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Research Article

RESEARCH ARTICLE

Endosymbiont diversity and prevalence in herbivorous spider mite populations in South-Western Europe

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One sentence summary: Endosymbiont diversity and prevalence in spider mite populations of South-Western Europe suggest horizontal transfers of *Cardinium* between host and facilitation by *Wolbachia*.

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†Isabelle Olivieri sadly passed away before the submission of this paper.

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ABSTRACT

Bacterial endosymbionts are known as important players of the evolutionary ecology of their hosts. However, their distribution, prevalence and diversity are still largely unexplored. To this aim, we investigated infections by the most common bacterial reproductive manipulators in herbivorous spider mites of South-Western Europe. Across 16 populations belonging to three *Tetranychus* species, *Wolbachia* was the most prevalent (ca. 61%), followed by *Cardinium* (12%–15%), while only few individuals were infected by *Rickettsia* (0.9%–3%), and none carried *Arsenophonus* or *Spiroplasma*. These endosymbionts are here reported for the first time in *Tetranychus evansi* and *Tetranychus ludeni*, and showed variable infection frequencies between and within species, with several cases of coinfections. Moreover, *Cardinium* was more prevalent in *Wolbachia*-infected individuals, which suggests facilitation between these symbionts. Finally, sequence comparisons revealed no variation of the *Wolbachia wsp* and *Rickettsia gtlA* genes, but some diversity of the *Cardinium 16S rRNA,* both between and within populations of the three mite species. Some of the *Cardinium* sequences identified belonged to distantly-related clades, and the lack of association between these sequences and spider mite mitotypes suggests repeated host switching of *Cardinium*. Overall, our results reveal a complex community of symbionts in this system, opening the path for future studies.

Keywords: endosymbiosis; maternally inherited bacteria; multiple infections; reproductive parasitism; two-spotted spider-mite; *Tetranychus urticae*

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INTRODUCTION

Terrestrial arthropods often harbor several bacterial microorganisms that occur in the cytoplasm of host cells, and are maternally inherited from mother to offspring through the egg cytoplasm (Moran, McCutcheon and Nakabachi 2008). In the last decade, intensive field surveys of distinct arthropod species revealed that the most common of these endosymbionts are *Wolbachia, Rickettsia*, *Cardinium*, *Arsenophonus* and *Spiroplasma* (Duron *et al.* 2008; Weinert *et al.* 2015). Whereas *Wolbachia* is present in most groups, *Rickettsia* and *Cardinium* have low incidence in the hexapoda relative to other groups, with the exception of hemiptera (Weeks, Velten and Stouthamer 2003; Zchori-Fein and Perlman 2004; Weinert *et al.* 2015). These three bacterial genera are also extremely prevalent in chelicerates, especially in spiders, mites and ticks (Duron *et al.* 2008; Weinert *et al.* 2015; Zhang *et al.* 2016a; Duron *et al.* 2017).

The maternal inheritance of endosymbiotic bacteria has selected for a variety of phenotypic changes in their hosts, such as cytoplasmic incompatibility (CI, i.e. embryonic mortality in crosses between infected males and uninfected females), feminization (i.e. genetic males that develop as females), thelytokous parthenogenesis (i.e. asexual daughter development), or male killing (i.e. infected males are eliminated during embryogenesis or late larval instars). These reproductive manipulations, by promoting the production and fitness of infected daughters (i.e. the transmitting sex) via negative effects on the fitness of individuals not involved in the transmission, enhance symbiont transmission and allow their spread within host populations (Werren, Baldo and Clark 2008; Engelstadter and Hurst 2009). Moreover, such manipulations may also facilitate the transmission of other vertically transmitted symbionts co-occurring in the same host (Frank 1998; Engelstadter, Telschow and Hammerstein 2004; Vautrin *et al.* 2008; Vautrin and Vavre 2009; Hellard *et al.* 2015).

Although traditionally viewed as vertically transmitted symbionts, evidence of horizontal transmission events stems from incongruences between host and symbiont phylogenies (Schilthuizen and Stouthamer 1997; Vavre *et al.* 1999; Raychoudhury *et al.* 2009; Gerth, Rothe and Bleidorn 2013). Indeed, a few recent studies have provided direct evidence for horizontal transmission, and suggest parasitism and feeding as the two main routes of transfer. For instance, *Wolbachia* can be transmitted between whiteflies via parasitoids (Ahmed *et al.* 2015), or to a parasitoid from its infected drosophila host (Heath *et al.* 1999), *Spiroplasma* may be transmitted between two *Drosophila* species via ectoparasitic mites (Jaenike *et al.* 2007; Brown and Lloyd 2015), and *Rickettsia* infecting *Bemisia tabaci* may be transferred to parasitoids, although it is not vertically transmitted in the latter (Chiel *et al.* 2009). Similarly, *Trichogramma* wasps of the same or different species have exchanged symbionts when infecting the same butterfly egg (Huigens *et al.* 2004). Symbionts may also be transmitted via the resource that their hosts feed on (but see Faria, Paulo and Sucena 2016). The most compelling evidence for this infection route comes from herbivorous arthropods, in which horizontal transmission via plant feeding has been shown for *Rickettsia* and *Wolbachia* in whiteflies (Caspi-Fluger *et al.* 2012; Li *et al.* 2017) and for *Cardinium* in leaf hoppers (Gonella *et al.* 2015). However, the incidence of horizontal transmission for many vertically transmitted arthropod endosymbionts is still under debate and other studies do not corroborate these results (e.g. Gualtieri *et al.* 2017).

Several studies have shown that herbivorous spider mites are often infected with *Wolbachia* and *Cardinium* (Gotoh, Noda and Hong 2003; Liu, Miao and Hong 2006; Gotoh *et al.* 2007b; Xie, Chen and Hong 2011; Ros *et al.* 2012; Suh *et al.* 2015; Zhang *et al.* 2016b) and occasionally with *Spiroplasma* (Zhang *et al.* 2016b; Staudacher *et al.* 2017) and *Rickettsia* (Hoy and Jeyaprakash 2005; Zhang *et al.* 2016b). In spider mites, *Wolbachia* and *Cardinium* may have diverse fitness effects. In particular, they may cause different degrees of CI (Gotoh, Noda and Hong 2003; Gotoh, Noda and Ito 2007a; Gotoh *et al.* 2007b; Ros and Breeuwer 2009; Xie, Chen and Hong 2011; Zhu *et al.* 2012; Suh *et al.* 2015; Xie *et al.* 2016). Moreover, these bacteria have been found in the same individual host (Ros *et al.* 2012; Zhu *et al.* 2012; Zhao, Zhang and Hong 2013a; Zhao *et al.* 2013b; Xie *et al.* 2016), and multiple strains of these symbionts can be found in spider-mite populations, although levels of nucleotide diversity depend on the host species and/or populations (Gotoh, Noda and Hong 2003; Liu, Miao and Hong 2006; Gotoh, Noda and Ito 2007a; Gotoh *et al.* 2007b; Yu *et al.* 2011; Ros *et al.* 2012; Zhang *et al.* 2016b).

This study aims to contribute to the knowledge of the symbiont community of spider mites, by characterizing the community of herbivorous spider-mite populations and their endosymbionts in South-West Europe, a relatively unstudied area. By unraveling the diversity and prevalence of endosymbionts in natural spider-mite populations, this study might provide some insight on potential facilitation in multiple infections and horizontal transfers among hosts.

MATERIAL AND METHODS

Spider-mite collection and population rearing

Populations of Tetranychidae mites were collected from September to December 2013 at several locations in Central Portugal (Table 1). In addition, two populations were collected from Southern Spain, and one population from Southern France for comparison with nearby countries. Each population in each location was collected from different leaves on different plants. Mite collection was done by placing infested leaves in closed paper bags. Subsequently, adult females (n = 25–600) from all populations were transferred to insect-proof cages containing bean cv. Contender seedlings (obtained from Germisem, Oliveira do Hospital, Portugal) immediately after collection from the field. After species identification for each of these populations, Solanaceae specialist spider mites (*Tetranychus evansi*) were transferred to tomato cv. Money Maker seedlings (obtained from Mr. Fothergill's Seeds, Kentford, UK). The colonies were then maintained in the laboratory under standard conditions (25 ± 2°C, 60% RH, 16/8 h L/D; Clemente *et al.* 2016).

Molecular species identification and screening for endosymbiont infection

From 0 to 100 days after collection, adult spider mite females (n = 11–16) were randomly picked from each colony and individually analyzed for the presence of reproductive manipulators and for species identification. Total genomic DNA was extracted from each individual spider mite using the Sigma-Aldrich protocol and materials (GenElute Mammalian Genomic DNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO, United States). Total DNA was eluted in the final step with 20 μl RNase-free water (Qiagen NV, Venlo, The Netherlands) in order to increase

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Table 1. Tetranychidae mite populations collected in Portugal (P), France (F) and Spain (S) from September to December 2013.

Name	Species	Collection date	Location	Coordinates	Host plant
FR	*T. urticae**	11/10/2013	Montpellier (F)	43.614951, 3.859846	*Solanum lycopersicum*
AlBe		09/11/2013	Almería (S)	36.855725, −2.320374	*Solanum melongena*
AlRo		09/11/2013	Almería (S)	36.855725, −2.320374	*Rosa spp.*
DC		10/09/2013	S. Domingos (P)	39.058742, −9.135427	*Cucurbita pepo*
DF		10/09/2013	S. Domingos (P)	39.058742, −9.135427	*Phaseolus vulgaris*
COL		08/09/2013	Colares (P)	38.799517, −9.448335	*Phaseolus vulgaris*
LOU		03/10/2013	Lourinhã (P)	39.248145, −9.276321	*Solanum melongena*
CH		31/10/2013	Casal Hortelão (P)	38.851962, −9.393918	*Solanum lycopersicum*
RF		04/11/2013	Ribeira de Fráguas (P)	39.366415, −8.851037	*Solanum lycopersicum*
AMP		18/11/2013	Aldeia da Mata Pequena (P)	38.534363, −9.191163	*Datura stramonium*
GH	*T. evansi*	03/10/2013	University of Lisbon (P)	38.757852, −9.158221	*Solanum lycopersicum*
QL		26/11/2013	Quinta das Lameiras (P)	39.085837, −8.991478	*Datura stramonium*
GRA		03/12/2013	Graça, Lisbon (P)	38.71392, −9.129537	*Solanum lycopersicum*
Assaf	*T. ludeni*	20/09/2013	Assafora (P)	38.904743, −9.408592	*Datura stramonium*
CVM		03/10/2013	Casal Vale do Medo (P)	39.248450, −9.294550	*Ipomoea purpurea*
Alval		05/11/2013	Alvalade, Lisbon (P)	38.75515, −9.14685	*Ipomoea purpurea*

*Red form (or *T. cinnabarinus*).

the concentration of DNA obtained from these very tiny animals (c.a. 300 μm long). The same DNA from each individual was used to screen for the presence of potential endosymbionts and to identify the spider-mite species. These identifications were performed by PCR amplification and then sequencing of a fragment of the nuclear ribosomal DNA (rDNA) ITS2 (internal transcribed spacer 2) region, which is the most commonly used marker to distinguish among spider-mite species (Ben-David *et al.* 2007; Hurtado *et al.* 2008), as the mitochondrial DNA (mtDNA) Cytochrome Oxidase subunit I (*COI*) is more polymorph in spider mites (Navajas *et al.* 1998). The presence of *Wolbachia*, *Cardinium, Rickettsia*, *Arsenophonus* and *Spiroplasma* in each sample was determined using genus-specific primers, and the mitochondrial DNA (mtDNA) Cytochrome Oxidase subunit I (*COI*) was amplified and sequenced for 75 samples chosen depending of their infection status with *Cardinium* and *Wolbachia*. The list of primers, for both hosts and symbionts, is given in Table S1 (Additional file 1 in the Supplementary data). For each of the genes under study, the PCRs were done along with both positive and negative controls, in a 10 μl reaction mixture containing 5 μl of 2X QIAGEN Multiplex PCR Master Mix (Qiagen NV, Venlo, The Netherlands), 2 μl RNase free water, 1 μl of each primer at 2 μM, and 1 μl of DNA solution. Amplification conditions were as follows: 15 min at 94°C, and then 35 cycles of 94°C for 30 s, annealing (see primer-specific temperatures in Table S1) for 1 min 30 s, 72°C for 1 min and a final step at 72°C for 10 min. PCR products were visualized on 2% agarose gel. To verify the identity of the amplified endosymbionts genes (for all *Cardinium*- and *Rickettsia*-infected individuals, and up to three *Wolbachia*-infected individuals per population haphazardly chosen within both single- and multiple-infected mites), the PCRs were repeated for positive samples in a 20 μl final volume reaction, the PCR products were purified using Qiagen protocol and materials (QIAquick PCR Purification Kit) and sent for sequencing (Stabvida, Caparica, Portugal). All ITS2 and *COI* genes were also sequenced using the same protocol. The chromatograms were checked manually using MEGA version 5.1 beta (Tamura *et al.* 2011) and the resulting sequences were run against the non-redundant nucleotide database using the BLAST algorithm of the National Center for Biotechnology Information. New sequences have been deposited in GenBank (see Table S2 and S3 in the Supplementary data, Additional file 1).

Phylogenetic analyses of *COI* mitochondrial haplotypes

The phylogenetic relationships between *Tetranychus* spp. haplotypes were reconstructed using maximum-likelihood (ML) analyses based on *COI* mitochondrial nucleotide sequences (fragments of 392 unambiguously aligned sites; best-fitting evolutionary model: HKY + I). The evolutionary model that best fitted the sequence data was determined using the Akaike information criterion with the program MEGA (Tamura *et al.* 2011). ML heuristic searches using a starting tree obtained by neighbor joining were conducted in MEGA (Tamura *et al.* 2011). Clade robustness was assessed by bootstrap analysis using 1000 replicates.

Statistical analyses

Analyses were carried out using the R statistical package (v. 3.3.2). The different statistical models built to analyze the effects of species, or of population within species, on the prevalence of *Wolbachia* and of *Cardinium* are described in the electronic supplementary material, Table S4 (Additional file 1, Supplementary data). The general procedure for building the statistical models was as follows: time (number of days after collection from the field at which DNA extraction was performed), species and the status of infection of the females by *Wolbachia* or *Cardinium* were fit as fixed explanatory variables, whereas population was either fit as a random explanatory variable when analyzing the complete dataset, or as a fixed effect when analyzing the differences between populations within each spider-mite species. The effect of spider-mite species (models 1 and 5) on the proportion of *Wolbachia*- or *Cardinium*-infected females, a binary response variable (1: infected, or 0: uninfected), was analyzed using a generalized linear mixed model with a binomial distribution (glmer, lme4 package), and the effect of population within species (models 2 to 4, and 6 to 8) was analyzed using a generalized linear model with a binomial distribution (glm). When the variables species or population were found to be significant, *a*

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Table 2. Mitochondrial *COI* alleles in spider mite populations. A total of 75 sequences were obtained from individual female spider mites. For each allele, we give the genbank accession number and the corresponding number of mites per population. We also give the total number of mites carrying each allele and the corresponding proportion relative to the total number of mites.

	Genbank accession	*T. ludeni*			*T. evansi*			*T. urticae*										Total number
Species	number [Ref]	Assaf	CVM	Alval	GRA	GH	QL	AlRo	AlBe	DF	LOU	COL	AMP	RF	DC	CH	FR	(proportion)
T. ludeni	GQ141911 [*]	3	5	5	–	–	–	–	–	–	–	–	–	–	–	–	–	13 (0.17)
T. evansi	MF428442 [**]	–	–	–	3	4	1	–	–	–	–	–	–	–	–	–	–	8 (0.11)
	MF428443 [**]	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	3 (0.04)
T. urticae	HM486513 [49]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1 (0.01)
	HM565899 [96]	–	–	–	1	1	–	–	–	–	–	–	–	–	–	–	1	3 (0.04)
	HM565901 [96]	–	–	–	–	–	–	5	5	–	–	–	–	–	–	–	–	10 (0.13)
	MF428440 [**]	1	–	–	1	–	1	–	–	4	4	4	4	5	4	5	3	36 (0.48)
	MF428441 [**]	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	1 (0.01)

[*] Unpublished.
[**] This study.

posteriori contrasts (Crawley 2007) were carried out by aggregating factor levels and testing the fit of the simplified model using ANOVA. For all analyses, maximal models were simplified by sequentially eliminating non-significant terms to establish a minimal model (Crawley 2007), and the significance of the explanatory variables was established using chi-squared tests (Bolker 2008). The significant X^2 values given in the text are for the minimal model (Crawley 2007). Finally, the effect of *Wolbachia* and *Cardinium* infection on sequence divergence among *COI* haplotypes, and, for *Cardinium*-infected individuals, the effect of *Cardinium* strains on *COI* sequence divergence were assessed by a hierarchical analysis of molecular variance (AMOVA; Excoffier, Smouse and Quattro 1992). The statistical significance of the variance components of the AMOVA was determined by nonparametric procedures using 10 000 random permutations. Full dataset are given in Additional files 2, 3 and 4 (Supplementary data).

RESULTS

Species identification: ITS ribosomal types and *COI* mitochondrial haplotypes

Amplification and sequencing of a portion of the ITS2 gene allowed assigning 3 out of the 16 populations collected to *T. evansi*, 3 populations to *T. ludeni* and the 10 remaining populations to *T. urticae* (red morph; Table 1). All sequences obtained from populations of *T. urticae* were strictly identical and matched (100% identity at the nucleotide level) a sequence obtained from *T. urticae* collected in southern Spain (e.g. GenBank: GU565314). Similarly, all sequences obtained for *T. ludeni* (GenBank: MF428439) were strictly identical and matched (99% identity) several sequences obtained for this species worldwide (e.g. New Zealand, Japan, India, or Israel; GenBank: KP744529, AB076371, KU759957 and DQ656451, respectively). For *T. evansi*, however, both ITS2 ribosomal type 1 (GenBank: FJ440674) and type 2 (GenBank: FJ440673; Boubou *et al.* 2011) were found in the population 'QL', but only the type 1 in 'GRA' and 'GH'.

Analysis of mitochondrial *COI* DNA sequences from 75 individuals revealed 64 polymorphic sites within 392 bases sequenced (Table S2), and allowed distinguishing eight different mtDNA haplotypes (each individual within the same haplotype sharing exactly the same *COI* sequence; Table 2; Fig. 1). In *T. urticae*, almost all mites collected in Spain and Portugal belonged to the same haplotype (GenBank: HM565901, and MF428440, respectively), and shared a high degree of similarity with each other (99% identity; the two sequences differed by one single nucleotide mutation; Table S2). One female from the population 'DC' collected in Portugal, however, belonged to a *COI* haplotype (GenBank: MF428441; Table 2) more similar to a haplotype found in the population 'FR' from France (GenBank: HM486513) than to the one from the Spanish populations (96% and 94% identity, respectively; Table S2). In the French population, two other haplotypes were found: the main haplotype found in Portugal (GenBank: MF428440), and another, highly similar to this one (99% identity; GenBank: HM565899). In *T. evansi*, two different haplotypes were found (GenBank: MF428442 and MF428443), which were strictly identical (100% identity, query cover: 91%; Table S2) to the *COI* haplotypes I and II, respectively, originally recorded in France and Spain (GenBank: FJ440676 and FJ440678; Gotoh *et al.* 2009), but also previously found in Portugal (Boubou *et al.* 2011). Conversely, only one haplotype of *T. ludeni* was found and was identical to a haplotype found in China (GenBank: GQ141911; Table 2). Importantly, one *T. ludeni* female from the population 'Assaf', and 4 females from all *T. evansi* populations (from both ITS types), harbored two different *T. urticae COI* haplotypes, but the reverse (*i.e. T. ludeni* or *T. evansi COI* haplotypes associated with *T. urticae* ITS type) was not observed (Table 2).

Inter- and intraspecific variation of endosymbionts prevalence and diversity

Overall, our sampling revealed infections by *Wolbachia*, *Cardinium* and *Rickettsia* within the three spider mites species, but none of the tested populations were found infected by *Arsenophonus* or *Spiroplasma* (Fig. 2).

Wolbachia was the most prevalent endosymbiont with an average of 60.7 ± 3.3% of infected females (Fig. 2). This prevalence differed significantly between host species (model 1, $X^2_2 = 5.92$, $P = 0.05$), with no significant difference between infection prevalence in *T. ludeni* and *T. evansi* (31.6 ± 7.6% and 41.5 ± 7.8%, respectively; $X^2_1 = 0.24$, $P = 0.63$), but the infection frequency in *T. urticae* being significantly higher than in these two species (74.3 ± 3.7%; $X^2_1 = 5.69$, $P = 0.02$). Within species, no significant difference was found in *Wolbachia* prevalence among populations of *T. evansi* (model 2, $X^2_2 = 3.62$, $P = 0.16$), but populations differed significantly for *Wolbachia* infection frequencies in *T. ludeni* and *T. urticae* (model 3, $X^2_2 = 11.51$, $P = 0.003$, and model 4, $X^2_9 = 93.58$, $P < 0.0001$, respectively). In *T. ludeni*, no *Wolbachia*-infected mites were found in 'Assaf', while both 'Alval' and 'CVM' harbored 46.2 ± 10.0% of infected mites. In *T. urticae*, the lowest prevalences were found in the populations collected in Spain (on average 14.8 ± 7.0%) and France (46.7 ± 13.3%; contrast between Spanish–French populations: $X^2_1 = 5.28$, $P = 0.02$), whereas *Wolbachia* infection was fixed in five out of seven of the populations

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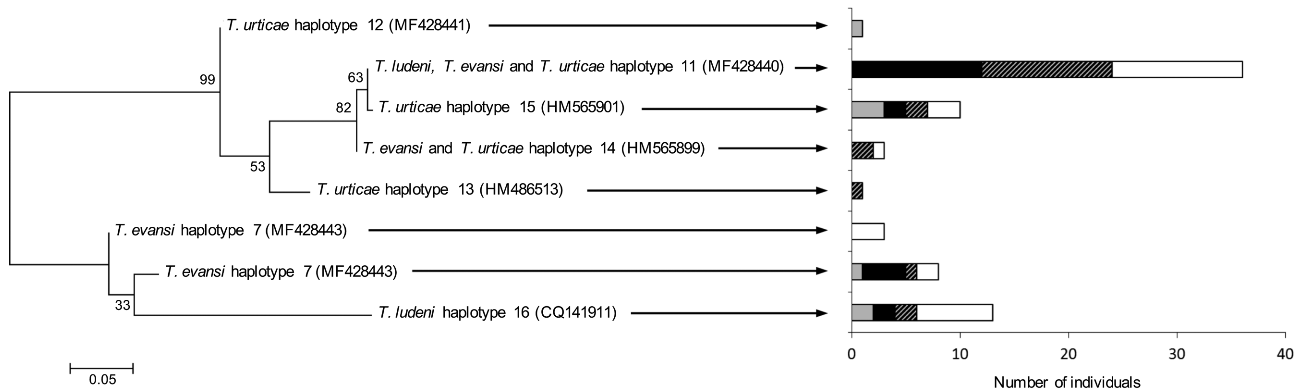

Figure 1. Phylogeny and distribution of *Wolbachia* and *Cardinium* (co)infections among *T. evansi*, *T. ludeni* and *T. urticae* mitochondrial *COI* haplotypes. The left panel shows the phylogeny of the 8 mtDNA haplotypes via maximum likelihood. Numbers on branches indicate percentage bootstrap support for major branches (1000 replicates). GenBank accession numbers are given between brackets. The scale bar is in units of substitutions/site. The right panel gives the distribution of symbiont infection observed with each mtDNA haplotype (black: *Wolbachia*-infected, n = 20; gray: *Cardinium*-infected, n = 7; dashed: *Wolbachia-Cardinium* coinfected individuals, n = 20; white: uninfected specimens, n = 28).

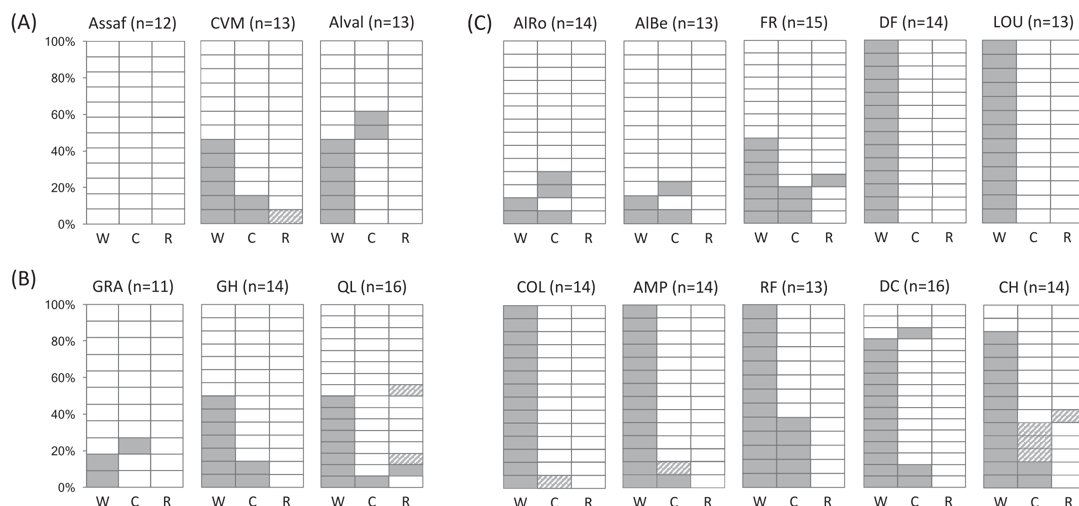

Figure 2. Prevalence of endosymbionts for each spider-mite population of (A) *T. ludeni*, (B) *T. evansi*, and (C) *T. urticae*. Each graph represents a population, in which the rows correspond to individual mites and the columns to their infection status by W: *Wolbachia*; C: *Cardinium*; and R: *Rickettsia*. White cells: uninfected; Gray cells: infected; Hatched cells: *Cardinium* and *Rickettsia* failed sequences but positive through PCR. None of the tested colonies were found infected by *Spiroplasma* or *Arsenophonus*.

collected in Portugal, and the remaining two Portuguese populations (CH and DC) had an average prevalence of 83.3 ± 6.9% (contrasts between CH-DC and the other Portuguese populations: $X^2{}_1 = 13.05$, $P < 0.001$; contrast between CH-DC and the French populations, $X^2{}_1 = 6.99$, $P = 0.008$).

At the molecular level, no diversity of *Wolbachia* was found (Table 3). Of the 42 *Wolbachia*-infected mites for which the *wsp* gene was sequenced, 41 were strictly identical and matched (100% identity at the nucleotide level) several sequences available in GenBank for *Wolbachia* infecting several *Tetranychus* species worldwide (e.g. GenBank: DQ910771 and JX844806 in *T. urticae*, AB096218 and AF358417 in *T. kanzawai*, AB096226 in *T. pueraricola*, AY585711 in *T. cinnabarinus*) and previously attributed to members of the *Wolbachia* B supergroup and Ori subgroup (Gotoh, Noda and Hong 2003). Only one of the sequences obtained (GenBank: MF428430), from a *Wolbachia*-infected mites of the *T. evansi* population 'GRA', differed from the others (82% identity at the nucleotide level), but was highly similar (99% identity at the nucleotide level) to many sequences found in diverse arthropods species such as fruit flies (GenBank: FJ403332, KU870673, DQ834379, or EU116319), beetles (GenBank: KX436089), moths (GenBank: GU166594) or head lice (GenBank: AY596782). Note, however, that strains with similar *wsp* sequences can have very different MLST allelic profiles (Baldo *et al.* 2006), but the amount of DNA obtained from each individual spider mite strongly restrained the possible number of PCRs and an in-depth study of *Wolbachia* variation within *Tetranychus* mites was beyond the scope of this study.

The prevalence of *Cardinium* was 14.6 ± 2.4% on average (total of 32 infected mites over 219). However, we could obtain the *16S rRNA* sequences only for 27 of 32 infected individuals: for five individuals we failed to amplify *Cardinium* through three successive PCR following the first diagnostic of infection. Two main possibilities could explain this failure: first, very low densities of *Cardinium* within these individuals could lead to PCR-based imperfect detection, as shown for other parasites (Zélé *et al.* 2014).

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Table 3. Occurrence and co-occurrence of *Wolbachia wsp*, *Cardinium 16S rRNA* and *Rickettsia gtlA* alleles. A total of 72 sequences were obtained from 80 positive samples through PCR. For each allele, we give the genbank accession number, the corresponding number of infected mites per population, and indicate the symbiont species involved in a multiple infection with superscripts (a: *Cardinium*, b: *Wolbachia* and c: *Rickettsia*). For each allele, we also give the total number of infected mites and the corresponding proportion relative to the total number of mites infected by the same endosymbiont.

Gene (fragment length)	Genbank accession	*T. ludeni*			*T. evansi*			*T. urticae*									Total number
	numbers [Ref]	CVM	Alval	GRA	GH	QL	AlRo	AlBe	DF	LOU	COL	AMP	RF	DC	CH	FR	(proportion)
Wolbachia wsp (524bp)	DQ910771 [72]	$3^{(1a)}$	3	1	$3^{(1a)}$	$3^{(1a1c)}$	$2^{(1a)}$	$2^{(1a)}$	3	3	$3^{(1a)}$	$3^{(2a)}$	$3^{(2a)}$	$3^{(2a)}$	$3^{(2a1c)}$	$3^{(1a1c)}$	41 (0.98)
	MF428430 [*]	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.02)
Cardinium 16S rRNA (325bp)	MF428431 [*]	–	–	–	–	–	1	–	–	–	–	–	–	–	1^{b}	1^{b}	3 (0.11)
	MF428432 [*]	$1^{b,c}$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.04)
	MF428433 [*]	–	–	1	–	–	1	–	–	–	–	1^{b}	–	–	–	–	3 (0.11)
	MF428434 [*]	–	2	–	$2^{(2b)}$	–	1^{b}	$2^{(1b)}$	–	–	–	–	$4^{(4b)}$	$3^{(2b)}$	1^{b}	$2^{(2b)}$	17 (0.63)
	MF428435 [*]	1^{b}	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1 (0.04)
	MF428436 [*]	–	–	–	–	–	–	–	–	–	–	–	1^{b}	–	–	–	1 (0.04)
	MF428437 [*]	–	–	–	–	1^{b}	–	–	–	–	–	–	–	–	–	–	1 (0.04)
Rickettsia gtlA (431bp)	MF428438 [*]	–	–	–	–	1^{b}	–	–	–	–	–	–	–	–	–	1^{b}	2 (1.00)

[*] This study.

Second, we cannot totally exclude the possibility of contaminations or of unspecific amplifications of other bacteria when using *16S rRNA* primers for *Cardinium*. For this latter reason, we did not consider these five individuals as infected in the following analyses. After correction, the prevalence of *Cardinium* was overall of 12.3 ± 2.2% (9.8 ± 4.7% in *T. evansi*, 10.5 ± 5.0% in *T. ludeni*, and 13.6 ± 2.9% in *T. urticae*) with no significant difference in prevalence among the three species (model 5, $X^2{}_2 = 0.32$, $P = 0.85$; Fig. 2). Within species, no differences among populations of *T. evansi* and *T. ludeni* were found (models 6 and 7, $X^2{}_2 = 0.55$, $P = 0.76$ and $X^2{}_2 = 3.25$, $P = 0.19$, respectively), but *Cardinium* prevalences in populations of *T. urticae* differed significantly (model 8, $X^2{}_9 = 31.19$, $P < 0.001$). Indeed, in three populations ('COL', 'DF' and 'LOU'), none of the mites tested were infected, but *a posteriori* contrast analyses among the remaining (infected) populations revealed no significant difference in *Cardinium* prevalence ($X^2{}_6 = 6.62$, $P = 0.36$).

At the molecular level, we found some degrees of inter-individual variation in the *Cardinium 16S rRNA* sequences (Table 3). Of the 27 *Cardinium 16S rRNA* sequences obtained, we could discriminate six different alleles, which, despite sharing a high degree of similarity (97%–99% identity at the nucleotide level), differed by single nucleotide mutations over 13 different nucleotide sites (Table S3). The most prevalent allele (GenBank: MF428434) was found in 17 *Cardinium*-infected mites over the three species (c.a. 63%; Table 3). The corresponding sequence is very close to several sequences found for *Cardinium* in diverse arthropod species, including spider mites (e.g. 99% identity at the nucleotide level with a sequence obtained in *T. urticae* in Japan; GenBank: AB241134; Gotoh, Noda and Ito 2007a). Three additional mites harbored highly similar *Cardinium* alleles (99% identity at the nucleotide level; GenBank: MF428435, MF428436, and MF428437, respectively; Tables 3 and S3). Interestingly, we also found alleles identical or highly similar (99% identity at the nucleotide level), to an allele previously found for *Cardinium* endosymbiont of *Scaphoideus titanus*, the American grapevine leafhopper (GenBank: AM042540; Marzorati *et al.* 2006), for two *T. urticae* (GenBank: MF428431), and one *T. ludeni* (GenBank: MF428432) individuals, respectively. Finally, four individuals (one *T. evansi* and three *T. urticae*) harbored *Cardinium* sequences (GenBank: MF428433) with double nucleotide peaks at the nucleotide positions that differ between AB241134 and MF428434 (Table S3). This result suggests co-infection with two different *Cardinium* strains within these mites.

Infection by *Rickettsia* was rare. Indeed, this symbiont was found in only 6 of 219 individuals belonging to the three spider mite species (2.7 ± 1.1%; Fig. 2): 7.3 ± 4.1% of *T. evansi* individuals were infected, belonging to a single population, 2.6 ± 2.6% of *T. ludeni* individuals, also from a single population, and 1.4 ± 1.0% of *T. urticae* individuals, stemming from two populations. However, for four of the six infected individuals we failed to obtain the *gtlA* sequences of *Rickettsia*. After correction, the prevalence of *Rickettsia* was overall of 0.9 ± 0.6% among the three mite species (2.4 ± 2.4% in *T. evansi*, 0% in *T. ludeni*, and 0.7 ± 0.7% in *T. urticae*). The two sequences obtained, one in *T. evansi*, and one in *T. urticae* (GenBank: MF428438; Table 3), were strictly identical to a sequence previously found in *T. urticae* in China (GenBank: KP828066; Zhang *et al.* 2016b). The most closely related sequences found in other arthropod hosts were from *Rickettsia* in the parasitoid wasp *Pnigalio sp.* (97% identity at the nucleotide level; GenBank: GU559856), and in *B. tabaci* (96% identity; GenBank: DQ077708).

Multiple infections and covariation of *Wolbachia* and *Cardinium* prevalence

Several individuals within the three mites species, and across different populations, were infected with more than one endosymbiont. We detected 25 mites (11.4 ± 2.2% in average) coinfected by *Wolbachia* and *Cardinium*, four mites (ca. 1.8 ± 0.9%) coinfected by *Wolbachia* and *Rickettsia* and one individual (ca. 0.5 ± 0.5%) infected by the three endosymbionts (population 'CVM'; Fig. 2). Moreover, our analyses revealed a co-variation between the prevalence of *Wolbachia* and that of *Cardinium*, both symbionts affecting significantly the probability of infection by each other (model 1, $X^2{}_1 = 4.99$, $P = 0.03$, and model 5, $X^2{}_1 = 4.56$, $P = 0.03$, respectively; Fig. 3). However, no particular pattern of association between *Cardinium 16S rRNA* gene sequences, *Wolbachia* infection, mites species or population emerged (Table 3).

Effects of *Wolbachia* and *Cardinium* infection on mtDNA variation

No particular pattern of association was found between the mitochondrial *COI* DNA sequences obtained from individual mites and their *Wolbachia* and/or *Cardinium* infection status ($F_{1,74} = 0.05$; $P = 0.07$, and $F_{1,74} = 0.009$; $P = 0.26$, respectively; Fig. 1), or between their mitochondrial *COI* DNA sequences and

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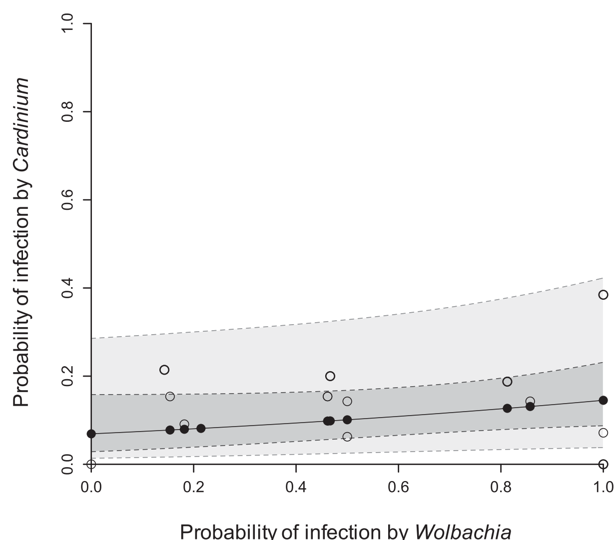

Figure 3. Mixed logistic regression of individual probability of *Wolbachia* infection on the probability of *Cardinium* infection. The solid line represents the mean predicted values; the dark shaded area represents the range of the 95% confidence intervals based on the fixed-effects uncertainty only, while the light shaded area also includes the variance of a random factor 'population'. The observed data at the individual level are not represented as they all take the values '0' or '1' for both *Wolbachia* and *Cardinium* infection. Open circles are the observed mean prevalence of *Cardinium* per population plotted against the observed mean prevalence of *Wolbachia*, while filled circles are their corresponding linear unbiased predictors.

the *Cardinium 16S rRNA* type ($F_{1,75}$ = 0.22; P = 0.18, Table 4). For instance, the most prevalent *Cardinium* sequence obtained (MF428434; c.a. 63% of *Cardinium* infected-mites; Table 3) was found for all mitotypes, and, reciprocally, several mitotypes harbored different *Cardinium* sequences (Table 4).

DISCUSSION

In this study, we analyzed the prevalence and diversity of South-West European populations of spider mites and their endosymbionts. The populations collected mainly belonged to three species of spider mites: *T. urticae*, *T. evansi* and *T. ludeni*, the most prevalent being *T. urticae*. Our sampling effort was such that we did not pre-select the plants sampled. Consequently, this result may reflect the host range of *T. urticae*, which is larger than that of *T. evansi* and *T. ludeni* (Migeon and Dorkeld 2006–2017). However, our results contrast with those of another study conducted in the Eastern part of the Iberian Peninsula, near Valencia, where *T. evansi* is the most abundant species (Ferragut, Garzon-Luque and Pekas 2013). A study on invasion scenarios of *T. evansi* predicts that populations around Valencia stem from an invasion route that differs from that leading to the establishment of *T. evansi* in Portugal (Boubou *et al.* 2012). Possibly, the invasive success of this species differs depending on the invasion event. The *T. evansi* clades that we recovered match those found earlier for the same region in Portugal (Boubou *et al.* 2011).

Analysis of mitochondrial and nuclear DNA revealed a high *COI* diversity in comparison to that of ITS for *T. urticae*, as found earlier (Navajas *et al.* 1998). Overall, these two markers yielded concordant species identifications in most individuals tested. However, we found mitochondrial DNA of *T. urticae* in the other two species, albeit at low frequencies. Screening contaminations seems unlikely, as mitochondrial and nuclear DNA were extracted from the same individuals, PCR controls were all correct and these unexpected ITS-*COI* associations occurred for two different mitotypes, and in four different populations (one of *T. ludeni* and the three *T. evansi*). Therefore, it seems that introgression may occur among these species, and this suggests that they co-occur on the same host plants and locations, although we did not detect species co-occurrence in this study. In laboratory studies using these species, viable hybrids have been found at very low frequencies as well (Sato, Alba and Sabelis 2014; Clemente *et al.* 2016, but see Clemente *et al.* 2018), and assortative mating was either low (Clemente *et al.* 2016) or absent (Sato, Alba and Sabelis 2014). These findings are compatible with the possible introgression found here. Nevertheless, the low frequencies observed here might also be due to the detection of F1 hybrids only, as in most cases such hybrids are sterile (Clemente *et al.* 2016). Curiously, however, this introgression seemed to be unidirectional, with the mtDNA of *T. ludeni* or *T. evansi* never being found in individuals with nuclear *T. urticae* DNA. It would thus be interesting to corroborate these results in future laboratory experiments, along with broader analyses of natural populations of these species.

Of the five endosymbionts screened, only *Wolbachia*, *Cardinium* and *Rickettsia* were found to infect spider mites, whereas none of the tested individuals harbored *Spiroplasma* or *Arsenophonus*. However, this is the first report of *Wolbachia*, *Cardinium* and *Rickettsia* in *T. evansi* and *T. ludeni*. Overall, *Wolbachia* prevalence was higher than that of the two other symbionts, even though it was lower in *T. evansi* and *T. ludeni*, and in non-Portuguese *T. urticae* populations. Such variable

Table 4. Co-occurrence of *Cardinium 16S rRNA* and mitochondrial *COI* alleles of spider mites. *Cardinium 16S rRNA* and mite *COI* sequences were obtained from a total of 29 *Cardinium*-infected individual female spider mites. For each *Cardinium 16S rRNA* allele, we give the corresponding number of infected mites per *COI* allele (Genbank accession numbers [Ref]), and the species to which they belong between brackets. Te: *T. evansi*, Tl: *T. ludeni*, Tu: *T. urticae*.

Cardinium 16S rRNA	Mites' COI						
	HM486513 [49]	HM565899 [96]	HM565901 [96]	MF428440 [*]	MF428441 [*]	MF428442 [*]	GQ141911 [**]
MF428431 [*]	–	1 (Tu)	1 (Tu)	–	–	–	–
MF428432 [*]	–	–	–	–	–	–	1 (Tl)
MF428433 [*]	–	–	1 (Tu)	2 (Tu)	–	1 (Te)	–
MF428434 [*]	1 (Tu)	2 (Te-Tu)	3 (Tu)	9 (Tu)	1 (Tu)	1 (Te)	2 (Tl)
MF428435 [*]	–	–	–	–	–	–	1 (Tl)
MF428436 [*]	–	–	–	1 (Tu)	–	–	–
MF428437 [*]	–	–	–	1 (Te)	–	–	–

[*] This study.
[**] Unpublished.

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levels of *Wolbachia* prevalence across species and populations have already been reported in other regions worldwide (Gotoh, Noda and Hong 2003; Gotoh *et al.* 2007b; Zhang *et al.* 2016b). Interestingly, however, we found *Wolbachia* in the invasive species *T. evansi*. This runs counter other studies that have shown the loss of *Wolbachia* in invasive populations of fire ants (Shoemaker *et al.* 2000), Argentine ants (Reuter, Pedersen and Keller 2005) and Australian thrips (Nguyen, Spooner-Hart and Riegler 2016). In invasive species, the loss of *Wolbachia* is expected because introduced populations are likely to harbor only a subset of the symbionts of their parental population(s), possibly because a bottleneck effect during invasion. Spider mites may thus be used to test hypotheses concerning the loss or maintenance of symbionts in invasive populations (see below). *Cardinium* was found in all three spider mites species, which corroborates previous findings of a relatively high occurrence of this symbiont in mites (Nakamura *et al.* 2009; Zhang *et al.* 2016a) compared with other arthropods groups (Weeks, Velten and Stouthamer 2003; Zchori-Fein and Perlman 2004; Duron *et al.* 2008; Nakamura *et al.* 2009). However, its prevalence was overall much lower than that of *Wolbachia*, a result not always found in spider mites (Zhang *et al.* 2016b). The prevalence of *Rickettsia* was very low, as found by Zhang *et al.* (2016b), but this study is the first to report *Rickettsia* infection in *T. ludeni* and *T. evansi*. Finally, although *Spiroplasma* has been previously found in *T. urticae* (Enigl and Schausberger 2007), we did not detect it in our study, as reported by Zhang *et al.* (2016b). Similarly, we did not find *Arsenophonus*. Although the latter has been found in the Acari (Clay *et al.* 2008; Dergousoff and Chilton 2010; Clayton *et al.* 2015; Duron *et al.* 2017), it has, to our knowledge, never been reported in spider mites.

The maintenance and spread of reproductive manipulators in nature, hence their prevalence, are mainly affected by the type and strength of reproductive manipulation, the fitness costs/benefits of the symbionts to their hosts, and the efficiency of vertical transmission (Werren, Baldo and Clark 2008; Engelstadter and Hurst 2009). Variability in any of these factors might explain the observed lower prevalence of *Cardinium* than that of *Wolbachia*. Both endosymbionts induce CI in spider mites (Gotoh, Noda and Ito 2007a; Gotoh *et al.* 2007b; Ros and Breeuwer 2009; Zhu *et al.* 2012; Zhao *et al.* 2013b). Although some studies found that *Cardinium* induces a higher level of CI than *Wolbachia* in several mites species (e.g. *T. pierci* and *Bryobia sarothamni*; Ros and Breeuwer 2009; Zhu *et al.* 2012), another showed that *Cardinium* may not induce CI in *T. urticae* (Gotoh, Noda and Ito 2007a). It is thus possible that its lower prevalence is due to low reproductive manipulation. In addition, *Cardinium* may exhibit imperfect maternal transmission, as found in thrips (Nguyen *et al.* 2017), or in the parasitic wasp *Encarsia pergandiella* (although near-perfect in this species; Perlman, Kelly and Hunter 2008). Conversely, the high prevalence of a single *Wolbachia* strain found here may be due to this strain inducing low fitness costs, strong reproductive manipulation and having nearly perfect maternal transmission. An in-depth study of the fitness effects of these endosymbionts, along with their ability to manipulate the reproduction of their host in these populations would thus allow testing these hypotheses.

These general predictions, coming from epidemiological modeling, may be tempered by environmental factors. Indeed, a large number of other factors have been identified, such as symbiont-host genotype interactions, host diet or temperature effects (e.g. Van Opijnen and Breeuwer 1999; Mouton *et al.* 2007; Wilkinson, Koga and Fukatsu 2007; Gotoh *et al.* 2007b; Pan *et al.* 2013; Zhang *et al.* 2013b; Boivin *et al.* 2014; Suh *et al.* 2015). For instance, Gotoh, Noda and Ito (2007a) showed that *Wolbachia* is much more sensitive to elevated temperature than *Cardinium* in *T. puerariçola*. In our study, the higher summer temperatures in Almeria (Spain; c.a. 26.7°C on average in August; http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos/valoresclimatologicos?l=6325O&k=and) compared to that of the region of Lisbon (Portugal; c.a. 23.5°C on average in August; http://www.ipma.pt/pt/oclima/normais.clima/1981-2010/012/) might explain the lower prevalence of *Wolbachia*, but not that of *Cardinium* in the *T. urticae* populations collected in this region. The fact that a single *Wolbachia wsp* sequence was found in this region, which contrasts with other studies of spider mites in other regions (Gotoh, Noda and Hong 2003; Gotoh *et al.* 2007b; Ros *et al.* 2012; Zhang *et al.* 2013a,b), may indicate specific adaptation of this strain to the environmental conditions of South-Western Europe. Host plants of herbivorous arthropods may also affect symbiont diversity and identity (e.g. Ferrari *et al.* 2004; Ahmed *et al.* 2010; Brady and White 2013; Guidolin and Consoli 2017; but see Ji *et al.* 2015). Moreover, bacterial endosymbionts may also be conditional mutualists in some host species, conferring advantages under certain environmental conditions. For instance, they may compensate for the nutritionally deficient diets of herbivore hosts and promote dietary specialization by affecting their performance on plants, they may also increase their ability to tolerate high temperatures, or may protect them against natural enemies (reviewed in Haine 2008; Moran, McCutcheon and Nakabachi 2008; Frago, Dicke and Godfray 2012). Unfortunately, our sampling design, largely unbalanced with respect to these factors, does not allow testing their relevance for the system under study.

Interaction between symbionts within a single host may also be an important factor affecting their prevalence, either negatively (competition) or positively (facilitation) (Vautrin *et al.* 2008; Vautrin and Vavre 2009; Hellard *et al.* 2015). As previously found in several spider mites species in China (Liu, Miao and Hong 2006; Zhao *et al.* 2013b; Zhang *et al.* 2016b), and in Northern Europe (Ros and Breeuwer 2009; Ros *et al.* 2012), in this study *Cardinium* prevalence was higher in populations also infected by *Wolbachia*, which may suggest facilitation between these endosymbionts. The maintenance of *Cardinium* in the spider mites populations collected in this study might thus be due to a hitchhiking effect, whereby *Wolbachia*-induced phenotypic changes favor their own transmission, but also that of other co-infecting symbionts (Frank 1998; Engelstadter, Telschow and Hammerstein 2004; Vautrin and Vavre 2009). Moreover, the effects of each symbiont on their host may be exacerbated in presence of the other. Indeed, several studies showed that *Wolbachia* can strengthen the induction of cytoplasmic incompatibility by *Cardinium* in several spider-mite (Zhu *et al.* 2012; Zhao, Zhang and Hong 2013a; Zhao *et al.* 2013b), or arthropods (White *et al.* 2009) species (but see Nguyen *et al.* 2017). Moreover, Ros and Breeuwer (2009) found a higher fecundity advantage conferred by *Wolbachia-Cardnium* co-infections in *Bryobia sarothamni* mites, than by *Wolbachia* or *Cardinium* single infections (although this result may be confounded by the host genetic background, as acknowledged by the authors). Studies of the fitness effects of both *Wolbachia* and *Cardinium*, in single and multiple infections in these populations would allow corroborating or rejecting this hypothesis.

Symbionts usually spread within species, but they may also cross species boundaries via two main routes. First, cytoplasmic introgression, through interspecific hybridization events, may favor the spread of vertically transmitted symbionts among species (Raychoudhury *et al.* 2009; Jackel, Mora and Dobler 2013).

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The incongruences between the mites *COI* and ITS sequences obtained for some individuals in this study (see above), go in that direction. Second, intimate ecological associations, such as trophic interactions and plant feeding in particular, may provide horizontal transmission opportunities. It has been shown, for instance, for *Rickettsia* and *Wolbachia* in whiteflies (Caspi-Fluger *et al*. 2012; Li *et al*. 2017) and for *Cardinium* in leaf hoppers (Gonella *et al*. 2015), and could explain the genetic diversity patterns observed here. Indeed, multiple events of horizontal transfer of *Cardinium* in the field would explain both its maintenance at low frequency within populations, the high sequence variation (i.e. potential strains) found for this symbiont, and the lack of association with the host mtDNA. In addition, phylogenetic similarity of donor and recipient host species should facilitate horizontal transmission (Boivin *et al*. 2014), and incongruences between phylogenies of host and parasites, suggesting occasional horizontal transmission of *Cardinium* and/or *Wolbachia* between hosts, have been found at many occasions in spider mites (Yu *et al*. 2011; Ros *et al*. 2012; Zhang *et al*. 2016b; but see Zhang *et al*. 2013a). However, although these symbionts might have unexpected tolerance towards stressful conditions (e.g. *Wolbachia* can be maintained alive for at least a week in synthetic liquid medium out of the host cells; Rasgon, Gamston and Ren 2006), the acquisition of a symbiont by horizontal transfer might be transient and not subsequently vertically transmitted in the new host species (Chiel *et al*. 2009). Further studies should thus be conducted to determine the occurrence of both intra- and interspecific horizontal transfers of these endosymbionts under controlled laboratory conditions in spider mites.

In conclusion, this study highlights the richness and complexity of the symbiont community in spider mites. Although the factors affecting the spread and persistence of these endosymbionts in host populations remain largely unexplored, this system has the potential to become a model system in the study of host-symbiont interactions.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Authors' contributions

Experimental conception and design: FZ, OD, MW, SM, IO; field collections: FZ, IS, SM; molecular analyses: FZ, IS; statistical analyses: FZ; phylogenetic analyses: OD; paper writing: FZ, SM, with input from all authors. Funding: SM, IO. All authors have read and approved the final version of the manuscript.

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