


Detection and localization of *Solitalea*-like and *Cardinium* bacteria in three *Acarus siro* populations (Astigmata: Acaridae)

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Abstract Bacteria associated with mites influence their fitness, nutrition and reproduction. Previously, we found *Solitalea*-like (*Sphingobacteriales*) and *Candidatus Cardinium* (*Cytophagales*) bacteria in the stored product mite *Acarus siro* L. by cloning and using pyrosequencing. In this study, taxon-specific primers targeting 16S rRNA gene were used to detect and quantify the bacteria in mites and eggs of three *A. siro* populations. The specific probes for fluorescent in situ hybridization (FISH) were used to localize *Solitalea*-like and *Cardinium* bacteria in mite bodies. The population growth as an indirect estimator of fitness was used to describe the mite-bacteria interactions on (1) control diet; (2) rifampicin supplemented diet; (3) tetracycline supplemented diet; (4) rifampicin pretreated mites; (5) tetracycline pretreated mites. *Solitalea*-like 16S rRNA gene sequences from *A. siro* formed a separate cluster together with sequences from *Tyrophagus putrescentiae*. qPCR analysis indicated that number of *Solitalea*-like bacteria 16S rRNA gene copies was ca. 100× higher than that of *Cardinium* and the numbers differed between populations. FISH analysis localized *Solitalea*-like bacteria in the parenchymal tissues, mesodeum and food bolus of larvae, nymphs and adults. *Solitalea*-like, but not *Cardinium* bacteria were detected by taxon-specific primers in mites and eggs of all three investigated populations. None of the antibiotic treatments eliminated *Solitalea*-like bacteria in the *A. siro* populations tested. Rifampicin pretreatment significantly decreased the population growth. The numbers of *Solitalea*-like bacteria did not correlate with the population growth as a fitness indicator. This study demonstrated that *A. siro* can host *Solitalea*-like bacteria either alone or together with *Cardinium*. We suggest that *Solitalea*-like bacteria are shared by vertical transfer in *A. siro* populations.

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Introduction

Acarus siro L. is a cosmopolitan mite species and one of the most common pests of stored grains and flour (Palyvos et al. 2008; Athanassiou et al. 2011). *Acarus siro* is found also in stored cheese and meat products (McClymont-Peace 1983; Armentia et al. 1994; Melnyk et al. 2010). The importance of *A. siro*, as well as other stored product mites, is in contamination of stored food and human environment with their bodies, excretory products and substances of allergenic potential with risks to consumers (Marx et al. 1993; Sanchez-Borges et al. 2009, 2013) and workers in food production, e.g. farmers (Musken et al. 2000) and bakers (Armentia et al. 1997). *Acarus siro* transfers microorganisms on the body surface or in the mite bodies (Hubert et al. 2004, 2012, 2016; Erban et al. 2016b).

Except of gut-associated bacteria, the mites host intracellular symbiotic or parasitic bacteria, e.g. *Candidatus Cardinium* (*Bacteroidetes*, *Cytophagales*, *Amoebophilaceae*) and *Wolbachia* spp. (*Alphaproteobacteria*, *Rickettsiales*, *Anaplasmataceae*). *Cand. Cardinium* was originally described as a manipulator of reproduction in insects and mites (Nakamura et al. 2012). However, the underlying mechanisms of cytoplasmic incompatibility (in parasitoid wasps, planthoppers, phytoseiids and spider mites) or feminization (in false spider mites) are still unknown. With exception of one diplodiploid insect host, a planthopper (Nakamura et al. 2012), host reproductive manipulations have been described only in haplodiploid and parahaploid hosts (Weeks et al. 2001; Zchori-Fein et al. 2004; Breeuwer et al. 2011; Wu and Hoy 2012a). However, no relevant observation was reported in diplodiploid Acari. The ultrastructure observations repeatedly showed *Cardinium* bacteria living in almost every single tissue of their insect, proturan, mite and tick hosts. Therefore, *Cardinium* is not exclusively associated to the reproductive tissues (Hess and Hoy 1982; Kurtti et al. 1996; Bigliardi et al. 2006; Kitajima et al. 2007; Sacchi et al. 2008; Dallai et al. 2011). Though *Cardinium* endosymbionts are prevalent in arthropods including several diplodiploid members of *Chelicerata*, no reproductive manipulations were uncovered so far (Chang et al. 2010; Perlman et al. 2010; Stefanini and Duron 2012; Kopecky et al. 2013). Interestingly, a relevant role in host nutrition was suggested by analysis of the *Cardinium* genome (Penz et al. 2012). For example, *C. hertigii* (strain cEper1) may provide biotin for insect growth and metamorphosis that cannot be synthesized by its insect host, the parasitoid wasp *Encarsia pergandiella* (Penz et al. 2012). Santos-Garcia et al. (2014) described the genome of *Cardinium* strain C1 from *Bemisia tabaci* (cBtQ1) containing a plasmid that carries a unique combination of four gliding genes *gldK*, *gldL*, *gldM* and *gldN*. It has shed some light on behavior of several *Bacteroidetes* species, which spread within different host tissues (Santos-Garcia et al. 2014). *Cardinium* ubiquity within chelicerates might be justified by a long-term interaction (Stefanini and Duron 2012; Duron 2013) due to a similar, predominantly nutritional role in diplodiploid chelicerates (Fukuzawa et al. 2008).

In the previous screening of cloned 16S rRNA gene sequences of *A. siro*, *Lepidoglyphus destructor*, *T. putrescentiae* and *Dermatophagoides farinae* from laboratory populations of Crop Research Institute, Prague, Czechia, the sequences of *Bacteroidetes* were found in *D. farinae* and *A. siro* (Hubert et al. 2012). In *A. siro*, they represented 34 % (20/170) of the

cloned sequences. The phylogenetic analyses of cloned 16S rRNA gene sequences indicated two clusters (1) ‘*Candidatus Cardinium*’ in *D. farinae* and (2) *Solitalea*-like clade within *Sphingobacteriales* in *A. siro* (Hubert et al. 2012). The genus *Solitalea* comprises free-living species found in soil samples (Weon et al. 2009). In another experiment, both *Cardinium* and *Solitalea* were found in the cloned universal bacterial 16S rRNA gene amplicon from the laboratory population of *A. siro* (Kopecky et al. 2014). The presence of *Cardinium* in this laboratory population and 12 other species of astigmatid mites was confirmed using *Cardinium* specific primers (Kopecky et al. 2013). Further, the pyrosequencing analysis of *A. siro* bacteriome showed that the bacteriome is formed by two dominant groups, i.e. *Bartonella*-like and *Solitalea*-like bacteria covering almost 75 and 25 % of sequences, respectively (Hubert et al. 2016). Additionally, *Solitalea*-like symbionts were identified in the cloned amplicons of bacterial 16S rRNA primers from Phillips and Dog populations of *T. putrescentiae* (Erban et al. 2016a). Using the taxon-specific primers, *Solitalea*-like bacteria were found in four populations of *T. putrescentiae* including the eggs indicating that the bacterium is transmitted vertically (Erban et al. 2016a). The results showed that *T. putrescentiae* populations varied in the amount of these bacteria. The data indicated that *Cardinium* and *Solitalea*-like bacteria can co-exist in both *A. siro* and *T. putrescentiae* (Erban et al. 2016a). However, the changes observed in the bacterial communities of *A. siro* laboratory populations may suggest that the two groups replace each other at the population level. The fact that the analyses were done on only one *A. siro* population was a weakness of the previous studies (Hubert et al. 2012, 2016; Kopecky et al. 2014).

In this study, we analyzed *Solitalea*-like and *Cardinium* bacteria associated with three populations of the stored product mite *A. siro*. Conventional PCR and qPCR were used to analyse samples of adults/juveniles and eggs. Fluorescent in situ hybridization (FISH) was used to localize *Solitalea*-like bacteria inside the mite bodies. The antibiotic treatment was used to suppress the symbiotic bacteria thereby showing strength of interaction between the symbionts and their hosts. We investigated temporary variations of the intracellular symbiotic bacteria after rifampicin and tetracycline treatments and pre-treatments. This study aimed to answer the following questions: (1) Do *Solitalea*-like bacteria form one or more clusters when 16S rRNA gene sequences are analyzed? (2) Are *Cardinium* and *Solitalea*-like bacteria present in various populations of *A. siro*? (3) Are *Solitalea*-like bacteria present in mite body and in the eggs? And (4) do the *Solitalea*-like bacteria affect the fitness of *A. siro*?

Materials and methods

Mites

Acarus siro populations and sampling design are given in Table 1. The mites were cultivated on a 25 cm² surface area in 70 mL tissue culture flasks (IWAKI flasks; Cat. No. 3100-025; Sterilin, Newport, UK). A constant 85 % RH was maintained with saturated KCl solutions in a Secador desiccator (Bel-Art Products, Pequannock, NJ, USA), which was in an air-conditioned, dark room maintained at 25 °C. The mites were reared on a wheat-derived diet designed for stored product mites (SPMd), which included a mixture of oat flakes, wheat germ and Pangamin-dried yeast extract (Rapeto, Bezdruzice, Czechia) (10:10:1 w/w) (Erban and Hubert 2008). The populations are maintained by transferring of

Table 1 Three populations of *Acarus siro* used for experiments

Population	Sampling conditions		Control	Treatments			
	Sampled/ obtained	Collector		Rifampicin	Riff. pre- treated	Tetracycline	Tet. pre- treated
LAB	Grain, Bustehrad, Czechia	E. Zdarkova	LAB-Con	LAB-Tre-Rif	LAB-Pre-Rif	LAB-Tre-Tet	LAB-Pre- Tet
ZVO	Oil rape seed debris, Zvoleneves, Czechia	M. Nesvorna	ZVO-Con	ZVO-Tre-Rif	ZVO-Pre-Rif	ZVO-Tre-Tet	ZVO-Pre- Tet
LET	Lettuce seed, Prague, Czechia	S. Tuckova	LET-Con				

All the mites were collected in Czechia, but from different sites and the former habitat is characterized by different food sources. Next, the mites were maintained for a different period. The laboratory (LAB) population is reared in the laboratory for 20 years. The lettuce (LET) population is used for mass rearing of the predatory mite *Cheyletus eruditus* on lettuce seeds. For this study, the LAB population was sampled in 12/2012 for FISH and 10/2014 for antibiotic experiment and in 12/2015 for eggs and adults/juveniles. The Zvoleneves (ZVO) population was sampled in 12/2014 for antibiotic experiment and 12/2015 eggs and adults/juveniles. LET population was sampled in 4/2016 for mites and eggs. The table provides the codes for the samples used in the experiment

ca. 1000 mites into the new diet and new flasks every month. The unsexed mites of known age were used for experiments. The mites were collected by a bush under STEMI 2000-C dissection microscope (Carl Zeiss, Jena, Germany). The samples of fresh mites were weighed using a microbalance, and a weight of 10 mg each was transferred to Eppendorf tubes in 6 replicates per population (LAB, ZVO, and LET in Table 1). The samples of mites were stored at -40°C until they were used for the extraction of DNA. The eggs were obtained using a modified method of Stepien and Rodriguez (1973). The food with mites from rearing flasks was placed on mesh with a size of $176\ \mu\text{m}$ under the water surface. All of the used mesh was polyamide (Silk & Progress, Brnec, Czechia). The females deposited eggs on the water surface after 48 h. The water was collected and filtrated through the mesh manifold (Stepien and Rodriguez 1973) using a vacuum pump. The mesh sizes in the manifold were in decreasing order: 411, 300, 206, 176, 139, 109, 86, $42\ \mu\text{m}$ diameter. Next, the eggs were cleaned with ddH_2O , Tween 20 (Cat No. P9416, Sigma-Aldrich, St Louis, MO, USA), bleach, and 80 % ethanol. The eggs were captured at 86 and $42\ \mu\text{m}$ mesh and removed by pipetting into Eppendorf tubes and stored in 80 % ethanol. Each sample consisted of 50 eggs. The design included three replicates per LAB population.

DNA extraction

One mL of bleach (5 % solution of sodium hypochlorite) was added to the Eppendorf tubes with the mites or eggs and left for 1 min. The sample was centrifuged at 3000g for 1 min. The bleach was replaced by 1 mL of pure ethanol for 1 min and centrifuged again at 3000g for 1 min. The ethanol was replaced by phosphate-buffered saline (PBST: 3.2 mM Na_2HPO_4 , 0.5 mM KH_2PO_4 , 1.3 mM KCl, and 135 mM NaCl) with 0.05 % w/w Tween

20 detergent to remove the surface microflora. Washing by PBST and Tween with subsequent centrifugation was repeated 3×. The mites or eggs were then homogenized in 100 µL of PBST in Tissue Grinders (Cat No. 440613, Radnoti, Monrovia, CA, USA). DNA was extracted from mite homogenates using tissue Genomic DNA Mini Kit (Cat No. GT100, Geneaid, New Taipei City, Taiwan) following the manufacturer's protocol. The eggs were extracted using the Exgene Genomic DNA micro Kit (Cat No. 118-050, Gene All, Biotechnology, Seoul, South Korea) according to the manufacturer's protocol. The extracted DNA was stored at −28 °C until analysis.

Standard PCR conditions

Based on the alignment of 20 16S rRNA gene sequences from *A. siro* (Hubert et al. 2012), specific primers for *Solitalea*-like bacteria were designed (see Table 2). To validate the prepared templates, PCR amplification of the 16S rRNA gene was performed with universal bacterial primers (Barbieri et al. 2001). Amplification was conducted in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a 25 µL PCR reaction mixture, containing 200 µM dNTPs, 3 mM MgCl₂, primers (100 nM each), 1.25 unit *Taq* polymerase (Top-Bio, Prague, Czechia) and 100–300 ng template DNA (including mite genomic DNA). The amplification conditions were: 3 min at 94 °C, 30 cycles of 110 s at 94 °C, 110 s at 50 °C for eubacterial primers and 56 °C for *Solitalea*-like bacteria specific primers

Table 2 List of primers and probes used for conventional PCR, quantities PCR (qPCR) and fluorescent in situ hybridization (FISH) in this study

Group	Name	Primer/probe (5–3)	AT (°C)	Size (bp)	References
Eubacteria, PCR	UF	AGAGTTTGATYMTGGC	50	1500	Barbieri et al. (2001)
	UR	GYTACCTGTTTACGACTT			
Eubacteria, qPCR	Com1	CAGCAGCCGCGGTAATAC	59	270	Dorn-In et al. (2015)
	769R	ATCCTGTTTGMTMCCCVCRC			
<i>Cardinium</i> , PCR	Card6	CTTAACGCTAGAACTGCGA	52	900	Kopecky et al. (2013)
	Card8	TCA AGTCTACCAACTCC			
<i>Cardinium</i> , qPCR	Card_6QF	TGCAAATCTCAAAAGCATGT	54	160	This study
	Card_6QR	TCAAGCTCTACCAACTCCCA			
<i>Cardinium</i> probe		CY3- TATCAATTGCAGTTCTAGCG			Matalon et al. (2007)
<i>Solitalea</i> -like, PCR	Soli F	TGCGACACAAAGAGCTGA	54	627	Erban et al. (2016a)
	Soli R	GCTGGCAACAGTACATGG			
<i>Solitalea</i> -like, qPCR	Soli_3QF	GCGAGACAAAGAGCTGAAG	60	180	This study
	Soli_3QR	GAGTTTCAAGGGCAGGC			
<i>Solitalea</i> -like, probe		CY55- CTTCTCCTTGCTTACGCAG	40		This study
<i>Wolbachia</i>	WpF	TTGTAGCCTGCTATGGTATAACT	52	900	O'Neill et al. (1992)
	WpR	GAATAGGTATGATTTTCATGT			
<i>Bartonella</i> -like	Bart 1F	TGTCWCCGAYCCAGCCK	63	920	Kopecky et al. (2014)
	Bart 2R	TGTCCTCGACCCAGCCT			

(see Table 2), and 60 s at 72 °C, and a final extension for 5 min at 72 °C. The resulting PCR products were purified with Wizard® SV Gel and the PCR product clean-up system Kit (Promega, Madison, WI, USA) and were cloned using the pGEM®-T Easy Vector (Promega). Selected clones were sequenced by Macrogen (Seoul, South Korea).

Quantitative PCR

DNA samples of the mites included control, and rifampicin or tetracycline treated and pre-treated mites. The samples were used for bacterial quantification via the quantitative PCR (qPCR). The primers are shown in Table 2. To avoid the influence of chloroplast DNA to analyse, we selected Com1 and 769R primers for quantification of total bacteria copies (Dorn-In et al. 2015). New taxon-specific primers were designed for quantification of *Solitalea*-like and *Cardinium* bacteria (Table 2). The qPCR standard was prepared from a cloned 16S rRNA gene amplicon (pGEM®-T Easy Vector, Promega) from the *Solitalea* and *Cardinium* specific primers. The competent bacterial cells with plasmids were inoculated in the LB medium (Himedia, Mumbai, India) with ampicillin 0.1 g/L (Cat No. A01104.0005, Duchefa Biochemie, Haarlem, The Netherlands) for 16 h at 37 °C. The plasmid was then purified with a Wizard Plus SV Mniipreps DNA purification system (Cat No. A1330, Promega) according to the manufacturer's protocol. Plasmids were linearized by SacI restriction (Cat No. R6061, Promega) and cleaned with a Wizard SV gel and PCR Clean-Up system (Cat No. A9285). The concentration of the cleaned product was measured on P330 Implen NanoPhotometer (Munich, Germany) and adjusted to 10 ng of DNA for each reaction. The standard of DNA was diluted by 1/10. Amplifications were conducted using a StepOnePlus™ Real-Time PCR System (Life Technologies, Grand Island, NY, USA). SYBR green (Bio-Rad Laboratories, The Netherlands) was used as a double-stranded DNA (dsDNA) binding dye. Baseline and threshold calculations were performed with the StepOnePlus software. The amplification consisted of 40 cycles, including a denaturation of 30 s at 95 °C, annealing for 35 s at 54–60 °C (Table 2), and elongation for 45 s at 72 °C. Melting curves were recorded to ensure qPCR specificity. The qPCR measurements were conducted in duplicate. We used design for six biological replicates each analyzed in two technical repeats.

Phylogenetic analyses

Partial 16S rRNA gene sequences of *Solitalea*-like bacteria were assembled with CodonCode Aligner, v.1.5.2 (CodonCode, Dedham, MA, USA) and assigned to bacterial taxonomy using the Ribosomal database project naive Bayesian classifier (Wang et al. 2007). The sequences were aligned using SILVA Incremental Aligner v.1.2.11 (Pruesse et al. 2012). The best-fit model of nucleotide substitution was selected using jModelTest v.2.1.7 (Guindon and Gascuel 2003; Darriba et al. 2012). Based on the selection, a model GTR with a proportion of invariable sites (+I) and a gamma distribution with four rate categories (+G) was used to infer phylogeny through Bayesian analysis with PhyloBayes-MPI v.1.4e (Jow et al. 2002; Lartillot et al. 2009; Rodrigue and Lartillot 2014) and maximum-likelihood analysis in PhyML v.3.0 (Guindon et al. 2010). The phylograms were finalized using Figtree v.1.4.2 (<http://tree.bio.ed.ac.uk/>).

Fluorescent in situ hybridization

FISH was performed using specific bacterial probes (Table 2) on LAB population. From the control diet group, ca. 30 mites were transferred into 4 % formaldehyde and whole mite specimens were hybridized according to a previously described protocol (Perotti et al. 2007). For the hybridizations, samples were incubated at 45 °C in darkness for a maximum of 20 h and then washed for 1 h with the same hybridization buffer, followed by washing in PBTA (phosphate buffer with Triton X-100 plus sodium azide) at room temperature. Samples were mounted in PBS/glycerol and examined under a microscope.

The bacterial probes Cy3 and Cy5 (Eurofins Genomics, Ebersberg, Germany) used in this experiment (Table 2) were equimolar mixed in the hybridization buffer (following remarks of ProBase). No probe and competition suppression controls using excess unlabeled probes were performed. The hybridized specimens were visualized using a Confocal Zeiss LSM510 microscope (Carl Zeiss) with a Coherent Multiphoton laser. Length measurements were performed using the Zeiss LSM Image Examiner.

The growth experiment with antibiotics

Antibiotic diets preparation

The diet was derived from the house dust mite diet (HDMd), which is composed of dog food (Ontario-pet, Placek, Podebrady, Czechia), wheat germ, dried fish food (LonBio, Aqua Tropic Lonsky, Prague, Czechia), Mauripan-dried yeast extract (Rapeto, Bezdruzice, Czechia) and gelatin (Serva Electrophoresis, Heidelberg, Germany) (10:10:3:2:1 w/w) (Erban and Hubert 2008). The antibiotics rifampicin (Cat No. R3501; Sigma-Aldrich) and tetracycline (Cat No. T3258; Sigma-Aldrich) were diluted in HPLC-grade methanol and incorporated into the diet to obtain concentrations of 0 (control), 0.1, 1, 5, 10, 50 and 100 mg g⁻¹. The resulting combination was properly mixed using a MS1 Minishaker (IKA, Staufen, Germany). The 50-mL centrifuge tubes containing the diet were covered with a filter, fixed with a vented cap and lyophilized in a PowerDry LL3000 lyophilizer (Thermo, Shanghai, China). The lyophilized material was ground into a powder in a pottery grinding mortar. Prior to the experiment, the material in the flasks was rehydrated 24 h before the experiment in desiccators using distilled water (Erban et al. 2012). The tested diets for the growth test were rifampicin or tetracycline diet at concentrations 0, 0.1, 1, 5, 10, 50 and 100 mg g⁻¹ on LAB and ZVO populations of mites. The growth test started by adding 100 ± 5 mg of the experimental diet and 10 unsexed mites into IWAKI flask. The flasks with the diets and mites were incubated in the dark for 21 days in desiccator cabinets at 85 % RH and 25 °C. The experiment was terminated with the addition of Oudemans fluid (8 mL of acetic acid, 5 mL of glycerol and 87 mL of 70 % ethanol) to the flasks. Mites were counted under a dissection microscope (Carl Zeiss).

Antibiotic treated mite

Ca. 300 adults/juveniles were added to flasks with 100 ± 50 mg of HDMd, containing 5 mg g⁻¹ of antibiotics or the control diet. The flasks were cultivated under the same conditions used for the population growth experiments. After 21 days, the mites were placed into sterile Eppendorf tubes. Samples of fresh weight of 10 ± 1 mg of mites were weighed using a microbalance. Six replicates per treatment were prepared. The samples

were coded as LAB-Rif-Tre, LAB-Tet-Tre, ZVO-Rif-Tre and ZVO-Tet-Tre (Table 1). The samples were stored in a deep freezer at $-40\text{ }^{\circ}\text{C}$ until use.

Antibiotic pre-treated mites: Ten mite adults originated from previous mass production, i.e. diets with 5 mg g^{-1} of rifampicin or tetracycline and control were transferred into IWAKI flasks on HDMd $100 \pm 5\text{ mg}$ and incubated for 21 days in the same conditions as growth experiments. The mites were collected during next moth according to the actual population density in the flask. The samples were coded as LAB-Rif-Pre, LAB-Tet-Pre, ZVO-Rif-Pre and ZVO-Tet-Pre (see Table 1). The samples were stored in a deep freezer at $-40\text{ }^{\circ}\text{C}$ until use.

Data analyses

Numbers of copies of bacteria, Solitalea and Cardinium 16S rRNA gene fragment

The design usually included six biological and two technical replicates. The data were log-transformed and analyzed by the Kruskal–Wallis nonparametric test, using Dunn postshot comparison and a Bonferroni correction.

Growth test

First, we compared the population growth of *A. siro* on HDMd diet, when the populations were factor, by *t* test. In the next step, the population numbers (*N*) of *A. siro* after 21 days of cultivation had a normal distribution, and data were analyzed by analysis of covariance (ANCOVA). Population numbers were dependent, and type of antibiotics, concentration, mite population and their interactions were independent variables. The ANCOVA models had higher R^2 values when the antibiotic concentration (*x*) was transformed according to the formula $\ln[x + 1.10^{-7}]$ than the models that were not transformed. Finally, the interaction between the final population number and the antibiotic concentration was analyzed separately for each antibiotic type and mite species using regression models. Effective doses of antibiotic concentrations that reduced the final population to 50 % (EC_{50}) compared to the control were estimated from the model with 95 % confidence intervals.

To evaluate the effect of antibiotic pretreatment on the fitness of *A. siro*, final population numbers (*N*) were analyzed using ANOVA separately for each mite population. In the model, the dependent variable was the final population size, and the factors were the control, rifampicin and tetracycline treatment and pretreatment. A Tukey post host comparison of means was used to identify samples with different population growth. The analyses were conducted in XLSTAT 2007 (Addinsoft, New York, NY, USA) and QC-Expert (TriloByte Statistical Software, Pardubice, Czechia).

Results

Phylogenetic analysis of *Solitalea*-like bacteria

The 16S rRNA sequences classified as *Solitalea*-like group were obtained in previous studies by cloning of nearly full-length 16S rRNA gene amplified from total DNA isolated from the whole-body homogenates of mites using universal bacterial primers (Hubert et al.

2012; Kopecky et al. 2014; Erban et al. 2016a). A set of 110 *Solitalea*-like clones were analyzed together with the reference sequences including 191 type-strains of the order *Sphingobacteriales*, 27 closest sequences retrieved by BLAST search in GenBank database, and 29 other strains and clones classified within the genus *Solitalea* in the Ribosomal database project database. Phylogeny was inferred by Bayesian and Maximum-likelihood analyses employing the GTR model with a proportion of invariable sites (+I) and a gamma distribution with four rate categories (+G) chosen according to the Akaike information criterion. All *Solitalea*-like clones formed a new distinct cluster separated from the neighboring clusters of the genus *Solitalea* and of uncultured bacteria associated with amoebae and sweet water or soil habitats. The phylogenetic analysis did not show a close relatedness of the three clusters, but it supported the common node shared with the other *Sphingobacteriaceae* genera (Fig. 1).

Differences in *Solitalea*-like and *Cardinium* bacteria in populations of *Acarus siro*

Based on the conventional PCR using taxon-specific primers the presence of *Solitalea*-like and *Cardinium* bacteria differed in the DNA samples from *A. siro* populations (Table 3). *Cardinium* was identified in LET population only, whereas *Solitalea*-like bacteria was present in the DNA samples from all populations. The *Cardinium* presence was confirmed by qPCR in all populations. The numbers of bacterial 16S rRNA copies differed among the populations based on qPCR with universal Com1 and 769R primers ($K_{33,2} = 17.573$, $P < 0.001$) (Fig. 2a). The numbers decreased in the order from LET, LAB to ZVO. The numbers of *Cardinium* and *Solitalea*-like bacterial 16S rRNA gene copies also differed among populations (Fig. 2a). The *Solitalea*-like bacteria copy numbers differed among populations ($K_{2,30} = 24.881$, $P < 0.001$) (Fig. 2a). The LET population had 10× higher *Solitalea*-like copy numbers than LAB and ZVO populations. The differences in *Cardinium* copy numbers were significant among the populations ($K_{2,33} = 31.135$, $P < 0.001$). ZVO population had 10× and 100× higher *Cardinium* copy numbers than LET and LAB populations, respectively (Fig. 2a).

Localization of *Solitalea*-like bacteria by FISH and detection of bacteria in eggs using PCR in LAB population

Cells of *Solitalea*-like bacteria were localized inside the digestive tract (i.e., cells of colon, diverticula, food bolus), reproductive tract and fat bodies in larvae, nymphs, males and females (Fig. 3). In multi-infected specimens, bacteria were observed at different locations. *Cardinium* bacteria were detected in reproductive tissues (Fig. 3b). In *A. siro* eggs, *Solitalea*-like bacteria were detected using the group-specific primers with the exception of LET population, whereas *Cardinium*-specific primers yielded no specific amplicon (Table 3).

The growth test

Mite growth in control (HDM) diet

Acarus siro population densities on HDMd significantly differed between ZVO and LAB populations ($T_{1,20} = 2.85$; $P = 0.01$). The population density of ZVO populations was

1.4× higher compare to LAB population. The antibiotic supplemented to the diet suppressed the growth of mites (ANCOVA model: $F_{10,133} = 37$, $P < 0.001$). The population density was influenced by antibiotic concentration ($F_{1,142} = 33$, $P < 0.001$), but not by population ($F_{1,142} = 0.82$, $P = 0.37$) or type of antibiotic ($F_{1,142} = 1.64$, $P = 0.20$). However, the interaction of antibiotic concentration with population had a significant effect ($F_{4,139} = 2.48$, $P < 0.05$). The effect of antibiotics on *A. siro* population density is shown in Fig. 4. EC₅₀ indicated that the LAB population was more susceptible to both antibiotics

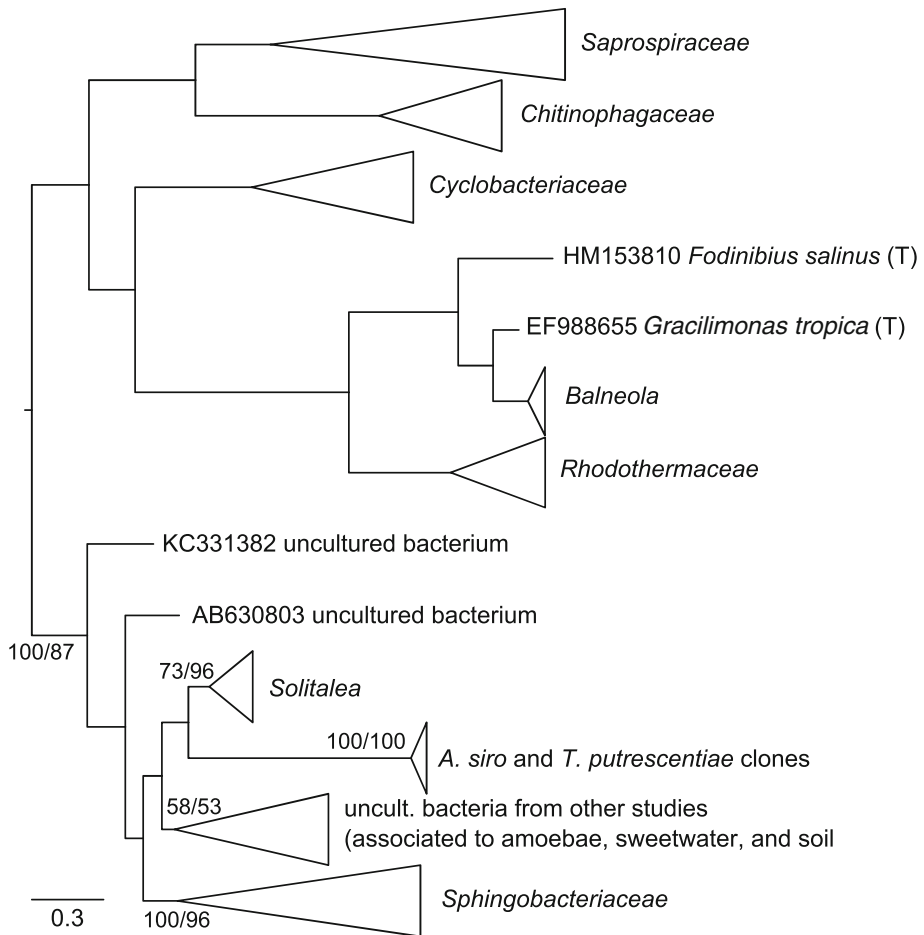


Fig. 1 Phylogenetic analysis of *Solitalea*-like clones from *Acarus siro* and *Tyrophagus putrescentiae*. Phylogeny was inferred by Bayesian analysis employing the GTR model with a proportion of invariable sites (+I) and a gamma distribution with four rate categories (+G). The initial alignment consisted of 110 nearly full-length sequences of the 16S rRNA gene from this study, 20 sequences from Hubert et al. (2012) and 247 reference sequences including 191 type-strains representing the order *Sphingobacteriales*, 27 closest sequences retrieved by BLAST search in the GenBank database, and 29 other sequences classified in the genus *Solitalea* in the Ribosomal database project database. Branch lengths correspond to mean posterior estimates of evolutionary distances (scale bar 0.3). Branch labels indicate the Bayesian posterior probability and supporting bootstrap value from maximum-likelihood analysis. The phylogram was rooted using *Cytophaga fermentans* sequence M58766 as an outgroup

Table 3 Presence or absence of amplicons of bacterial 16S rRNA generated with universal and taxon-specific primers in different populations of *Acarus siro*

Population	Source	Bacteria	<i>Cardinium</i>	<i>Solitalea</i>	<i>Wolbachia</i>	<i>Bartonella</i>
LAB	Adults/juveniles	+	–	+	–	+
	Eggs	+	–	+	–	–
ZVO	Adults/juveniles	+	–	+	–	+
	Eggs	+	–	+	–	–
LET	Adults/juveniles	+	+	+	–	–
	Eggs	+	–	–	–	–

LAB laboratory, ZVO Zvoleneves, LET lettuce

+ presence, – absence

than ZVO (Fig. 4b). EC_{50} was found 13 \times and 40 \times higher in ZVO-Tet-Tre and ZVO-Rif-Tre than in LAB-Tet-Tre and LAB-Rif-Tre populations, respectively.

Effect of antibiotic pretreatment on the mite growth on the control (HDMd) diet

Pretreatment by antibiotics (5 mg g⁻¹) significantly influenced the population growth of mites on the HDMd of LAB and ZVO populations ($F_{2,32} = 15.489$ and $F_{2,31} = 26.844$, both $P < 0.001$) (Fig. 2b). In the LAB population, rifampicin pretreatment (LAB-Rif-Pre) decreased the population density to 89 % of the control (LAB-Con). In the ZVO population, rifampicin pretreatment (ZVO-Rif-Pre) decreased the population density to 72 % of the control (ZVO-Con).

Effect of antibiotic treatment on numbers of bacteria and *Solitalea*-like bacteria

The numbers of bacteria and *Solitalea*-like bacteria 16S rRNA copies were compared in the mites on control diet, treated mites by 5 mg g⁻¹ of rifampicin or tetracycline and in the mites pretreated by 5 mg g⁻¹ of rifampicin or tetracycline. The effects of treatment and pretreatment to the numbers of bacteria and *Solitalea*-like bacteria were different. The pretreatment of mites significantly reduced the numbers of bacteria in both populations ($K_{4,64} = 35.57$ and 23.92, both $P < 0.001$, for LAB and ZVO, respectively) (Fig. 2b). However, no effect of antibiotic treatment and pretreatment was observed on *Solitalea*-like bacteria in both populations ($K_{4,22} = 3.896$, $P = 0.44$ and $K_{4,22} = 7.225$, $P = 0.12$, for LAB and ZVO, respectively) (Fig. 2b).

Discussion

***Solitalea*-like bacteria in astigmatid mites**

Among the neighboring *Solitalea* taxa, according to our phylogenetic analysis (Fig. 1), the known *Solitalea* species *S. koreenis* and *S. canadensis* are considered free-living bacteria with representatives isolated from the soil (Weon et al. 2009). *Solitalea* has an ability to hydrolyze gelatin, starch and maltose (Weon et al. 2009; Liu et al. 2016). Members of

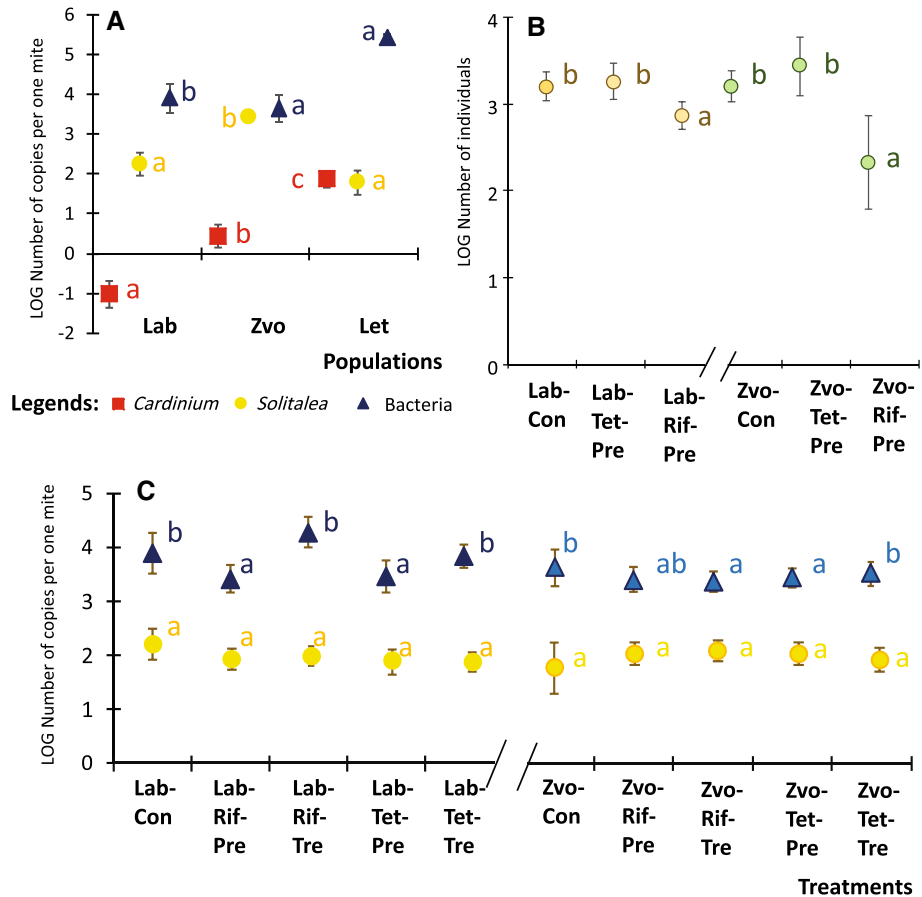
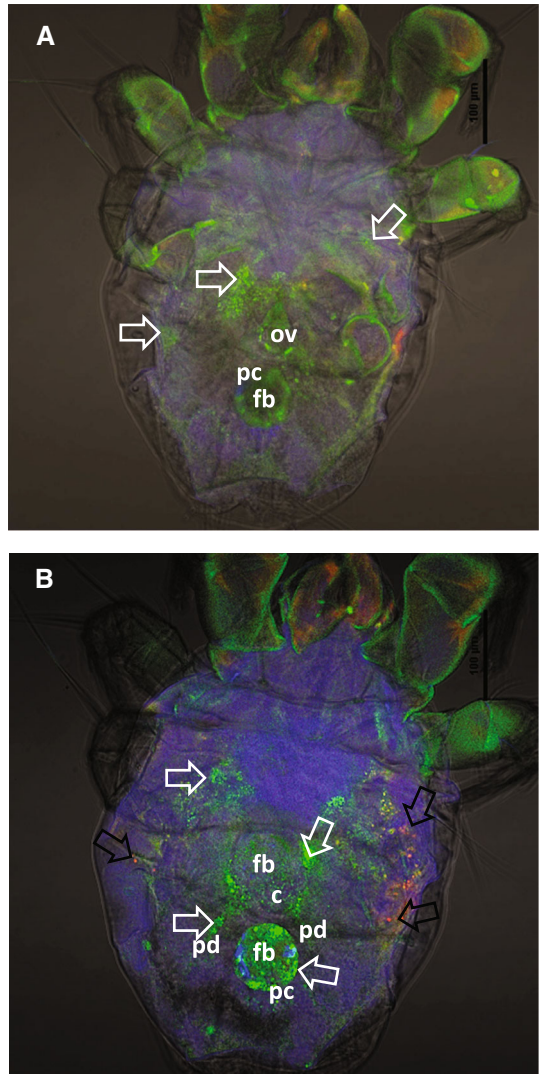


Fig. 2 The quantification of bacteria, *Solitalea*-like and *Cardinium* 16S rRNA copies per one mite by qPCR in samples and the effect of antibiotic pretreatment to mite growth. The *points* are means, *bars* indicate standard deviations, and the *letters* indicated significant differences ($P < 0.05$) based Kruskal–Wallis nonparametric test, using Dunn postshot comparison and a Bonferroni correction. **a** The numbers of Bacteria, *Solitalea*-like and *Cardinium* in samples of three populations of *Acarus siro*; **b** the effect of antibiotic pretreatment (5 mg g^{-1}) on the final population density of *A. siro* reared on HDMd; **c** the effect of antibiotic treatment (5 mg g^{-1}) and antibiotic pretreatment (5 mg g^{-1}) to the numbers of bacteria and *Solitalea*-like bacteria in the samples of *A. siro* from the laboratory (LAB) and Zvoleneves (ZVO) populations. *Let* Lettuce populations, *Con* control (HDMd diets without any antibiotics), *Tet* tetracycline, *Rif* rifampicin, *Tre* treatment (antibiotic in the diet), *Pre* pretreatment (after antibiotic application on HDMd diet)

another *Sphingobacteriaceae* genus, *Pedobacter*, were reported to be associated with the glassy-winged sharpshooter and bees (Mohr and Tebbe 2006; Lacava et al. 2007). *Sphingobacterium* spp. were reported from the gut of longhorn beetles (*Batocera horsfieldi*) and are suggested as symbionts and xylanase producers (Zhou et al. 2009). A hypothetical ability of *Solitalea*-like bacteria to hydrolyze starch-like substrates would correspond to the digestion of starch-type substrates in *A. siro* (Hubert et al. 2005; Erban et al. 2009). Bacteria could compete for or participate in utilization of plant starch from the grain diets. It would be supported by enzyme profiles of known *Solitalea* species (Weon et al. 2009; Liu et al. 2016). The important findings of this analysis is that *Solitalea*-like bacteria from

Fig. 3 Localization of *Solitalea*-like bacteria in the body of *Acarus siro* using fluorescent in situ hybridization. **a** Single infection by *Solitalea*-like bacteria indicated by white arrows. **b** Multiple infection by *Solitalea*-like (green color, white arrows)/*Cardinium* bacteria (red color, black arrows). The *Solitalea*-like probe is Cy5, whereas Cy3 was a probe for *Cardinium*. **c** colon, **fb** food bolus, **ov** ovipositor, **pc** postcolon, **pd** diverticula



two populations of *A. siro* and two populations of *T. putrescentiae* are closely related forming a cluster separated from other *Sphingobacteriaceae* genera. However for verification, future isolation and genome characterization of the *Solitalea*-like bacteria would be necessary.

Both *Solitalea*-like and *Cardinium* bacteria co-occur in mite populations

The present study has demonstrated the co-occurrence of *Solitalea*-like and *Cardinium* bacteria in all investigated populations of *A. siro* at the population and individual levels. At the population level, the numbers of *Solitalea*-like bacteria are much higher than those of *Cardinium*. Since *Solitalea*-like bacteria appear as new symbionts, to our knowledge, no information is available about the co-infection of these bacteria. Co-infection has

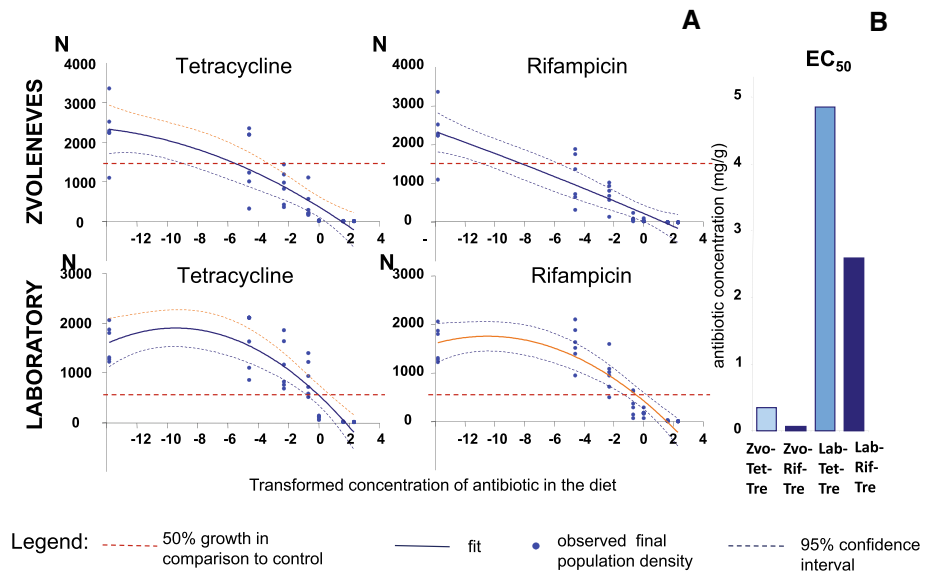


Fig. 4 The effect of addition of the antibiotics tetracycline (Tet) or rifampicin (Rif) to HDMd on the final population size (N) of *Acarus siro*. The regression models described the interaction between $\ln(x + 1 \times 10^{-7})$ transformed antibiotic concentration and population density (a). Fitted values of EC₅₀ of antibiotic concentration reduced the final population density by 50 % compared to the control (b). LAB laboratory, ZVO Zvoleneves, LET Lettuce populations, Con control (HDMd diets without any antibiotics)

been reported for *Cardinium* and *Wolbachia* (Weeks et al. 2003; Zchori-Fein and Perlman 2004; Gotoh et al. 2007; Duron et al. 2008). For example, a *Tetranychus phaselus* spider mite population was simultaneously infected by *Cardinium* and two distinct lineages of *Wolbachia* (Zhao et al. 2013). In some cases of multi-infested mites, only one symbiont is present in the eggs and is vertically transmitted. Both *Wolbachia* and *Cardinium* were found in the predatory mite *Metaseiulus occidentalis*, whereas in the eggs, only *Cardinium* were detected (Wu and Hoy 2012b). Here we found 100× lower numbers of *Cardinium* than of members of the *Solitalea*-like group in *A. siro*. In addition, we did not detect *Cardinium* in the eggs by taxon-specific primers, whereas *Solitalea*-like bacteria were present in the eggs. It means that *Cardinium* were rare in bodies of *A. siro* and under the detection limits in the mite bodies and eggs by conventional PCR. An alternative explanation of the absence in eggs might be that *Cardinium* were not vertically transmitted in *A. siro* populations used in the study. A further study employing high numbers of individuals would be necessary to solve the question regarding the vertical transmission.

Changes in symbiotic bacterial populations during long-term growth in the rearing facility

Our data indicated the disappearance of *Cardinium* infection in the laboratory strain of *A. siro* and replacement by *Solitalea*-like bacteria during a long-term cultivation lasting for several years in the rearing facility. An investigation of the LAB population in 2010 indicated the presence of both *Cardinium* and *Solitalea*-like bacteria in that population

(Kopecky et al. 2014). However, a more recent study of the same population in 2012 showed *Solitalea*-like and *Bartonella*-like bacteria predominating, whereas *Cardinium* was represented only by one sequence in a subsample dataset (Hubert et al. 2016). Later, in 2014, almost complete replacement of *Cardinium* by *Solitalea*-like bacteria was observed in this study. In summary, these results suggest that during 4 years of observation within one laboratory-reared population, *Cardinium* were replaced by another group of *Solitalea*-like bacteria. Further experiments are necessary to address such symbiont replacements in mites, and the physiological studies should uncover whether and how such replacements may or may not be beneficial for the host.

Localization of *Solitalea*-like and *Cardinium* bacteria

Based on the FISH localization, *Solitalea*-like bacteria were present in the digestive tract, reproductive organs and fat bodies. Such distribution is similar to the known distribution of *Cardinium* in *Brevipalpus* mites (Kitajima et al. 2007). We also found *Solitalea*-like bacteria in both sexes and all developmental stages. The presence of bacteria in the eggs provided the first evidence that these bacteria were vertically transmitted. The eggs were surface cleaned by bleaching a method recommended for symbiont detection in eggs by PCR (Wu and Hoy 2012b). The localization of *Solitalea*-like infection in reproductive organs, where the infection is transferred to the offspring and the presence of it in the eggs confirms transovarial transmission and rules out external contamination or horizontal transmission. Here we found *Solitalea*-like bacteria inside the gut, but also in the internal organs. Previously, it was found that *Candidatus* Erwinia dacicola resided intracellularly in the gastric ceca of the larval midgut but extracellularly in the lumen of the foregut and ovipositor diverticulum of adult olive flies (*Bactrocera oleae*) (Estes et al. 2009). Our results indicated that such distribution is also possible for *Solitalea*-like bacteria.

In specimens of *A. siro* with multiple infections of *Solitalea*-like bacteria and *Cardinium*, we observed a spatial separation of bacteria in their bodies. However, in some individuals, multiple infections were detected in reproductive organs. The reproductive organs are the source of the vertical transfer of bacteria, which is known to be the case for *Cardinium* (Ros et al. 2012).

Antibiotic treatment did not eliminate *Solitalea*-like bacteria

Antibiotic treatment is suggested as a useful tool for elimination of parasitic bacteria in mites and observation of cytoplasmic incompatibility, for instance, in *Tetranychus urticae* and *T. turkestanii* (Breeuwer 1997). For example, tetracycline treatment eliminated *Wolbachia* and *Cardinium* in *Neoseiulus paspalivorus* females (Sourassou et al. 2014) and penicillin-cured females of *Eotetranychus suginamensis* were free of *Cardinium* (Gotoh et al. 2007). Tetracycline eliminated *Cardinium* in *Brevipalpus phoenicis* (Groot and Breeuwer 2006) and *Tetranychus piercei* (Zhu et al. 2012). Antibiotics added to the diet of *Pieris rapae* larvae (Lepidoptera) were used to detect previously unobserved bacterial species after perturbation of the gut bacterial community (Robinson et al. 2010). The dose 5 mg g⁻¹ of tetracycline and rifampicin in the diet did not eliminate *Solitalea*-like bacteria in *A. siro*. Similar results were reported previously, when we found that *Solitalea*-like bacteria were still present in the clones of bacteria from *A. siro* after neomycin and streptomycin treatments (1 mg g⁻¹) of *A. siro* (Kopecky et al. 2014). Here, we found that in antibiotic pretreated *A. siro* the total number of bacteria is 10× lower than on control diet. It means that the relative proportion of *Solitalea*-like bacteria is increasing in *A. siro*

bacteriome after antibiotic treatment. We also observed different responses in terms of population growth suppression between populations and antibiotics. The fitted values of antibiotic concentration for 50 % suppression of the population growth of mites were at least $5\times$ lower for ZVO population than that of LAB population. We observed $1.4\times$ higher population growth, suggested as an indirect estimator of fitness (Erban and Hubert 2008), of ZVO population than of LAB population. The pretreatment of mites by rifampicin and tetracycline (5 mg g^{-1}) did not increase fitness, contrary to the previously observed effect of streptomycin and neomycin (10 mg g^{-1}) (Kopecky et al. 2014). Moreover, the pretreatment by rifampicin resulted in the decrease of population fitness in comparison to tetracycline pretreatment and the control situation in both *A. siro* populations. It seemed likely that rifampicin eliminated some beneficial symbionts in *A. siro*, whereas neomycin and streptomycin suppressed parasitic or pathogenic bacteria. ZVO population was found to have higher fitness, higher sensitivity to antibiotics and higher numbers of *Solitalea*-like bacteria. With respect to the higher effect of antibiotics on *Solitalea*-like bacterial numbers and fitness decrease, the suggestion is that the *Solitalea*-like group might be more important in the ZVO than in the LAB *A. siro* population.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

- Armentia A, Fernandez A, Perez-Santos C, de la Fuente R, Sanchez P, Sanchis F, Mendez J, Stolle R (1994) Occupational allergy to mites in salty ham, chorizo and cheese. *Allergol Immunopathol* 22:152–154
- Armentia A, Martinez A, Castrodeza R, Martinez J, Jimeno A, Mendez J, Stolle R (1997) Occupational allergic disease in cereal workers by stored grain pests. *J Asthma* 34:369–378
- Athanassiou CG, Kavallieratos NG, Sciarretra A, Palyvos NE, Trematerra P (2011) Spatial associations of insects and mites in stored wheat. *J Econ Entomol* 104:1752–1764
- Barbieri E, Paster BJ, Hughes D, Zurek L, Moser DP, Teske A, Sogin ML (2001) Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg capsules of the squid *Loligo pealei* (Cephalopoda: Loliiginidae). *Environ Microbiol* 3:151–167
- Bigliardi E, Sacchi L, Genchi M, Alma A, Pajoro M, Daffonchio D, Marzorati M, Avanzati AM (2006) Ultrastructure of a novel *Cardinium* sp. symbiont in *Scaphoideus titanus* (Hemiptera: Cicadellidae). *Tissue Cell* 38:257–261
- Breeuwer JAJ (1997) *Wolbachia* and cytoplasmic incompatibility in the spider mites *Tetranychus urticae* and *T. turkestanii*. *Heredity* 79:41–47
- Breeuwer H, Ros VID, Groot TVM (2011) *Cardinium*: the next addition to the family of reproductive parasites. In: Zchori-Fein E, Bourtzis K (eds) Manipulative tenants: bacteria associated with arthropods. CRC Press, Boca Raton, pp 207–224
- Chang J, Masters A, Avery A, Werren JH (2010) A divergent *Cardinium* found in daddy long-legs (Arachnida: Opiliones). *J Invertebr Pathol* 105:220–227
- Dallai R, Mercati D, Giusti F, Gottardo M, Carapelli A (2011) A *Cardinium*-like symbiont in the proturan *Acerella muscorum* (Hexapoda). *Tissue Cell* 43:151–156
- Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* 9:772
- Dorn-In S, Bassitta R, Schwaiger K, Bauer J, Holzel CS (2015) Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA. *J Microbiol Methods* 113:50–56

- Duron O (2013) Lateral transfers of insertion sequences between *Wolbachia*, *Cardinium* and *Rickettsia* bacterial endosymbionts. *Heredity* 111:330–337
- Duron O, Hurst GDD, Hornett EA, Josling JA, Engelstadter J (2008) High incidence of the maternally inherited bacterium *Cardinium* in spiders. *Mol Ecol* 17:1427–1437
- Erban T, Hubert J (2008) Digestive function of lysozyme in synanthropic acaridid mites enables utilization of bacteria as a food source. *Exp Appl Acarol* 44:199–212
- Erban T, Erbanova M, Nesvorna M, Hubert J (2009) The importance of starch and sucrose digestion in nutritive biology of synanthropic acaridid mites: alpha-amylases and alpha-glucosidases are suitable targets for inhibitor-based strategies of mite control. *Arch Insect Biochem Physiol* 71:139–158
- Erban T, Rybansky J, Hubert J (2012) The efficacy of four avermectins on the synanthropic mite *Lepidoglyphus destructor* under laboratory conditions. *Exp Appl Acarol* 58:43–50
- Erban T, Klimov PB, Smrz J, Phillips TW, Nesvorna M, Kopecky J, Hubert J (2016a) Populations of stored product mite *Tyrophagus putrescentiae* differ in their bacterial communities. *Front Microbiol* 7:1046
- Erban T, Rybanska D, Harant K, Hortova B, Hubert J (2016b) Feces derived allergens of *Tyrophagus putrescentiae* reared on dried dog food and evidence of the strong nutritional interaction between the mite and *Bacillus cereus* producing protease bacillolysins and exo-chitinases. *Front Physiol* 7:53
- Estes AM, Hearn DJ, Bronstein JL, Pierson EA (2009) The olive fly endosymbiont, ‘*Candidatus* Erwinia dacicola’, switches from an intracellular existence to an extracellular existence during host insect development. *Appl Environ Microbiol* 75:7097–7106
- Fukuzawa AH, Vellutini BC, Lorenzini DM, Silva PI Jr, Mortara RA, da Silva JM, Daffre S (2008) The role of hemocytes in the immunity of the spider *Acanthoscurria gomesiana*. *Dev Comp Immunol* 32:716–725
- Gotoh T, Noda H, Ito S (2007) *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. *Heredity* 98:13–20
- Groot TVM, Breeuwer JAJ (2006) *Cardinium* symbionts induce haploid thelytoky in most clones of three closely related *Brevipalpus* species. *Exp Appl Acarol* 39:257–271
- Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696–704
- Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59:307–321
- Hess RT, Hoy MA (1982) Microorganisms associated with the spider mite predator *Metaseiulus* (= *Typhlodromus*) *occidentalis*: electron microscope observations. *J Invertebr Pathol* 40:98–106
- Hubert J, Stejskal V, Munzbergova Z, Kubatova A, Vanova M, Zdarkova E (2004) Mites and fungi in heavily infested stores in the Czech Republic. *J Econ Entomol* 97:2144–2153
- Hubert J, Doleckova-Maresova L, Hyblova J, Kudlikova I, Stejskal V, Mares M (2005) *In vitro* and *in vivo* inhibition of alpha-amylases of stored-product mite *Acarus siro*. *Exp Appl Acarol* 35:281–291
- Hubert J, Kopecky J, Perotti MA, Nesvorna M, Braig HR, Sagova-Mareckova M, Macovei L, Zurek L (2012) Detection and identification of species-specific bacteria associated with synanthropic mites. *Microb Ecol* 63:919–928
- Hubert J, Kopecky J, Sagova-Mareckova M, Nesvorna M, Zurek L, Erban T (2016) Assessment of bacterial communities in thirteen species of laboratory-cultured domestic mites (Acari: Acaridida). *J Econ Entomol*. 109:1887–1896
- Jow H, Hudelot C, Rattray M, Higgs P (2002) Bayesian phylogenetics using an RNA substitution model applied to early mammalian evolution. *Mol Biol Evol* 19:1591–1601
- Kitajima EW, Groot TV, Novelli VM, Freitas-Astua J, Alberti G, de Moraes GJ (2007) *In situ* observation of the *Cardinium* symbionts of *Brevipalpus* (Acari: Tenuipalpidae) by electron microscopy. *Exp Appl Acarol* 42:263–271
- Kopecky J, Perotti MA, Nesvorna M, Erban T, Hubert J (2013) *Cardinium* endosymbionts are widespread in synanthropic mite species (Acari: Astigmata). *J Invertebr Pathol* 112:20–23
- Kopecky J, Nesvorna M, Mareckova-Sagova M, Hubert J (2014) The effect of antibiotics on associated bacterial community of stored product mites. *PLoS ONE* 9:e112919
- Kurtti TJ, Munderloh UG, Andreadis TG, Magnarelli LA, Mather TN (1996) Tick cell culture isolation of an intracellular prokaryote from the tick *Ixodes scapularis*. *J Invertebr Pathol* 67:318–321
- Lacava PT, Parker J, Andreote FD, Dini-Andreote F, Ramirez JL, Miller TA (2007) Analysis of the bacterial community in glassy-winged sharpshooter heads. *Entomol Res* 37:261–266
- Lartillot N, Lepage T, Blanquart S (2009) PhyloBayes 3: a Bayesian software package for phylogenetic reconstruction and molecular dating. *Bioinformatics* 25:2286–2288
- Liu FF, Kulinich A, Du YM, Liu L, Voglmeir J (2016) Sequential processing of mannose-containing glycans by two alpha-mannosidases from *Solitalea canadensis*. *Glycoconj J* 33:159–168

- Marx JJ Jr, Twigg JT, Ault BJ, Merchant JA, Fernandez-Caldas E (1993) Inhaled aeroallergen and storage mite reactivity in a Wisconsin farmer nested case-control study. *Am Rev Respir Dis* 147:354–358
- Matalon Y, Katzir N, Gottlieb Y, Portnoy V, Zchori-Fein E (2007) *Cardinium* in *Plagiomerus diaspidis* (Hymenoptera: Encyrtidae). *J Invertebr Pathol* 96:106–108
- McClymont-Peace D (1983) Reproductive success of the mite *Acarus siro* L. on stored cheddar cheese of different ages. *J Stored Prod Res* 19:97–104
- Melnik JP, Smith A, Scott-Dupree C, Marcone MF, Hill A (2010) Identification of cheese mite species inoculated on Mimolette and Milbenkase cheese through cryogenic scanning electron microscopy. *J Dairy Sci* 93:3461–3468
- Mohr KI, Tebbe CC (2006) Diversity and phylotype consistency of bacteria in the guts of three bee species (Apoidea) at an oilseed rape field. *Environ Microbiol* 8:258–272
- Musken H, Franz JT, Wahl R, Paap A, Cromwell O, Masuch G, Bergmann KC (2000) Sensitization to different mite species in German farmers: clinical aspects. *J Investig Allergol Clin Immunol* 10:346–351
- Nakamura Y, Yukuhiro F, Matsumura M, Noda H (2012) Cytoplasmic incompatibility involving *Cardinium* and *Wolbachia* in the white-backed planthopper *Sogatella furcifera* (Hemiptera: Delphacidae). *Appl Entomol Zool* 47:273–283
- O'Neill SL, Giordano R, Colbert AME, Karr TL, Robertson HM (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc Natl Acad Sci USA* 89:2699–2702
- Palyvos NE, Emmanouel NG, Saitanis CJ (2008) Mites associated with stored products in Greece. *Exp Appl Acarol* 44:213–226
- Penz T, Schmitz-Esser S, Kelly SE, Cass BN, Muller A, Woyke T, Malfatti SA, Hunter MS, Horn M (2012) Comparative genomics suggests an independent origin of cytoplasmic incompatibility in *Cardinium hertigii*. *PLoS Genet* 8:e1003012
- Perlman SJ, Magnus SA, Copley CR (2010) Pervasive associations between *Cybaeus* spiders and the bacterial symbiont *Cardinium*. *J Invertebr Pathol* 103:150–155
- Perotti MA, Allen JM, Reed DL, Braig HR (2007) Host-symbiont interactions of the primary endosymbiont of human head and body lice. *FASEB J* 21:1058–1066
- Pruesse E, Peplies J, Glockner FO (2012) SINA: accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* 28:1823–1829
- Robinson CJ, Schloss P, Ramos Y, Raffa K, Handelsman J (2010) Robustness of the bacterial community in the cabbage white butterfly larval midgut. *Microb Ecol* 59:199–211
- Rodrigue N, Lartillot N (2014) Site-heterogeneous mutation-selection models within the PhyloBayes-MPI package. *Bioinformatics* 30:1020–1021
- Ros VID, Fleming VM, Feil EJ, Breeuwer JAJ (2012) Diversity and recombination in *Wolbachia* and *Cardinium* from *Bryobia* spider mites. *BMC Microbiol* 12:S13
- Sacchi L, Genchi M, Clementi E, Bigliardi E, Avanzati AM, Pajoro M, Negri I, Marzorati M, Gonella E, Alma A, Daffonchio D, Bandi C (2008) Multiple symbiosis in the leafhopper *Scaphoideus titanus* (Hemiptera: Cicadellidae): details of transovarial transmission of *Cardinium* sp. and yeast-like endosymbionts. *Tissue Cell* 40:231–242
- Sanchez-Borges M, Suarez-Chacon R, Capriles-Hulett A, Caballero-Fonseca F, Iraola V, Fernandez-Caldas E (2009) Pancake syndrome (oral mite anaphylaxis). *World Allergy Organ J* 2:91–96
- Sanchez-Borges M, Suarez-Chacon R, Capriles-Hulett A, Caballero-Fonseca F, Fernandez-Caldas E (2013) Anaphylaxis from ingestion of mites: pancake anaphylaxis. *J Allergy Clin Immunol* 131:31–35
- Santos-Garcia D, Rollat-Farnier P-A, Beitia F, Zchori-Fein E, Vavre F, Mouton L, Moya A, Latorre A, Silva FJ (2014) The genome of *Cardinium* cBtQ1 provides insights into genome reduction, symbiont motility, and its settlement in *Bemisia tabaci*. *Genome Biol Evol* 6:1013–1030
- Sourassou NF, Hanna R, Breeuwer JAJ, Negloh K, de Moraes GJ, Sabelis MW (2014) The endosymbionts *Wolbachia* and *Cardinium* and their effects in three populations of the predatory mite *Neoseiulus paspalivorus*. *Exp Appl Acarol* 64:207–221
- Stefanini A, Duron O (2012) Exploring the effect of the *Cardinium* endosymbiont on spiders. *J Evol Biol* 25:1521–1530
- Stepien Z, Rodriguez JG (1973) Collecting large quantities of acarid mites. *Ann Entomol Soc Am* 66:478–480
- Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73:5261–5267
- Weeks AR, Marec F, Breeuwer JAJ (2001) A mite species that consists entirely of haploid females. *Science* 292:2479–2482

- Weeks AR, Velten R, Stouthamer R (2003) Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proc R Soc Lond B* 270:1857–1865
- Weon HY, Kim BY, Lee CM, Hong SB, Jeon YA, Koo BS, Kwon SW (2009) *Solitalea koreensis* gen. nov., sp. nov. and the reclassification of [*Flexibacter*] *canadensis* as *Solitalea canadensis* comb. nov. *Int J Syst Evol Microbiol* 59:1969–1975
- Wu K, Hoy MA (2012a) *Cardinium* is associated with reproductive incompatibility in the predatory mite *Metaseiulus occidentalis* (Acari: Phytoseiidae). *J Invertebr Pathol* 110:359–365
- Wu K, Hoy MA (2012b) Extended starvation reduced and eliminated *Wolbachia*, but not *Cardinium*, from *Metaseiulus occidentalis* females (Acari: Phytoseiidae): a need to reassess *Wolbachia*'s status in this predatory mite? *J Invertebr Pathol* 109:20–26
- Zchori-Fein E, Perlman SJ (2004) Distribution of the bacterial symbiont *Cardinium* in arthropods. *Mol Ecol* 13:2009–2016
- Zchori-Fein E, Perlman SJ, Kelly SE, Katzir N, Hunter MS (2004) Characterization of a '*Bacteroidetes*' symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of '*Candidatus Cardinium heritigii*'. *Int J Syst Evol Microbiol* 54:961–968
- Zhao D-X, Chen D-S, Ge C, Gotoh T, Hong X-Y (2013) Multiple infections with *Cardinium* and two strains of *Wolbachia* in the spider mite *Tetranychus phaseelus* Ehara: revealing new forces driving the spread of *Wolbachia*. *PLoS ONE* 8:e54964
- Zhou J, Huang H, Meng K, Shi P, Wang Y, Luo H, Yang P, Bai Y, Zhou Z, Yao B (2009) Molecular and biochemical characterization of a novel xylanase from the symbiotic *Sphingobacterium* sp. TN19. *Appl Microbiol Biotechnol* 85:323–333
- Zhu L-Y, Zhang K-J, Zhang Y-K, Ge C, Gotoh T, Hong X-Y (2012) *Wolbachia* strengthens *Cardinium*-induced cytoplasmic incompatibility in the spider mite *Tetranychus piercei* McGregor. *Curr Microbiol* 65:516–523