	IAB69	GU573524	<i>C.luteum</i> 1P10AnD (98%)	<i>C. luteum</i> X77437 (92%)
			IAB74	GU573527	<i>C. flaccumfaciens</i> SMW-8 (99%)	<i>C. luteum</i> X77437 (97%)
	IAB34		GU573497	<i>C. luteum</i> 1P10AnD (96%)	<i>Curtobacterium</i> sp. AB046364 (87%)	
	IAB75		GU573528	<i>C. flaccumfaciens</i> pv. <i>basellae</i> (97%)	<i>Curtobacterium</i> sp. AB046364 (91%)	
	IAB79		GU573531	<i>C. citreum</i> LCR27 (98%)	<i>Curtobacterium</i> sp. AY688357 (97%)	
	IAB80		GU573532	<i>Curtobacterium</i> sp. LCR25 (98%)	<i>Curtobacterium</i> sp. AB046364 (92%)	
	IAB41		GU573502	<i>C. flaccumfaciens</i> SMW-8 (97%)	<i>C. luteum</i> X77437 (93%)	
	IAB42		GU573503	<i>C. citreum</i> LCR27 (97%)	<i>Curtobacterium</i> sp. AY688357 (91%)	
	IAB83		GU573534	<i>C. luteum</i> 1P10AnD (98%)	<i>Curtobacterium</i> sp. AB046364 (93%)	
	IAB70		GU573525	<i>C.luteum</i> 1P10AnD (98%)	<i>Curtobacterium</i> sp. AB046364 (94%)	
	Others	IAB33	GU573496	<i>C. luteum</i> X77437 (97%)	<i>C. luteum</i> X77437 (93%)	
		IAB91	GU57353	<i>Brevibacillus brevis</i> NBRC 100599 (98%)	<i>Brevibacillus agri</i> AB112716 (95%)	
IAB48		GU573508	<i>Sphingomonas phyllosphaerae</i> FA1 (97%)	<i>Sphingomonas phyllosphaerae</i> AY453855 (94%)		
<i>A. citrina</i>		II	IAB63	GU573520	<i>C.luteum</i> 1P10AnD (98%)	<i>C. flaccumfaciens</i> EF063717 (92%)
			IAB85	GU573535	<i>C.luteum</i> 1P10AnD (97%)	<i>Curtobacterium</i> sp. AB046364 (95%)
		Others	IAB53	GU573513	<i>Bacillus bataviensis</i> PBA (98%)	<i>Bacillus</i> sp. DQ275174 (93%)
			IAB56	GU573515	<i>B. megaterium</i> DQ447778 (98%)	<i>Bacillus</i> sp. EU276114 (96%)
Others		IAB58	GU573516	<i>Brevundimonas</i> sp SB4 (97%)	<i>Brevundimonas</i> sp. DQ981459 (94%)	
	IAB46	GU573506	<i>B. firmus</i> SK20 (97%)	<i>Bacillus firmus</i> AJ717384 (93%)		
	IAB87	GU573537	<i>Brachybacterium paraconglomeratum</i> 13636D (95%)	<i>Brachybacterium paraconglomeratum</i> AB098579 (89%)		
	IAB86	GU573536	<i>Brachybacterium phenoliresistens</i> phenol-A (99%)	<i>Brachybacterium</i> sp. EU249983 (96%)		
	IAB62	GU573519	<i>Staphylococcus pasteurii</i> BCCS 018 (99%)	<i>Staphylococcus</i> sp. AJ276810 (95%)		
	IAB59	GU573517	<i>Pseudoclavibacter helvolus</i> BH16SR (97%)	<i>Pseudoclavibacter helvolus</i> X77440 (88%)		
<i>D. costalimai</i>	Others	IAB50	GU573510	<i>Nocardia corynebacterioides</i> NRRL 21057 (97%)	<i>Nocardia beijingensis</i> AB094641 (91%)	
		IAB51	GU573511	<i>Rhodococcus</i> sp. lxb-6 (96%)	<i>Rhodococcus</i> sp. AJ002093 (89%)	
		IAB45	GU573505	<i>Bacillus firmus</i> UST981101-007 (98%)	<i>Bacillus firmus</i> AJ717383 (95%)	
		IAB81	GU573533	<i>Paenibacillus ginsengagri</i> (97%)	<i>Paenibacillus</i> sp. AF395029 (96%)	

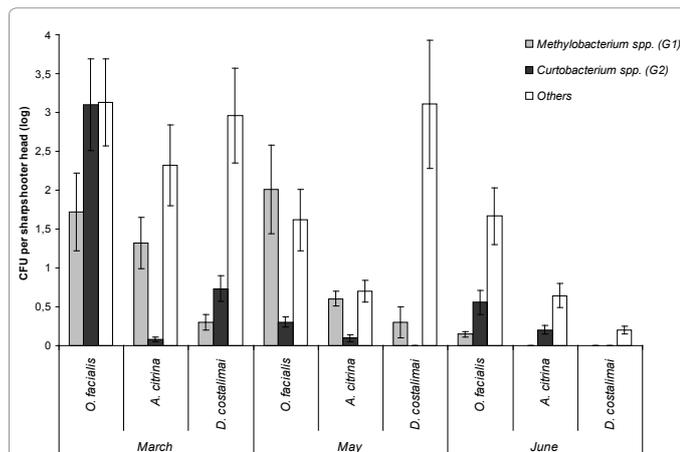
**Table 2:** Identification of distinct ribotypes obtained from the cleavage patterns of the 16S rDNA gene from bacterial isolates from sharpshooter (Insect Associated Bacteria). Sequences classifications were made by closest Blast match and by classification at Ribosomal Data Project.

Analyzed parameters	Sharpshooter species		
	<i>O. facialis</i>	<i>A. citrina</i>	<i>D. costalimai</i>
Frequency of <i>Methylobacterium</i> spp. isolation (G1) <sup>1</sup>	47	8	60.4
Frequency of <i>Curtobacterium</i> spp. isolation (G2) <sup>1</sup>	450	0.36	1.6
Percentage of positive specific PCR for <i>Methylobacterium</i> spp. <sup>2</sup>	51.7%	20.0%	8.7%
Percentage of positive specific PCR for <i>Curtobacterium</i> spp. <sup>2</sup>	89.6%	70.0%	39.1%
Number of isolates with ribotype I ( <i>Methylobacterium</i> spp.) (out of 120)	10	1	0
Number of isolates with ribotype II ( <i>Curtobacterium</i> spp.) (out of 120)	32	6	0
Transmission efficiency of Xfp (according to Krüchner et al., 2000)	1%	2%	5%

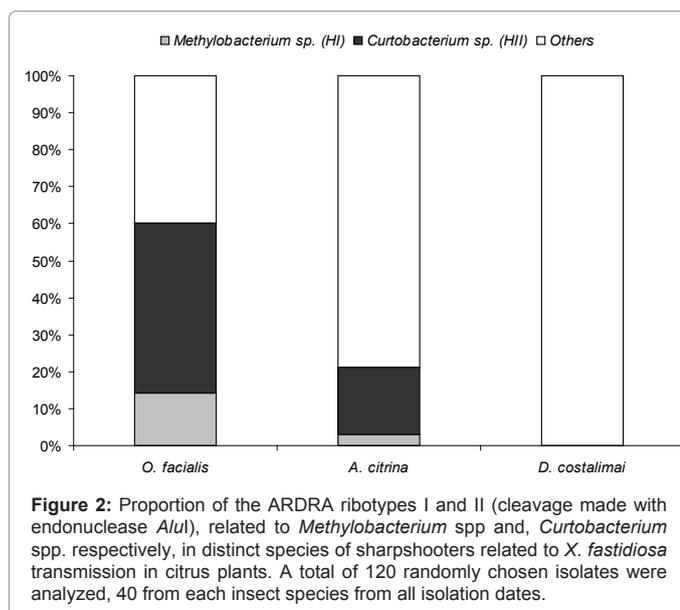
<sup>1</sup>Average of the number of bacteria isolated from each insect species in all sampling dates. CFU/head.

<sup>2</sup>Percentage of positive specific PCR for the detection of *Curtobacterium* or *Methylobacterium* using specific primers in a nested PCR. To each insect species 15 individuals were tested from each sampling date resulting in 45 insects tested.

**Table 3:** A comparative analysis of the *Curtobacterium* spp. and *Methylobacterium* spp. isolation and the efficiency of the sharpshooter species in transmitting *Xylella fastidiosa* subsp. *pauca* (Xfp), previously determined (Krüchner et al., 2000).



**Figure 1:** Abundance of heterotrophic bacteria, obtained in TSB (5% of recommended concentration) solid medium in distinct samplings and species. Due to the easy recognition of *Curtobacterium* and *Methylobacterium* affiliated colonies, they were separately quantified, besides the total number of colonies in each plate. Bars indicate the standard error of the average.



**Figure 2:** Proportion of the ARDRA ribotypes I and II (cleavage made with endonuclease *AluI*), related to *Methylobacterium* spp and *Curtobacterium* spp. respectively, in distinct species of sharpshooters related to *X. fastidiosa* transmission in citrus plants. A total of 120 randomly chosen isolates were analyzed, 40 from each insect species from all isolation dates.

R1378 (Table 1), was performed as described below. Second, two microliters of the first reaction was re-amplified in a nested PCR performed with primers MMV6 and R1378 (Table 1), which generated a product of 300bp when DNA from *M. mesophilicum* is present. Similarly, the detection of *C. flaccumfaciens* was made with primers CF16S and 530R (Table 1), which generated a product of 400bp. The PCR reactions consisted of an initial denaturation step of 94°C for 7 min, followed by 25 cycles of 94°C for 1 min, 56°C and 62°C (for *M. mesophilicum* and *C. flaccumfaciens* respectively) for 1 min, and 72°C for 1 min and a final extension of 10 min at 72°C. The percentage of positive results was determined.

### Assessment of the bacterial community in sharpshooter heads by DGGE

The total DNA extracted from insect heads was subjected to DGGE analysis. A nested approach was applied as previously described for specific PCR procedures. First amplification was performed using primers PO27F and R1378 (Table 1). The second amplification was

made with DGGE primers R1378 and U968GC (Table 1) as previously described [38]. A PCR mixture was made up of 1 µl of extracted DNA (approx. 5 ng) from the insect heads, 5 µl of 10X Stoffel buffer (10 mM Tris-HCl [pH 8.3], 10 mM KCl), 20 pmol of each primer (Table 1), 200 µmol of each deoxynucleoside triphosphate, 3.75 mM MgCl<sub>2</sub>, 0.5 µl of formamide and 2.5 U of Taq DNA polymerase Invitrogen (Carlsbad, CA, USA) in a 50 µl final volume. For the amplification protocol, denaturation was carried out at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension of 10 min at 72°C. Five microliters of the PCR product was analyzed by electrophoresis in a 1.4% agarose gel with 0.5X TBE buffer before running the DGGE gel.

DGGE was performed as previously described [33,39] using the INGENY phor U2 apparatus (INGENY, Goes, The Netherlands). PCR samples were loaded onto 6.0% polyacrylamide gels in 0.5X TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA, pH 7.4). The polyacrylamide gels were made with denaturing gradients ranging from 45 to 65% (where the 100% denaturant contained 7 M urea and 40% formamide). The gels were run for 16 h at 100 V and 60°C, after which the gels were soaked for 30 min in SYBR Green I nucleic acid stain (1:10,000 dilution; Molecular Probes, Leiden, The Netherlands) and immediately photographed under UV light.

In order to compare the DGGE fingerprinting, gels were submitted to analysis by the software GelcomparII (Applied Maths, Kortrijk, Belgium), where images were converted, normalized and band patterns were compared. Cluster analysis of DGGE patterns was performed using the UPGMA method (unweighted pair group method with arithmetic mean) based on the similarity calculated by densitometric Pearson correlation.

## Results

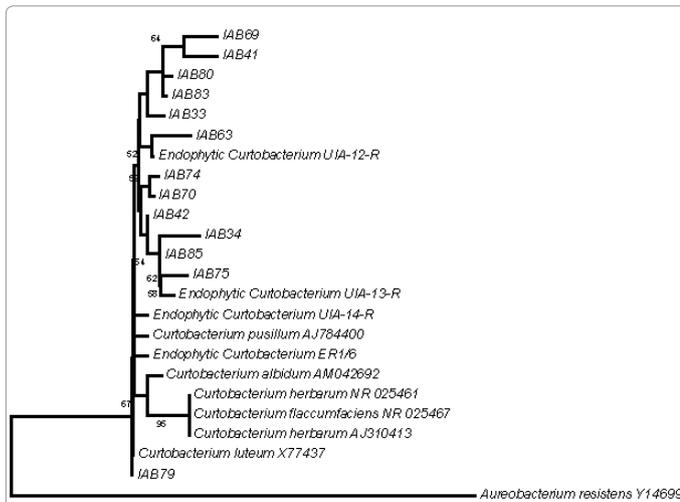
### Isolation of cultivable bacteria from insects

A total of 17,230 bacterial colonies were counted in all isolations from different species and at distinct sampling time. These colonies were classified into three distinct morphological groups; G1) pink-pigmented colonies which appear on plates after seven days of incubations, typically *Methylobacterium* spp.; G2) dark pink or orange fast grower colonies, typically *Curtobacterium* spp.; G3) other colonies. The concentration of bacteria (CFU/insect head) belonging to each morphological group is represented in (Figure 1).

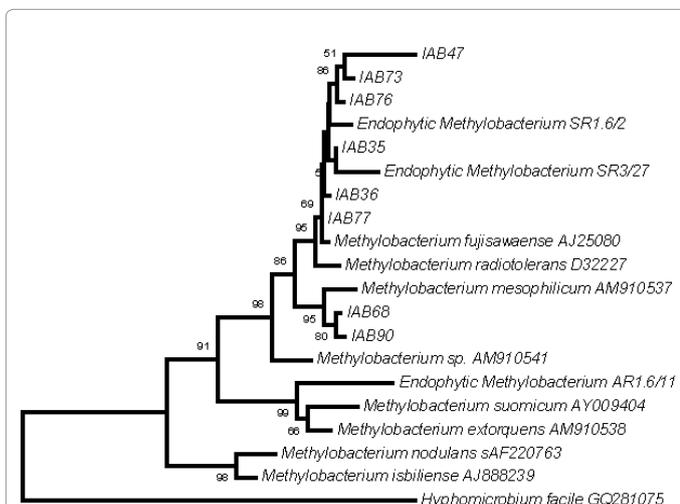
The results show a differential composition of the heterotrophic bacterial communities according to the sharpshooter species and depending on the month of sampling. Abundance of total bacteria was higher from samples taken in March compared to numbers from May and June. Also specific groups varied in frequency along the period of samplings. The populations of group G1 (*Methylobacterium*) remained constant for *O. facialis* and *D. costalimai* in March and May, decreasing in June. From *A. citrina* the presence of G1 bacteria decreased from May. Group 2 (*Curtobacterium*) was higher in March for *O. facialis* and *D. costalimai*, decreased in May and was undetected in *D. costalimai* in June. The population of *Curtobacterium* spp. in *A. citrina* remained constant during the period of analysis (Figure 1).

### ARDRA and sequencing

A subsample of the total number of colonies obtained (120 colonies, 40 isolated from each insect species) was subjected to the genotypic characterization by ARDRA. In total, 16 cleavage patterns were observed, determining the ribotypes constituting the heterotrophic bacterial communities from sharpshooter heads.



**Figure 3:** Neighbor-joining clustering among sharpshooters (Insect Associated Bacteria –IAB) and plant associated bacteria sequences with types strains of *Curtobacterium* spp. A partial fragment of the 16SrDNA gen was used for comparisons. Besides the insect-associated and endophytic sequences, the tree encompasses sequences from type strains of each species, represented by an average of 650 bases. The sequence from *Aureobacterium resistens* is presented as out-group. Also, a bootstrap analysis was performed with 1000 repetitions and values indicate the percentage of clustering matching. The bar in the bottom of the figure scales the number of differences in base composition amongst sequences.



**Figure 4:** Neighbor-joining clustering among sharpshooters (Insect Associated Bacteria-IAB) and plant associated bacteria sequences with types strains of *Methylobacterium* spp. A partial fragment of the 16SrDNA gen was used for comparisons. Besides the insect-associated and endophytic comparisons, the tree encompasses sequences from type strains of each species, represented by an average of 650 bases. The sequence from *Hyphomicrobium facile* is presented as out-group. Also, a bootstrap analysis was performed with 1000 repetitions and values indicate the percentage of clustering matching. The bar in the bottom of the figure scales the number of differences in base composition amongst sequences.

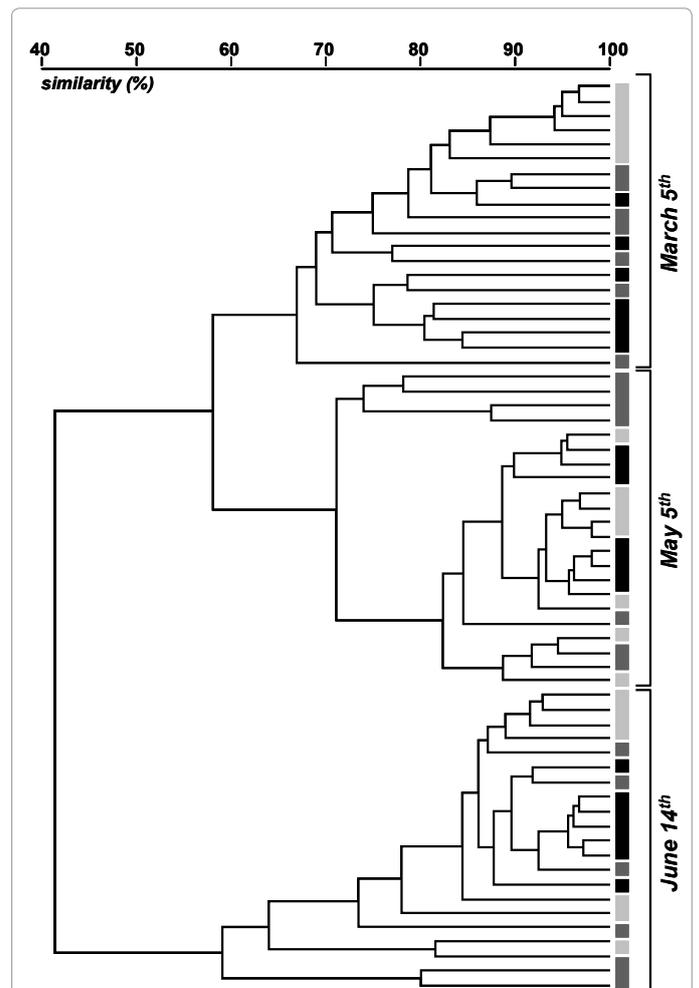
Among these ribotypes, the colonies from the two targeted groups, *Curtobacterium* spp. and *Methylobacterium* spp., has revealed a fidelity in the pattern of the 16S rDNA gene cleavage, allowing this approach to measure the proportion of these bacteria within the sampled colonies

(Figure 2). Other bacterial haplotypes were characterized randomly and sequences have shown the affiliation of the isolates to the genera *Bacillus*, *Brachybacterium*, *Brevibacillus*, *Brevundimonas*, *Nocardia*, *Paenibacillus*, *Pseudoclavibacter*, *Rhodococcus*, *Sphingomonas* and *Staphylococcus* (Table 2).

Correlating the number of ribotypes found in colonies from each insect species, we observed a higher occurrence of the ribotype II (*Curtobacterium* spp.) in species *O. facialis* and *A. citrina*, and a similar trend but in lower number for the occurrence of *Methylobacterium* spp. (ribotype I) (Figure 2).

### Confirmation of the *Curtobacterium* spp. and *Methylobacterium* spp. colonization by specific PCR

DNA samples directly extracted from insect heads were used for specific amplification of DNA from bacteria affiliated with the genera *Curtobacterium* and *Methylobacterium*. DNA from 15 heads of each insect species was extracted at each sampling date. The number of positive samples was unique to each sharpshooter species. The detection of DNA from *Curtobacterium* spp. was positive in 89.6% of the samples from *O. facialis*, 39.1% of *D. costalimai* and 70% of *A.*



**Figure 5:** Clustering of DGGE fingerprinting generated by UPGMA based on the similarity calculated by the Pearson correlation. Sharpshooters species are represented by dark gray bars (*Oncometopia facialis*), light gray (*Acrogonia citrina*) and black bars (*Dilobopterus costalimai*). Sampling dates are indicated on the vertical axis.

*citrina*. In a similar analysis for *Methylobacterium* spp. the numbers of positive PCR were 51.7% for *O. facialis*, 8.7% for *D. costalimai* and 20% of the *A. citrina*. The results from isolation and ARDRA ribotyping are presented in comparison to previous work in the (Table 3).

### Phylogeny of sharpshooter isolates and similarity to citrus-endophytic bacteria

Another point found in this work was the comparative phylogeny of isolates from both targeted groups (Insect Associated Bacteria - IAB) with endophytic isolates obtained in previous works. UIA-12-R, UIA-13-R, UIA-14-R and ER1.6 are 16S rDNA sequences from *Curtobacterium flaccumfaciens* and AR1.6/11, SR1.6/2 and SR3/27 are from *Methylobacterium* species previously isolated from citrus [17]. One can observe the clustering of IAB isolated in this work with endophytic strains isolated previously from citrus trees, more specifically *C. flaccumfaciens* strain UIA-12-R (Figure 3) and *Methylobacterium* strains SR1.6/2 and SR3/27 (Figure 4). In general, bacteria isolated in this study clustered together.

### Analysis of the bacterial communities of sharpshooter heads by DGGE

The DGGE analysis showed variable fingerprinting according to the period when insects were collected. The first separation was observed for samples collected in June, with a further sub-branching for samples from May and March (Figure 5). These variations were based on changes in the abundance of bands and also in the intensity of similar bands found in distinct samples. Also, within each major branching it is possible to separate the DGGE pattern according to the vector species, with a more deterministic separation for the species *A. citrina* (Figure 5). For other species, *D. costalimai* and *O. facialis*, the species-selection was lower, as evidenced by the mixing of samples from these species of sharpshooters.

### Discussion

The role of associated microbial community in the pathogenic potential of *X. fastidiosa* has been discussed in the literature [12,14,17,18,37], where authors state that these microorganisms can partially inhibit or promote the growth of *X. fastidiosa* inside the citrus plants. However, there was a lack of knowledge about the occurrence and frequency of endophytic bacteria commonly found inside the insect vectors, which transmit pathogens from plant-to-plant. In order to fill this gap this study was conducted, using three insect species described as *X. fastidiosa* vectors, generating the first insights in this important aspect, allowing further focused studies on the biological control or symbiotic control of this pathogen in citrus groves. We focused our approaches in detecting and quantifying two major citrus endophytes in the selected insects. However, a culture-independent approach was also used to describe changes in bacterial communities associated with these vectors.

Differences in bacterial communities associated with insects suggest complex interactions between insects, host plants, bacteria and environment. In March, when the temperatures were around 25°C and the precipitation 160 mm the bacterial community was denser with a higher number of bacterial groups, when compared to the months of May and June when the temperatures and the precipitation were lower (20°C, 15°C and 80 mm, 60mm respectively). Each insect contained an associated bacterial community that was altered in time and in different environmental conditions. Climate shifts affect not only the bacterial community in plants but also the behavior of insects [31], which choose alternative host plants during different periods of the

year, and host plant conditions (citrus or adjacent ones), which change depending on temperature and precipitation on the area.

The DGGE technique has been used to study bacterial communities [39]. Reeson et al. [40] described the bacterial communities associated with wasps (*Vespula germanica*) by applying DGGE-based methods as well as Lacava et al. [33] who used DGGE to characterize microbe in glassy-winged sharpshooter (GWSS), the main insect vector of *X. fastidiosa* subsp. *piercei* [6] causing Pierce's disease (PD) in the USA [22]. Lacava et al. [33] found *M. extorquens* and *C. flaccumfaciens* as part of the bacterial community of GWSS in the USA. In addition, Kirkpatrick and Wilhelm [41] have also isolated strains of *C. flaccumfaciens* as part of endophytic bacterial community of grapevine in California, USA. Also, *C. flaccumfaciens* was consistently isolated as an endophytic bacterium from citrus plants in Brazil [17,18].

In the present study, *Curtobacterium* sp. was the most abundant bacteria colonizing insect heads. *Curtobacterium flaccumfaciens* was implicated in playing an important role in the prevention of CVC symptoms in citrus trees [17,18,33]. The presence of the citrus endophyte, *Curtobacterium* sp., colonizing the insect heads could explain why the transmission efficiency of *X. fastidiosa* subsp. *pauca* by vectors is low (5 to 10%), when compared to the transmission of *X. fastidiosa* subsp. *fastidiosa* by GWSS, which cause Pierce's disease in grapevines (45%) [26,31]. Table 3 illustrates that the genus *Curtobacterium*, can play an important role in the transmission of *X. fastidiosa* subsp. *pauca*. The presence of this *Curtobacterium* has been cited in several works where the interaction of these bacteria with *X. fastidiosa* had been studied. High population of *Curtobacterium* were observed in asymptomatic citrus plant [19,20], A less severe colonization of plants by *X. fastidiosa* was observed in plants previously colonized by *C. flaccumfaciens* [22] and in this study, a high population of *Curtobacterium* was observed associated to insects with lower transmission rate of *X. fastidiosa*. These observations show a colonization pattern what suggest an interaction between both species where *Curtobacterium* would affect negatively the growth of *X. fastidiosa* and so the transmission and development of CVC symptoms. *Curtobacterium* could be influencing pathogen adhesion to the vector foregut or inhibiting growth of the pathogen.

The bacterial communities associated with vector insects and plants differ in abundance through the yearly season. Endophytic bacteria could influence disease development by reducing the insect transmission efficiency due to competition with pathogens in host plants and also in insect foreguts. In addition the bacterial communities in the foregut of insect vectors of *X. fastidiosa* subsp. *pauca* changed with time, environmental conditions and in different insect species. However, members of the genus *Curtobacterium* were consistently detected in the sharpshooters foregut and are commonly isolated from the xylem of citrus plants [17], and because of this, they may be candidates for biological control.

In a recent review, Lacava et al. [15] suggested *C. flaccumfaciens* is a potential candidate for biological control of CVC because an antagonism between *C. flaccumfaciens* and *X. fastidiosa* subsp. *pauca* was strongly indicated *in vitro* and *in vivo* [17,18,33] including inhibition of growth of *X. fastidiosa* subsp. *pauca* and reduced severity of the disease symptoms in the presence of this phytopathogen. The ability demonstrated by *C. flaccumfaciens* to colonize plant tissues in the presence of *X. fastidiosa* subsp. *pauca* and the reduction of disease symptoms caused by *X. fastidiosa* subsp. *pauca* [19] are prerequisites for the use of this endophytic bacterium as a biocontrol agent. Since members of the genus *Curtobacterium* were consistently detected in *Acrogonia citrina* Marucci & Cavichioli and *Oncometopia facialis*

(Signoret), two of the main insect vectors of *X. fastidiosa* subsp. *pauca* as demonstrated in the present study, they fulfill another requirement of candidates for biological control of *X. fastidiosa* subsp. *pauca* [19], i.e. they can colonize both the insect vectors of *X. fastidiosa* subsp. *pauca* and citrus plants.

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