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

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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 *T. evansi* and *T. 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* *gltA* 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*.

1 **Introduction**

2 Terrestrial arthropods often harbour several bacterial microorganisms that occur in the
3 cytoplasm of host cells, and are maternally inherited from mother to offspring through the
4 egg cytoplasm (Moran *et al.*, 2008). In the last decade, intensive field surveys of distinct
5 arthropod species revealed that the most common of these endosymbionts are *Wolbachia*,
6 *Rickettsia*, *Cardinium*, *Arsenophonus* and *Spiroplasma* (Duron *et al.*, 2008, Weinert *et al.*,
7 2015). Whereas *Wolbachia* is present in most groups, *Rickettsia* and *Cardinium* have low
8 incidence in the hexapoda relative to other groups, with the exception of hemiptera (Weeks
9 *et al.*, 2003, Zchori-Fein & Perlman, 2004, Weinert *et al.*, 2015). These three bacterial
10 genera are also extremely prevalent in chelicerates, especially in spiders, mites and ticks
11 (Duron *et al.*, 2008, Weinert *et al.*, 2015, Zhang *et al.*, 2016a, Duron *et al.*, 2017).

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

24 Although traditionally viewed as vertically-transmitted symbionts, evidence of
25 horizontal transmission events stems from incongruences between host and symbiont
26 phylogenies (Schilthuizen & Stouthamer, 1997, Vavre *et al.*, 1999, Raychoudhury *et al.*,
27 2009, Gerth *et al.*, 2013). Indeed, a few recent studies have provided direct evidence for
28 horizontal transmission, and suggest parasitism and feeding as the two main routes of
29 transfer. For instance, *Wolbachia* can be transmitted between whiteflies via parasitoids
30 (Ahmed *et al.*, 2015), or to a parasitoid from its infected drosophila host (Heath *et al.*, 1999),
31 *Spiroplasma* may be transmitted between two *Drosophila* species via ectoparasitic mites
32 (Jaenike *et al.*, 2007, Brown & Lloyd, 2015), and *Rickettsia* infecting *Bemisia tabaci* may
33 be transferred to parasitoids, although it is not vertically transmitted in the latter (Chiel *et*

34 *al.*, 2009). Similarly, *Trichogramma* wasps of the same or different species have
35 exchanged symbionts when infecting the same butterfly egg (Huigens *et al.*, 2004).
36 Symbionts may also be transmitted via the resource that their hosts feed on (but see Faria
37 *et al.*, 2016). The most compelling evidence for this infection route comes from
38 herbivorous arthropods, in which horizontal transmission via plant feeding has been shown
39 for *Rickettsia* and *Wolbachia* in whiteflies (Caspi-Fluger *et al.*, 2012, Li *et al.*, 2017) and
40 for *Cardinium* in leaf hoppers (Gonella *et al.*, 2015). However, the incidence of horizontal
41 transmission for many vertically-transmitted arthropods endosymbionts is still under
42 debate and other studies do not corroborate these results (e.g. Gualtieri *et al.*, 2017).

43 Several studies have shown that herbivorous spider mites are often infected with
44 *Wolbachia* and *Cardinium* (Gotoh *et al.*, 2003, Liu *et al.*, 2006, Gotoh *et al.*, 2007b, Xie *et al.*
45 *al.*, 2011, Ros *et al.*, 2012, Suh *et al.*, 2015, Zhang *et al.*, 2016b) and occasionally with
46 *Spiroplasma* (Zhang *et al.*, 2016b, Staudacher *et al.*, 2017) and *Rickettsia* (Hoy &
47 Jeyaprakash, 2005, Zhang *et al.*, 2016b). In spider mites, *Wolbachia* and *Cardinium* may
48 have diverse fitness effects. In particular, they may cause different degrees of CI (Gotoh *et al.*
49 *al.*, 2003, Gotoh *et al.*, 2007a, Gotoh *et al.*, 2007b, Ros & Breeuwer, 2009, Xie *et al.*,
50 2011, Zhu *et al.*, 2012, Suh *et al.*, 2015, Xie *et al.*, 2016). Moreover, these bacteria have
51 been found in the same individual host (Ros *et al.*, 2012, Zhu *et al.*, 2012, Zhao *et al.*,
52 2013b, Zhao *et al.*, 2013a, Xie *et al.*, 2016), and multiple strains of these symbionts can be
53 found in spider-mite populations, although levels of nucleotide diversity depend on the
54 host species and/or populations (Gotoh *et al.*, 2003, Liu *et al.*, 2006, Gotoh *et al.*, 2007a,
55 Gotoh *et al.*, 2007b, Yu *et al.*, 2011, Ros *et al.*, 2012, Zhang *et al.*, 2016b).

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

62

63 **Material and methods**

64 *Spider-mite collection and population rearing*

65 Populations of Tetranychidae mites were collected from September to December 2013 at
66 several locations in Central Portugal (Table 1). In addition, two populations were collected

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

77

78 *Molecular species identification and screening for endosymbiont infection*

79 From 0 to 100 days after collection, adult spider mite females (n=11-16) were randomly
80 picked from each colony and individually analyzed for the presence of reproductive
81 manipulators and for species identification. Total genomic DNA was extracted from each
82 individual spider mite using the Sigma-Aldrich protocol and materials (GenElute™
83 Mammalian Genomic DNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO, United States).
84 Total DNA was eluted in the final step with 20µl RNase-free water (Qiagen NV, Venlo, The
85 Netherlands) in order to increase the concentration of DNA obtained from these very tiny
86 animals (c.a. 300µm long). The same DNA from each individual was used to screen for the
87 presence of potential endosymbionts and to identify the spider-mite species. These
88 identifications were performed by PCR amplification then sequencing of a fragment of the
89 nuclear ribosomal DNA (rDNA) ITS2 (internal transcribed spacer 2) region, which is the
90 most commonly used marker to distinguish among spider-mite species (Ben-David *et al.*,
91 2007, Hurtado *et al.*, 2008), as the mitochondrial DNA (mtDNA) Cytochrome Oxidase
92 subunit I (*COI*) is more polymorph in spider mites (Navajas *et al.*, 1998). The presence of
93 *Wolbachia*, *Cardinium*, *Rickettsia*, *Arsenophonus* and *Spiroplasma* in the each sample was
94 determined using genus-specific primers, and the mitochondrial DNA (mtDNA) Cytochrome
95 Oxidase subunit I (*COI*) was amplified and sequenced for 75 samples chosen depending of
96 their infection status with *Cardinium* and *Wolbachia*. The list of primers, for both hosts and
97 symbionts, is given in Table S1 (Additional file 1 in the Supplementary data). For each of the
98 genes under study, the PCRs were done along with both positive and negative controls, in a
99 10µl reaction mixture containing 5µl of 2X QIAGEN Multiplex PCR Master Mix (Qiagen
100 NV, Venlo, The Netherlands), 2µl RNase free water, 1µl of each primer at 2µM, and 1µl of

101 DNA solution. Amplification conditions were as follows: 15min at 94°C, then 35 cycles of
102 94°C for 30s, annealing (see primer-specific temperatures in Table S1) for 1min30s, 72°C for
103 1min and a final step at 72°C for 10min. PCR products were visualized on 2% agarose gel.
104 To verify the identity of the amplified endosymbionts genes (for all *Cardinium*- and
105 *Rickettsia*-infected individuals, and up to three *Wolbachia*-infected individuals per population
106 haphazardly chosen within both single- and multiple-infected mites), the PCRs were repeated
107 for positive samples in a 20µl final volume reaction, the PCR products were purified using
108 Qiagen protocol and materials (QIAquick PCR Purification Kit) and sent for sequencing
109 (Stabvida, Caparica, Portugal). All ITS2 and *COI* genes were also sequenced using the same
110 protocol. The chromatograms were checked manually using MEGA version 5.1 beta (Tamura
111 *et al.*, 2011) and the resulting sequences were run against the non-redundant nucleotide
112 database using the BLAST algorithm of the National Center for Biotechnology Information
113 (NCBI). New sequences have been deposited in GenBank (see Table S2 and S3 in the
114 Supplementary data, Additional file 1).

115

116 *Phylogenetic analyses of COI mitochondrial haplotypes*

117 The phylogenetic relationships between *Tetranychus* spp. haplotypes were reconstructed
118 using maximum-likelihood (ML) analyses based on *COI* mitochondrial nucleotide sequences
119 (fragments of 392 unambiguously-aligned sites; best-fitting evolutionary model: HKY+I).
120 The evolutionary model that best fitted the sequence data was determined using the Akaike
121 information criterion with the program MEGA (Tamura *et al.*, 2011). ML heuristic searches
122 using a starting tree obtained by neighbor joining were conducted in MEGA (Tamura *et al.*,
123 2011). Clade robustness was assessed by bootstrap analysis using 1000 replicates.

124

125 *Statistical analyses*

126 Analyses were carried out using the R statistical package (v. 3.3.2). The different statistical
127 models built to analyse the effects of species, or of population within species, on the
128 prevalence of *Wolbachia* and of *Cardinium* are described in the electronic supplementary
129 material, Table S4 (Additional file 1, Supplementary data). The general procedure for
130 building the statistical models was as follows: time (number of days after collection from the
131 field at which DNA extraction was performed), species and the status of infection of the
132 females by *Wolbachia* or *Cardinium* were fit as fixed explanatory variables, whereas
133 population was either fit as a random explanatory variable when analysing the complete
134 dataset, or as a fixed effect when analysing the differences between populations within each

135 spider-mite species. The effect of spider-mite species (models 1 and 5) on the proportion of
136 *Wolbachia*- or *Cardinium*-infected females, a binary response variable (1: infected, or 0:
137 uninfected), was analysed using a generalized linear mixed model with a binomial
138 distribution (glmer, lme4 package), and the effect of population within species (models 2 to
139 4, and 6 to 8) was analysed using a generalized linear model with a binomial distribution
140 (glm). When the variables species or population were found to be significant, *a posteriori*
141 contrasts (Crawley, 2007) were carried out by aggregating factor levels and testing the fit of
142 the simplified model using ANOVA. For all analyses, maximal models were simplified by
143 sequentially eliminating non-significant terms to establish a minimal model (Crawley, 2007),
144 and the significance of the explanatory variables was established using chi-squared tests
145 (Bolker, 2008). The significant X^2 values given in the text are for the minimal model
146 (Crawley, 2007). Finally, the effect of *Wolbachia* and *Cardinium* infection on sequence
147 divergence among *COI* haplotypes, and, for *Cardinium*-infected individuals, the effect of
148 *Cardinium* strains on *COI* sequence divergence, were assessed by a hierarchical analysis of
149 molecular variance (AMOVA; Excoffier *et al.*, 1992). The statistical significance of the
150 variance components of the AMOVA was determined by nonparametric procedures using
151 10000 random permutations. Full dataset are given in Additional files 2, 3 and 4
152 (Supplementary data).

153

154 **Results**

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

156 Amplification and sequencing of a portion of the ITS2 gene allowed assigning 3 out of the 16
157 populations collected to *T. evansi*, 3 populations to *T. ludeni* and the 10 remaining
158 populations to *T. urticae* (red morph; Table 1). All sequences obtained from populations of *T.*
159 *urticae* were strictly identical and matched (100% identity at the nucleotide level) a sequence
160 obtained from *T. urticae* collected in southern Spain (e.g. GenBank: GU565314). Similarly
161 all sequences obtained for *T. ludeni* (GenBank: MF428439) were strictly identical and
162 matched (99% identity) several sequences obtained for this species worldwide (e.g. New
163 Zealand, Japan, India, or Israel; GenBank: KP744529, AB076371, KU759957 and
164 DQ656451, respectively). For *T. evansi*, however, both ITS2 ribosomal type 1 (GenBank:
165 FJ440674) and type 2 (GenBank: FJ440673; Boubou *et al.*, 2011) were found in the
166 population “QL”, but only the type 1 in “GRA” and “GH”.

167 Analysis of mitochondrial *COI* DNA sequences from 75 individuals revealed 64

168 polymorphic sites within 392 bases sequenced (Table S2), and allowed distinguishing eight
169 different mtDNA haplotypes (each individual within the same haplotype sharing exactly the
170 same *COI* sequence; Table 2; Fig. 1). In *T. urticae*, almost all mites collected in Spain and
171 Portugal belonged to the same haplotype (GenBank: HM565901, and MF428440,
172 respectively), and shared a high degree of similarity with each other (99% identity; the two
173 sequences differed by one single nucleotide mutation; Table S2). One female from the
174 population “DC” collected in Portugal, however, belonged to a *COI* haplotype (GenBank:
175 MF428441; Table 2) more similar to a haplotype found in the population “FR” from France
176 (GenBank: HM486513) than to the one from the Spanish populations (96% and 94% identity,
177 respectively; Table S2). In the French population, two other haplotypes were found: the main
178 haplotype found in Portugal (GenBank: MF428440), and another, highly similar to this one
179 (99% identity; GenBank: HM565899). In *T. evansi*, two different haplotypes were found
180 (GenBank: MF428442 and MF428443), which were strictly identical (100% identity, query
181 cover: 91%; Table S2) to the *COI* haplotypes I and II, respectively, originally recorded in
182 France and Spain (GenBank: FJ440676 and FJ440678; Gotoh *et al.*, 2009), but also
183 previously found in Portugal (Boubou *et al.*, 2011). Conversely, only one haplotype of *T.*
184 *ludeni* was found and was identical to a haplotype found in China (GenBank: GQ141911;
185 Table 2). Importantly, one *T. ludeni* female from the population “Assaf”, and 4 females from
186 all *T. evansi* populations (from both ITS types), harbored two different *T. urticae COI*
187 haplotypes, but the reverse (*i.e.*, *T. ludeni* or *T. evansi COI* haplotypes associated to *T. urticae*
188 ITS type) was not observed (Table 2).

189

190 *Inter- and intraspecific variation of endosymbionts prevalence and diversity*

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

194 *Wolbachia* was the most prevalent endosymbiont with an average of $60.7 \pm 3.3\%$ of
195 infected females (Fig. 2). This prevalence differed significantly between host species (model
196 1, $X^2_2 = 5.92$, $p=0.05$), with no significant difference between infection prevalence in *T.*
197 *ludeni* and *T. evansi* ($31.6 \pm 7.6\%$ and $41.5 \pm 7.8\%$, respectively; $X^2_1 = 0.24$, $p=0.63$), but the
198 infection frequency in *T. urticae* being significantly higher than in these two species ($74.3 \pm$
199 3.7% ; $X^2_1 = 5.69$, $p=0.02$). Within species, no significant difference was found in *Wolbachia*
200 prevalence among populations of *T. evansi* (model 2, $X^2_2 = 3.62$, $p=0.16$), but populations
201 differed significantly for *Wolbachia* infection frequencies in *T. ludeni* and *T. urticae* (model

202 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
203 *Wolbachia*-infected mites were found in “Assaf”, while both “Alval” and “CVM” harbored
204 46.2 ± 10.0 % of infected mites. In *T. urticae*, the lowest prevalences were found in the
205 populations collected in Spain (on average 14.8 ± 7.0 %) and France (46.7 ± 13.3 %; contrast
206 between Spanish-French populations: $X^2_1 = 5.28$, $p=0.02$), whereas *Wolbachia* infection was
207 fixed in 5 out of 7 of the populations collected in Portugal, and the remaining two Portuguese
208 populations (CH and DC) had an average prevalence of 83.3 ± 6.9 % (contrasts between CH-
209 DC and the other Portuguese populations: $X^2_1 = 13.05$, $p<0.001$; contrast between CH-DC
210 and the French populations, $X^2_1 = 6.99$, $p=0.008$).

211 At the molecular level, no diversity of *Wolbachia* was found (Table 3). Of the 42
212 *Wolbachia*-infected mites for which the *wsp* gene was sequenced, 41 were strictly identical
213 and matched (100% identity at the nucleotide level) several sequences available in GenBank
214 for *Wolbachia* infecting several *Tetranychus* species worldwide (e.g. GenBank: DQ910771
215 and JX844806 in *T. urticae*, AB096218 and AF358417 in *T. kanzawai*, AB096226 in *T.*
216 *pueraricola*, AY585711 in *T. cinnabarinus*) and previously attributed to members of the
217 *Wolbachia* B supergroup and Ori subgroup (Gotoh *et al.*, 2003). Only one of the sequences
218 obtained (GenBank: MF428430), from a *Wolbachia*-infected mites of the *T. evansi*
219 population “GRA”, differed from the others (82% identity at the nucleotide level), but was
220 highly similar (99% identity at the nucleotide level) to many sequences found in diverse
221 arthropods species such as fruit flies (GenBank: FJ403332, KU870673, DQ834379, or
222 EU116319), beetles (GenBank: KX436089), moths (GenBank: GU166594), or head lice
223 (GenBank: AY596782). Note, however, that strains with similar *wsp* sequences can have
224 very different MLST allelic profiles (Baldo *et al.*, 2006), but the amount of DNA obtained
225 from each individual spider mite strongly restrained the possible number of PCRs and an in-
226 depth study of *Wolbachia* variation within *Tetranychus* mites was beyond the scope of this
227 study.

228 The prevalence of *Cardinium* was 14.6 ± 2.4 % on average (total of 32 infected mites
229 over 219). However, we could obtain the *16S rRNA* sequences only for 27 of 32 infected
230 individuals: for 5 individuals we failed to amplify *Cardinium* through three successive PCR
231 following the first diagnostic of infection. Two main possibilities could explain this failure:
232 First, very low densities of *Cardinium* within these individuals could lead to PCR-based
233 imperfect detection, as shown for other parasites (Zélé *et al.*, 2014). Second, we cannot
234 totally exclude the possibility of contaminations or of unspecific amplifications of other
235 bacteria when using *16S rRNA* primers for *Cardinium*. For this latter reason, we did not

236 consider these five individuals as infected in the following analyses. After correction, the
237 prevalence of *Cardinium* was overall of $12.3 \pm 2.2\%$ ($9.8 \pm 4.7\%$ in *T. evansi*, $10.5 \pm 5.0\%$
238 in *T. ludeni*, and $13.6 \pm 2.9\%$ in *T. urticae*) with no significant difference in prevalence
239 among the three species (model 5, $X^2_2 = 0.32$, $p=0.85$; Fig. 2). Within species, no differences
240 among populations of *T. evansi* and *T. ludeni* were found (models 6 and 7, $X^2_2 = 0.55$, $p=0.76$
241 and $X^2_2 = 3.25$, $p=0.19$, respectively), but *Cardinium* prevalences in populations of *T. urticae*
242 differed significantly (model 8, $X^2_9 = 31.19$, $p<0.001$). Indeed, in 3 populations (“COL”,
243 “DF” and “LOU”), none of the mites tested were infected, but *a posteriori* contrast analyses
244 among the remaining (infected) populations revealed no significant difference in *Cardinium*
245 prevalence ($X^2_6 = 6.62$, $p=0.36$).

246 At the molecular level, we found some degrees of inter-individual variations in the
247 *Cardinium 16S rRNA* sequences (Table 3). Of the 27 *Cardinium 16S rRNA* sequences
248 obtained, we could discriminate 6 different alleles, which, despite sharing a high degree of
249 similarity (97-99% identity at the nucleotide level), differed by single nucleotide mutations
250 over 13 different nucleotide sites (Table S3). The most prevalent allele (GenBank:
251 MF428434) was found in 17 *Cardinium*-infected mites over the three species (c.a. 63%;
252 Table 3). The corresponding sequence is very close to several sequences found for *Cardinium*
253 in diverse arthropod species, including spider mites (e.g. 99% identity at the nucleotide level
254 with a sequence obtained in *T. urticae* in Japan; GenBank: AB241134; Gotoh *et al.*, 2007a).
255 Three additional mites harbored highly similar *Cardinium* alleles (99% identity at the
256 nucleotide level; GenBank: MF428435, MF428436, and MF428437, respectively; Tables 3
257 and S3). Interestingly, we also found alleles identical or highly similar (99% identity at the
258 nucleotide level), to an allele previously found for *Cardinium* endosymbiont of *Scaphoideus*
259 *titanus*, the American grapevine leafhopper (GenBank: AM042540; Marzorati *et al.*, 2006),
260 for two *T. urticae* (GenBank: MF428431), and one *T. ludeni* (GenBank: MF428432)
261 individuals, respectively. Finally, four individuals (one *T. evansi* and three *T. urticae*)
262 harbored *Cardinium* sequences (GenBank: MF428433) with double nucleotide peaks at the
263 nucleotide positions that differ between AB241134 and MF428434 (Table S3). This result
264 suggests co-infection with two different *Cardinium* strains within these mites.

265 Infection by *Rickettsia* was rare. Indeed, this symbiont was found in only 6 of 219
266 individuals belonging to the three spider mite species ($2.7 \pm 1.1\%$; Fig. 2): $7.3 \pm 4.1\%$ of *T.*
267 *evansi* individuals were infected, belonging to a single population, $2.6 \pm 2.6\%$ of *T. ludeni*
268 individuals, also from a single population, and $1.4 \pm 1.0\%$ of *T. urticae* individuals,
269 stemming from two populations. However, for 4 of the 6 infected individuals we failed to

270 obtain the *gltA* sequences of *Rickettsia*. After correction, the prevalence of *Rickettsia* was
271 overall of 0.9 ± 0.6 % among the three mite species (2.4 ± 2.4 % in *T. evansi*, 0% in *T. ludeni*,
272 and 0.7 ± 0.7 % in *T. urticae*). The two sequences obtained, one in *T. evansi*, and one in *T.*
273 *urticae* (GenBank: MF428438; Table 3), were strictly identical to a sequence previously
274 found in *T. urticae* in China (GenBank: KP828066; Zhang *et al.*, 2016b). The most closely
275 related sequences found in other arthropod hosts were from *Rickettsia* in the parasitoid wasp
276 *Pnigalio sp.* (97% identity at the nucleotide level; GenBank: GU559856), and in *Bemisia*
277 *tabaci* (96% identity; GenBank: DQ077708).

278

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

280 Several individuals within the three mites species, and across different populations, were
281 infected with more than one endosymbiont. We detected 25 mites (11.4 ± 2.2 % in average)
282 coinfecting by *Wolbachia* and *Cardinium*, 4 mites (ca. 1.8 ± 0.9 %) coinfecting by *Wolbachia*
283 and *Rickettsia* and one individual (ca. 0.5 ± 0.5 %) infected by the three endosymbionts
284 (population “CVM”; Fig. 2). Moreover, our analyses revealed a co-variation between the
285 prevalence of *Wolbachia* and that of *Cardinium*, both symbionts affecting significantly the
286 probability of infection by each other (model 1, $X^2_1 = 4.99$, $p=0.03$, and model 5, $X^2_1 = 4.56$,
287 $p=0.03$, respectively; Fig. 3). However, no particular pattern of association between
288 *Cardinium 16S rRNA* gene sequences, *Wolbachia* infection, mites species or population
289 emerged (Table 3).

290

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

292 No particular pattern of association was found between the mitochondrial *COI* DNA
293 sequences obtained from individual mites and their *Wolbachia* and/or *Cardinium* infection
294 status ($F_{1,74} = 0.05$; $p = 0.07$, and $F_{1,74} = 0.009$; $p = 0.26$, respectively; Fig. 1), or between
295 their mitochondrial *COI* DNA sequences and the *Cardinium 16S rRNA* type ($F_{1,75} = 0.22$;
296 $p = 0.18$, Table 4). For instance, the most prevalent *Cardinium* sequence obtained
297 (MF428434; c.a. 63% of *Cardinium* infected-mites; Table 3) was found for all mitotypes,
298 and, reciprocally, several mitotypes harbored different *Cardinium* sequences (Table 4).

299

300 **Discussion**

301 In this study, we analysed the prevalence and diversity of South-West European populations
302 of spider mites and their endosymbionts. The populations collected mainly belonged to three

303 species of spider mites: *T. urticae*, *T. evansi* and *T. ludeni*, the most prevalent being *T.*
304 *urticae*. Our sampling effort was such that we did not pre-select the plants sampled.
305 Consequently, this result may reflect the host range of *T. urticae*, which is larger than that of
306 *T. evansi* and *T. ludeni* (Migeon & Dorkeld, 2006-2017). However, our results contrast with
307 those of another study conducted in the Eastern part of the Iberian Peninsula, near Valencia,
308 where *T. evansi* is the most abundant species (Ferragut *et al.*, 2013). A study on invasion
309 scenarios of *T. evansi* predicts that populations around Valencia stem from an invasion route
310 that differs from that leading to the establishment of *T. evansi* in Portugal (Boubou *et al.*,
311 2012). Possibly, the invasive success of this species differs depending on the invasion
312 event. The *T. evansi* clades that we recovered match those found earlier for the same region
313 in Portugal (Boubou *et al.*, 2011).

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

333 Of the five endosymbionts screened, only *Wolbachia*, *Cardinium* and *Rickettsia* were
334 found to infect spider mites, whereas none of the tested individuals harbored *Spiroplasma* or
335 *Arsenophonus*. However, this is the first report of *Wolbachia*, *Cardinium* and *Rickettsia* in *T.*
336 *evansi* and *T. ludeni*. Overall, *Wolbachia* prevalence was higher than that of the two other

337 symbionts, even though it was lower in *T. evansi* and *T. ludeni*, and in non-portuguese *T.*
338 *urticae* populations. Such variable levels of *Wolbachia* prevalence across species and
339 populations have already been reported in other regions worldwide (Gotoh *et al.*, 2003, Gotoh
340 *et al.*, 2007b, Zhang *et al.*, 2016b). Interestingly, however, we found *Wolbachia* in the
341 invasive species *T. evansi*. This runs counter other studies that have shown the loss of
342 *Wolbachia* in invasive populations of fire ants (Shoemaker *et al.*, 2000), Argentine ants
343 (Reuter *et al.*, 2005), and Australian thrips (Nguyen *et al.*, 2016). In invasive species, the loss
344 of *Wolbachia* is expected because introduced populations are likely to harbor only a subset of
345 the symbionts of their parental population(s), possibly because a bottleneck effect during
346 invasion. Spider mites may thus be used to test hypotheses concerning the loss or
347 maintenance of symbionts in invasive populations (see below). *Cardinium* was found in all
348 three spider mites species, which corroborates previous findings of a relatively high
349 occurrence of this symbiont in mites (Nakamura *et al.*, 2009, Zhang *et al.*, 2016a) compared
350 with other arthropods groups (Weeks *et al.*, 2003, Zchori-Fein & Perlman, 2004, Duron *et al.*,
351 2008, Nakamura *et al.*, 2009). However, its prevalence was overall much lower than that of
352 *Wolbachia*, a result not always found in spider mites (Zhang *et al.*, 2016b). The prevalence of
353 *Rickettsia* was very low, as found by Zhang *et al.* (2016b), but this study is the first to report
354 *Rickettsia* infection in *T. ludeni* and *T. evansi*. Finally, although *Spiroplasma* has been
355 previously found in *T. urticae* (Enigl & Schausberger, 2007), we did not detect it in our
356 study, as reported by Zhang *et al.* (2016b). Similarly, we did not find *Arsenophonus*.
357 Although the latter has been found in the Acari (Clay *et al.*, 2008, Dergousoff & Chilton,
358 2010, Clayton *et al.*, 2015, Duron *et al.*, 2017), it has, to our knowledge, never been reported
359 in spider mites.

360 The maintenance and spread of reproductive manipulators in nature, hence their
361 prevalence, are mainly affected by the type and strength of reproductive manipulation, the
362 fitness costs/benefits of the symbionts to their hosts, and the efficiency of vertical
363 transmission (Werren *et al.*, 2008, Engelstadter & Hurst, 2009). Variability in any of these
364 factors might explain the observed lower prevalence of *Cardinium* than that of *Wolbachia*.
365 Both endosymbionts induce CI in spider mites (Gotoh *et al.*, 2007a, Gotoh *et al.*, 2007b, Ros
366 & Breeuwer, 2009, Zhu *et al.*, 2012, Zhao *et al.*, 2013b). Although some studies found that
367 *Cardinium* induces a higher level of CI than *Wolbachia* in several mites species (e.g. *T. pierci*
368 and *Bryobia sarothamni*; Ros & Breeuwer, 2009, Zhu *et al.*, 2012) , another showed that
369 *Cardinium* may not induce CI in *T. urticae* (Gotoh *et al.*, 2007a). It is thus possible that its
370 lower prevalence is due to low reproductive manipulation. In addition, *Cardinium* may

371 exhibit imperfect maternal transmission, as found in thrips (Nguyen *et al.*, 2017), or in the
372 parasitic wasp *Encarsia pergandiella* (although near-perfect in this species; Perlman *et al.*,
373 2008). Conversely, the high prevalence of a single *Wolbachia* strain found here may be due
374 to this strain inducing low fitness costs, strong reproductive manipulation and having nearly
375 perfect maternal transmission. An in-depth study of the fitness effects of these
376 endosymbionts, along with their ability to manipulate the reproduction of their host in these
377 populations would thus allow testing these hypotheses.

378 These general predictions, coming from epidemiological modelling, may be tempered
379 by environmental factors. Indeed, a large number of other factors have been identified, such
380 as symbiont-host genotype interactions, host diet, or temperature effects (e.g. Van Opijnen &
381 Breeuwer, 1999, Gotoh *et al.*, 2007b, Mouton *et al.*, 2007, Wilkinson *et al.*, 2007, Pan *et al.*,
382 2013, Zhang *et al.*, 2013b, Boivin *et al.*, 2014, Suh *et al.*, 2015). For instance, Gotoh *et al.*
383 (2007a) showed that *Wolbachia* is much more sensitive to elevated temperature than
384 *Cardinium* in *T. pueraricola*. In our study, the higher summer temperatures in Almeria
385 (Spain; c.a. 26.7°C on average in August; [http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos/valoresclimatologicos?l=632](http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos/valoresclimatologicos?l=63250&k=and)
386 [50&k=and](http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos/valoresclimatologicos?l=63250&k=and)) compared to that of the region of Lisbon (Portugal; c.a. 23.5°C on average in
387 August; <http://www.ipma.pt/pt/oclima/normais.clima/1981-2010/012/>) might explain the
388 lower prevalence of *Wolbachia*, but not that of *Cardinium* in the *T. urticae* populations
389 collected in this region. The fact that a single *Wolbachia* *wsp* sequence was found in this
390 region, which contrasts with other studies of spider mites in other regions (Gotoh *et al.*, 2003,
391 Gotoh *et al.*, 2007b, Ros *et al.*, 2012, Zhang *et al.*, 2013a, Zhang *et al.*, 2013b), may indicate
392 specific adaptation of this strain to the environmental conditions of South-Western Europe.
393 Host plants of herbivorous arthropods may also affect symbiont diversity and identity (e.g.
394 Ferrari *et al.*, 2004, Ahmed *et al.*, 2010, Brady & White, 2013, Guidolin & Consoli, 2017; but
395 see Ji *et al.*, 2015). Moreover, bacterial endosymbionts may also be conditional mutualists in
396 some host species, conferring advantages under certain environmental conditions. For
397 instance, they may compensate for the nutritionally deficient diets of herbivore hosts and
398 promote dietary specialization by affecting their performance on plants, they may also
399 increase their ability to tolerate high temperatures, or may protect them against natural
400 enemies (reviewed in Haine, 2008, Moran *et al.*, 2008, Frago *et al.*, 2012). Unfortunately, our
401 sampling design, largely unbalanced with respect to these factors, does not allow testing their
402 relevance for the system under study.

403
404 Interaction between symbionts within a single host may also be an important factor

405 affecting their prevalence, either negatively (competition), or positively (facilitation) (Vautrin
406 *et al.*, 2008, Vautrin & Vavre, 2009, Hellard *et al.*, 2015). As previously found in several
407 spider mites species in China (Liu *et al.*, 2006, Zhao *et al.*, 2013b, Zhang *et al.*, 2016b), and
408 in Northern Europe (Ros & Breeuwer, 2009, Ros *et al.*, 2012), in this study
409 *Cardinium* prevalence was higher in populations also infected by *Wolbachia*, which may
410 suggest facilitation between these endosymbionts. The maintenance of *Cardinium* in the
411 spider mites populations collected in this study might thus be due to a hitchhiking effect,
412 whereby *Wolbachia*-induced phenotypic changes favor their own transmission, but also that
413 of other co-infecting symbionts (Frank, 1998, Engelstadter *et al.*, 2004, Vautrin & Vavre,
414 2009). Moreover, the effects of each symbiont on their host may be exacerbated in presence
415 of the other. Indeed, several studies showed that *Wolbachia* can strengthen the induction of
416 cytoplasmic incompatibility by *Cardinium* in several spider-mite (Zhu *et al.*, 2012, Zhao *et al.*
417 *et al.*, 2013b, Zhao *et al.*, 2013a), or arthropods (White *et al.*, 2009) species (but see Nguyen *et al.*
418 *et al.*, 2017). Moreover, Ros & Breeuwer (2009) found a higher fecundity advantage conferred
419 by *Wolbachia-Cardinium* co-infections in *Bryobia sarothamni* mites, than by *Wolbachia* or
420 *Cardinium* single infections (although this result may be confounded by the host genetic
421 background, as acknowledged by the authors). Studies of the fitness effects of both
422 *Wolbachia* and *Cardinium*, in single and multiple infections in these populations would allow
423 corroborating or rejecting this hypothesis.

424 Symbionts usually spread within species, but they may also cross species boundaries
425 via two main routes. First, cytoplasmic introgression, through interspecific hybridization
426 events, may favor the spread of vertically-transmitted symbionts among species
427 (Raychoudhury *et al.*, 2009, Jackel *et al.*, 2013). The incongruences between the mites *COI*
428 and *ITS* sequences obtained for some individuals in this study (see above), go in that
429 direction. Second, intimate ecological associations, such as trophic interactions and plant
430 feeding in particular, may provide horizontal transmission opportunities. It has been
431 shown, for instance, for *Rickettsia* and *Wolbachia* in whiteflies (Caspi-Fluger *et al.*, 2012,
432 Li *et al.*, 2017) and for *Cardinium* in leaf hoppers (Gonella *et al.*, 2015), and could explain
433 the genetic diversity patterns observed here. Indeed, multiple events of horizontal transfer of
434 *Cardinium* in the field would explain both its maintenance at low frequency within
435 populations, the high sequence variation (*i.e.*, potential strains) found for this symbiont, as
436 well as the lack of association with the host mtDNA. In addition, phylogenetic similarity of
437 donor and recipient host species should facilitate horizontal transmission (Boivin *et al.*,
438 2014), and incongruences between phylogenies of host and parasites, suggesting occasional

439 horizontal transmission of *Cardinium* and/or *Wolbachia* between hosts, have been found at
440 many occasions in spider mites (Yu *et al.*, 2011, Ros *et al.*, 2012, Zhang *et al.*, 2016b; but see
441 Zhang *et al.*, 2013a). However, although these symbionts might have unexpected tolerance
442 towards stressful conditions (e.g. *Wolbachia* can be maintained alive for at least a week in
443 synthetic liquid medium out of the host cells; Rasgon *et al.*, 2006), the acquisition of a
444 symbiont by horizontal transfer might be transient and not subsequently vertically transmitted
445 in the new host species (Chiel *et al.*, 2009). Further studies should thus be conducted to
446 determine the occurrence of both intra- and interspecific horizontal transfers of these
447 endosymbionts under controlled laboratory conditions in spider mites.

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

452

453 **Authors' contributions**

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

458

459 **Conflicts of interest**

460 We declare that we do not have any conflicts of interest.

461

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

*red form (or *T. cinnabarinus*)

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.

Species	Genbank accession number [Ref]	<i>T. ludeni</i>			<i>T. evansi</i>			<i>T. urticae</i>									Total number (proportion)	
		Assaf	CVM	Alval	GRA	GH	QL	AIro	AlBe	DF	LOU	COL	AMP	RF	DC	CH		FR
<i>T. ludeni</i>	GQ141911 [*]	3	5	5	-	-	-	-	-	-	-	-	-	-	-	-	-	13 (0.17)
<i>T. evansi</i>	MF428442 [**]	-	-	-	3	4	1	-	-	-	-	-	-	-	-	-	-	8 (0.11)
	MF428443 [**]	-	-	-	-	-	3	-	-	-	-	-	-	-	-	-	-	3 (0.04)
<i>T. urticae</i>	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

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 numbers [Ref]	<i>T. ludeni</i>		<i>T. evansi</i>			<i>T. urticae</i>									Total number (proportion)		
		CVM	Alval	GRA	GH	QL	AIro	AlBe	DF	LOU	COL	AMP	RF	DC	CH		FR	
<i>Wolbachia wsp</i> (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 ^(2a)	3 ^(2a1c)	3 ^(1a1c)	41 (0.98)
	MF428430 [*]	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cardinium 16S rRNA</i> (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)
<i>Rickettsia gtlA</i> (431bp)	MF428438 [*]	-	-	-	-	1 ^b	-	-	-	-	-	-	-	-	-	-	1 ^b	2 (1.00)

* this study

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: *Tetranychus evansi*, Tl: *T. ludeni*, Tu: *T. urticae*.

<i>Cardinium</i> 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

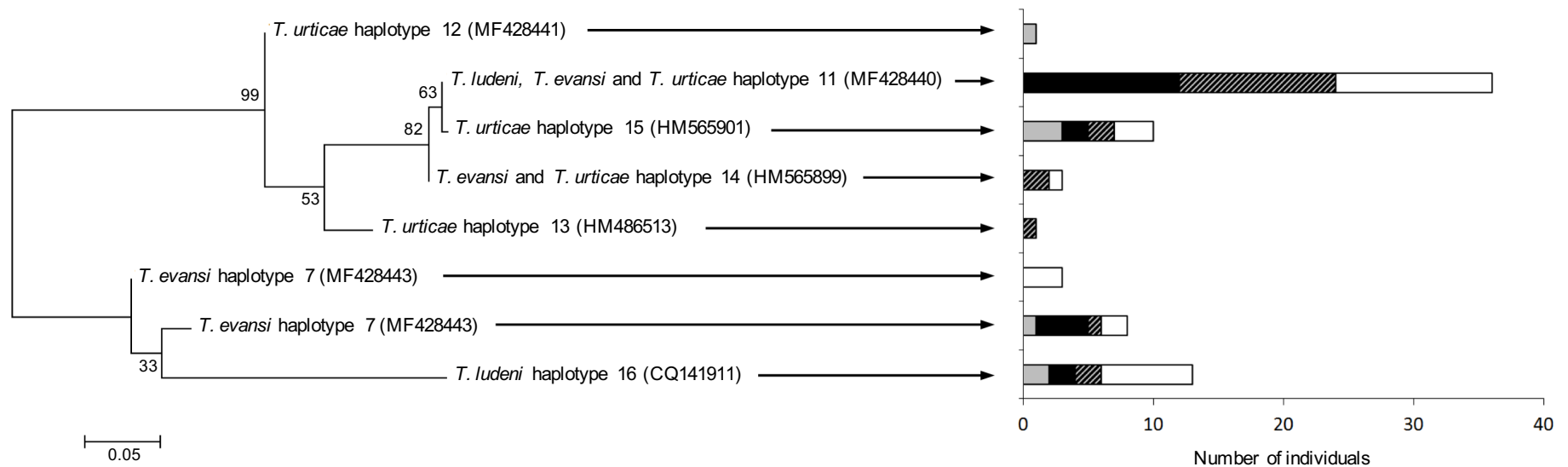


Figure 1. Phylogeny and distribution of *Wolbachia* and *Cardinium* (co)infections among *T. evansi*, *T. ludeni* and *T. urticae* mitochondrial *COI* haplotypes. The left panel shows the phylogeny of the 8 mtDNA haplotypes via maximum likelihood. Numbers on branches indicate percentage bootstrap support 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; grey: *Cardinium*-infected, n = 7; dashed: *Wolbachia*-*Cardinium* coinfecting individuals, n = 20; white: uninfected specimens, n = 28).

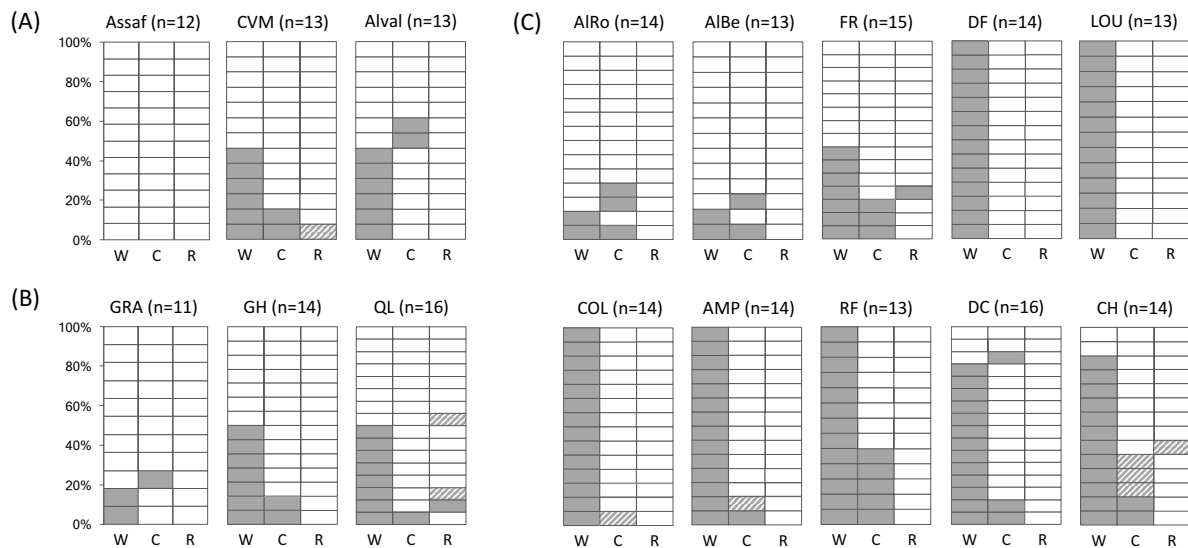


Figure 2. Prevalence of endosymbionts for each spider-mite population of (A) *T. ludeni*, (B) *T. evansi*, and (C) *T. urticae*. Each graph represents a population, in which the rows correspond to individual mites and the columns to their infection status by W: *Wolbachia*; C: *Cardinium*; and R: *Rickettsia*. White cells: uninfected; Grey 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*.

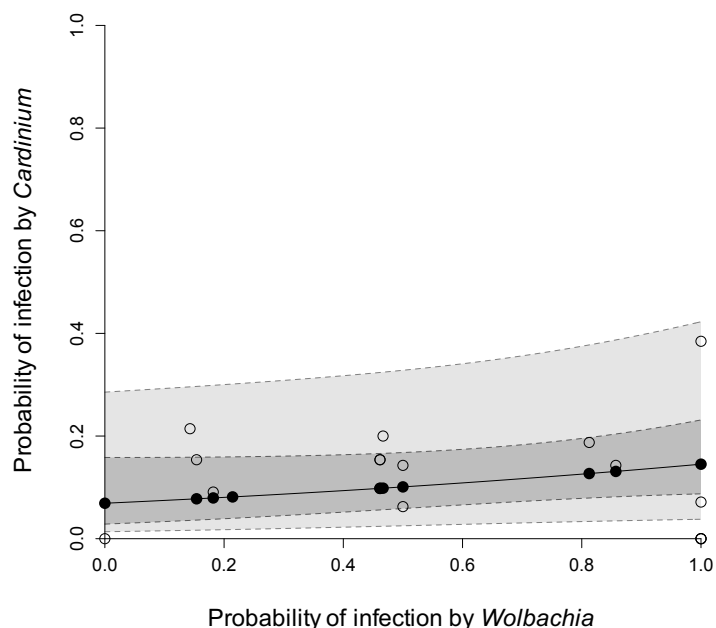


Figure 3. Mixed logistic regression of individual probability of *Wolbachia* infection on the probability of *Cardinium* infection. The solid line represents the mean predicted values; the dark shaded area represents the range of the 95% confidence intervals based on the fixed-effects uncertainty only, 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.

Additional file 1: Electronic supplementary materials (Tables S1 to S4).

Additional file 2: Complete dataset for the inter- and intraspecific variations of endosymbiont prevalences.

Additional file 3: Complete *COI* sequences dataset. Sequences name: species_population_*COI* GenBank accession number_*Wolbachia* infection status (W+: infected; W-: uninfected)_ *Cardinium* infection status (C+: infected; C-: uninfected); *Cardinium 16S rRNA* GenBank accession number for infected individuals.

Additional file 4: Complete *Cardinium 16S rRNA* sequences dataset. Sequences name: species_population_*COI* GenBank accession number_*Wolbachia* infection status (W+: infected; W-: uninfected)_ *Cardinium* infection status (C+: infected; C-: uninfected); *Cardinium 16S rRNA* GenBank accession number for infected individuals.