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Identification of spider-mite species and their endosymbionts using multiplex PCR

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Abstract Spider mites of the genus Tetranychidae are severe crop pests. In the Mediterranean a few species coexist, but they are difficult to identify based on morphological characters. Additionally, spider mites often harbour several species of endosymbiotic bacteria, which may affect the biology of their hosts. Here, we propose novel, cost-effective, multiplex diagnostic methods allowing a quick identification of spider-mite species as well as of the endosymbionts they carry. First, we developed, and successfully multiplexed in a single PCR, primers to identify *Tetranychus urticae*, *T. evansi* and *T. ludeni*, some of the most common tetranychids found in southwest Europe. Moreover, we demonstrated that this method allows detecting multiple species in a single pool, even at low frequencies (up to 1/100), and can be used on entire mites without DNA extraction. Second, we developed another set of primers to detect spider-mite endosymbionts, namely *Wolbachia*, *Cardinium* and *Rickettsia* in a multiplex PCR, along with a generalist spider-mite primer to control for potential failure of DNA amplification in each PCR. Overall, our method represents a simple, cost-effective and reliable method to identify spider-mite species and their symbionts in natural field populations, as well as to detect contaminations in laboratory rearings. This method may easily be extended to other species.

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Introduction

Spider mites (Tetranychidae) are ubiquitous herbivorous crop pests that occur on several plant species (Migeon and Dorkeld 2006–2017). In South-Western Europe, the most prevalent species found in natural populations are *T. urticae*, *T. evansi* and *T. ludeni* (Zélé et al. 2018). Whereas *T. urticae* is a generalist species, occurring on more than 900 host-plant species, *T. ludeni* is mainly found on plants of the order Solanales and *T. evansi* is a specialist of plants in the family Solanaceae (Migeon and Dorkeld 2006–2017). Thus, they often co-occur in the same field and even on the same individual plant (Ferragut et al. 2013). Given their minute size, phenotypic similarity, and scarcity of diagnose characters, the identification of these organisms at the species level is often a difficult task (Matsuda et al. 2013; Skoracka et al. 2015). To overcome this, several molecular methods, based on the diversity of the internal transcribed spacer region 2 of nuclear ribosomal DNA (ITS2), have been proposed to distinguish among spider-mite species and are currently under use (Ben-David et al. 2007; Hurtado et al. 2008; de Mendonça et al. 2011; Arimoto et al. 2013; Matsuda et al. 2013; Li et al. 2015; Shim et al. 2016). However, all methods described so far are relatively costly or time-consuming, as they require either sequencing the PCR products or using Restricted Fragment Length Polymorphisms (RFLP). The development of multiplex PCR, a reliable, cheap and quick method, to routinely identify arthropod species (e.g., Hosseini et al. 2007; Staudacher et al. 2011), including within the Acari (Perez-Sayas et al. 2015), appears to be an excellent solution to overcome these issues.

Spider mites (and the Acari in general) are often colonized by several endosymbiotic bacteria, at high incidence relative to most other arthropod groups (Weinert et al. 2015). Indeed, herbivorous spider mites are commonly infected with *Wolbachia* and *Cardinium* (Gotoh et al. 2003, 2007b; Liu et al. 2006; Xie et al. 2011; Ros et al. 2012; Suh et al. 2015; Zhang et al. 2016; Zélé et al. 2018) and occasionally with *Spiroplasma* (Zhang et al. 2016; Staudacher et al. 2017) and *Rickettsia* (Hoy and Jeyaprakash 2005; Zhang et al. 2016; Zélé et al. 2018). *Spiroplasma* and *Rickettsia* are by far the least common (*Spiroplasma* has, to our knowledge, never been found in South-Western Europe; Zélé et al. 2018), and their phenotypic effects on spider mites are as yet unknown. In contrast, several studies have shown that *Wolbachia* and *Cardinium* have diverse fitness effects on their spider-mite hosts. In particular, they may manipulate the reproduction of their hosts via the induction of different degrees of cytoplasmic incompatibility (Vala et al. 2002; Gotoh et al. 2003, 2007a, b; Ros and Breeuwer 2009; Xie et al. 2011, 2016; Zhu et al. 2012; Suh et al. 2015), a mechanism by which the cross between an infected male and an uninfected female results in embryonic mortality (Werren et al. 2008; Engelstadter and Hurst 2009; Ma et al. 2014). Apart from this common phenotypic effect, both bacteria can induce other types of reproductive manipulation such as feminization (i.e., genetic males developing as females) by *Cardinium* in the false spider-mite *Brevipalpus phoenicis* (Weeks et al. 2001), parthenogenesis induction (i.e., asexual daughter development) by *Wolbachia* in the genus *Bryobia* (Weeks and Breeuwer 2001), and hybrid breakdown by *Wolbachia* in *T. urticae* (Vala et al. 2000). Moreover, based on phenotypic characterisation and sequence comparisons (usually the *Wolbachia wsp* gene and the *Cardinium* 16S rDNA), multiple strains of these symbionts can be found in spider-mite populations (Gotoh et al. 2003, 2007a, b; Liu et al.

2006; Yu et al. 2011; Ros et al. 2012; Zhang et al. 2016) and these two bacterial genera have been found infecting the same individual host (Ros et al. 2012; Zhu et al. 2012; Zhao et al. 2013a, b; Xie et al. 2016). In addition, *Wolbachia* and *Cardinium* can induce variable fitness effects in spider mites. For example, they can either decrease (Perrot-Minnot et al. 2002; Ros and Breeuwer 2009; Suh et al. 2015), increase (Vala et al. 2002; Gotoh et al. 2007b; Xie et al. 2011; Zhao et al. 2013b), or not affect (Breeuwer 1997; Vala et al. 2000, 2002; Perrot-Minnot et al. 2002; Gotoh et al. 2007a, b) the fecundity of their spider-mite hosts. These endosymbionts are thus likely to affect the distribution of spider mites and characterising their community in natural host populations is a timely issue. Although such symbionts can easily be identified using specific primers, attempts to develop a multiplex method to detect them are surprisingly scarce. Indeed, we found a report of two multiplex diagnostic methods allowing to identify up to seven symbionts in whiteflies: *Portiera*, *Hemipteriphilus*, *Cardinium* and *Rickettsia* on the one hand, and *Portiera*, *Hamiltonella*, *Arsenophonus* and *Wolbachia* on the other (Kurata et al. 2016); and another study allowing to identify *Wolbachia* and *Cardinium* in spider mites (Chen et al. 2009). However, to our knowledge, a multiplex allowing the simultaneous detections of *Wolbachia*, *Cardinium* and *Rickettsia* has to date not been developed. Moreover, none of these studies have performed the simultaneous amplification of host DNA, which allows the exclusion of false negatives for each single PCR.

Here we present a simple, cost-effective and reliable method to identify spider-mite species and their symbiotic community, focussing on the mite species we find in the Iberian Peninsula. This method can be applied to characterise field communities as well as to detect contaminations in laboratory populations, and may be extended to other species in the future.

Materials and methods

Spider-mite populations

A total of 22 spider-mite populations, belonging to three different species (*T. urticae* green and red forms, *T. ludeni* and *T. evansi*), were used in this study. Some of these populations were collected by our group in 2013 (Zélé et al. 2018), others were kindly provided by A. Janssen and T. Van Leeuwen (see Electronic supplementary materials, Table S1, for a full description of these populations). The populations were maintained in our laboratory under standard conditions (25 ± 2 °C, 60% RH, 16/8 h L/D; Clemente et al. 2016) in mass-rearing insect-proof cages containing either bean cv. Contender seedlings (obtained from Germisem, Oliveira do Hospital, Portugal) for *T. urticae* and *T. ludeni*, or tomato cv. Moneymaker seedlings (obtained from Mr. Fothergill's Seeds, Kentford, UK) for *T. evansi*. The species and infection status of each population was known prior to this experiment (based on earlier sequencing of the *wsp* gene of *Wolbachia*, of the 16S rDNA of *Cardinium*, of the *gtlA* gene of *Rickettsia*, and of complete sequences of spider-mite rDNA region including ITS1, 5.8S rRNA, ITS2 and partial fragments of 18S and 28S rRNA from individuals and pools of mites from each population following at least 1 year of laboratory rearing; data not shown). Some of the populations infected by *Wolbachia* and/or *Rickettsia* were treated with tetracycline hydrochloride (0.1%, w/v) for three successive generations (Breeuwer 1997) to obtain their uninfected counterparts (the suffix '.tet' has been added to the name of the population in this case). During the treatment, mites were maintained

Table 1 List of primers used in multiplex to identify *Tetranychus urticae*, *T. ludeni* and *T. evansi*

Target gene	Primer name	Sequence (5′ → 3′)	Concentration (µM)	Product size (bp)
Tetranychus 5.8S	TspG_F	TAATCGGTGCGAATTGCAGG	2	–
T. urticae 28S	TuS_R	ATGTTTATTTGTGTTGTTTGCAAGC	2	550
T. ludeni ITS2	TeS_R	GAATGAAATAGATACTATTTGTGATTC	3	454
T. evansi ITS2	TlS_R	GATTCATGTATACAYATATAAATATATGC	3	189

We used a *Tetranychus*-generalist forward primer but three different species-specific reverse primers. 'Concentration' corresponds to the concentration of each primer in the final mix

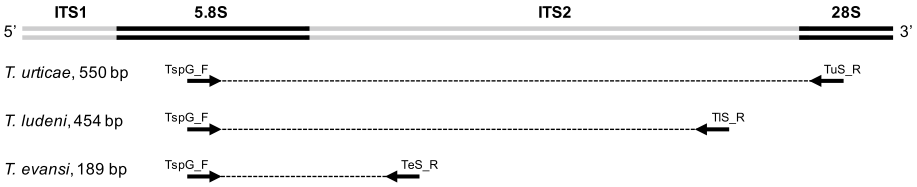

Fig. 1 Schematic overview of *Tetranychus*-generalist and species-specific primers targeting the partial 5.8S rDNA, ITS2 and 28S gene sequences (full primer sequences are listed in Table 1), and corresponding PCR products allowing the identification of *T. urticae*, *T. ludeni* and *T. evansi* in a multiplex PCR. '_F' and '_R' denote forward and reverse primers, respectively

in Petri dishes containing bean (or tomato) leaf fragments placed on cotton wet with the solution. At each generation, 50 adult mated daughters were transferred to a new Petri dish containing fresh leaf fragments on tetracycline. At the third generation after treatment, 14 individual females and a pool of 100 females were taken from each colony and checked by PCR to confirm that they were uninfected. These colonies were maintained in a mass-rearing environment without antibiotics for about five generations before this study, to avoid (or limit) potential side effects of antibiotic treatment (Ballard and Melvin 2007; Zeh et al. 2012; O'Shea and Singh 2015).

Primer design and multiplex PCR for spider-mite identification

We developed a multiplex PCR to identify the three spider-mite species commonly found in the field samples that we have collected in South-Western Europe, namely *T. urticae*, *T. ludeni* and *T. evansi* (Zélé et al. 2018). A *Tetranychus*-generalist forward primer and three different species-specific reverse primers (Table 1) were designed manually using an alignment of 38 complete *Tetranychus* sequences of the rDNA region including ITS1, 5.8S rRNA, ITS2 and partial fragments of 18S and 28S rRNA from GenBank and belonging to 18 species (Supplementary Table S2). Across several sets of primers designed to meet the criteria of a multiplex PCR (Henegariu et al. 1997; Markoulatos et al. 2002; Sint et al. 2012), the best set of spider-mite generalist primers obtained allow the amplification of partial 5.8S rDNA, ITS2 and 28S rDNA gene sequences (Fig. 1). Primer concentrations in the mix (Table 1) were empirically adjusted to ensure an even amplification of all targeted DNA fragments (Sint et al. 2012). PCRs were performed using QIAGEN Multiplex PCR

materials (Qiagen, Venlo, The Netherlands) and optimized for PCR using DNA extracts in a 10 µl final reaction volume (Table 2). Total genomic DNA was extracted either from individual or pooled mites using the Sigma-Aldrich protocol (GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma-Aldrich, St. Louis, MO, USA). Amplification conditions were as follows: 15 min at 95 °C, followed by 35 cycles of 94 °C for 30 s, 58 °C for 1 min 30 s, 72 °C for 1 min and a final step at 72 °C for 10 min. Following the PCR, 5 µL of each PCR product was electrophoresed on a 2% agarose gel, stained with Envision™ DNA Dye as Loading Buffer (Amresco, Solon, OH, USA), and photographed under ultraviolet light.

Primer design and multiplex PCR to detect simultaneously *Wolbachia*, *Cardinium* and *Rickettsia*

To detect infection by *Wolbachia*, *Cardinium* and *Rickettsia*, bacterial-specific primers (Table 3) were designed using PrimerPlex (Multiplex PCR Primer Design Software; PREMIER Biosoft International, Palo Alto, CA, USA), on the basis of the *wsp* gene of *Wolbachia*, of the 16S rDNA of *Cardinium*, and of the *gtlA* gene of *Rickettsia*, along with spider-mite generalist primers (to control the DNA quality and discriminate uninfected individuals from PCR failure) on the basis of complete *Tetranychus* sequences of the rDNA region including ITS1, 5.8S rRNA, ITS2 and partial fragments of 18S and 28S rRNA genes as above. After designing and testing several primers sets, the best set obtained locates in the ITS1 region (Table 3). DNA extraction, PCRs (Table 2), amplification conditions (with an annealing temperature of 65 °C), and gel electrophoresis, were performed as previously described for species identification. Tests were done in *T. urticae*, as this was the only mite species harboring endosymbionts in our laboratory populations at the time of this study. Template DNAs came from pools of *T. urticae* females known to be uninfected, infected by *Wolbachia* only, by *Cardinium* only, or co-infected with *Wolbachia* and *Rickettsia* (we were unable to find mites infected with the latter only). To obtain other amplification profiles (e.g., *Wolbachia-Cardinium* co-infection or triple infection; Fig. 3), equal volumes of different DNA extracts were mixed prior to performing the PCR.

Sensitivity tests

We tested the sensitivity of both multiplex PCRs on DNA extracts from different sizes of female pools (10, 25, 50 and 100 individuals) in which only one female from a different species or with a different infection status (i.e., by *Wolbachia*, *Rickettsia*, or both) was

Table 2 Reaction set-up using QIAGEN multiplex PCR materials

Component	DNA extract (µL)	1 mite (µL)
2 × QIAGEN multiplex PCR MasterMix	5	5
Primers mix	2	2
Q-solution	2	1
DEPC-water	–	2
DNA	1	–

The volume per reaction of each component is given for PCRs using either DNA extracts or entire mites

Table 3 List of primers used in multiplex for simultaneous detection of *Wolbachia*, *Cardinium* and *Rickettsia* infections

Target gene	Primer name	Sequence (5′ → 3′)	Concentration (μM)	Product size (bp)
Spider mite partial ITS1	ITS1G_F	AGGTGAACCTGCGGAAGGATCATTAACG	0.5	365–385
	ITS1G_R	CCTTCTTTAAACCTTGCCGTCAGCATAAGC	0.5	
Rickettsia gtlA	RICTG_F	AGGCTAATGGGCTTTGGTCATCGTGTAT	2	293
	RICTG_R	TGTGCCATCCAGCCTACTGTTCTTGC	2	
Wolbachia wsp	WSPTG_F	GTTGGTGTTGGTGCAGCGTATGTAAGC	2	222
	WSPTG_R	AGTGCTGTAAAGAACTTTGATTCCGCCATC	2	
*Cardinium*16S rRNA	CARDTG_F	GGCGGCTTATTAAGTCAGTTGTGAAATCCT	3	152
	CARDTG_R	GCTGCCTACGCTATTGGTATTCCTTATGAT	3	

Spider-mite generalist primers were used to control for DNA quality, thus to distinguish uninfected individuals from PCR failure. ‘Concentration’ corresponds to the concentration of each primer in the final mix

added. We then checked whether two profiles, one corresponding to the original pool, the other to the introduced female, could be detected.

Application to non-DNA extracted individuals

We tested the efficiency of the multiplex PCRs for endosymbiont detection at the individual level on non-DNA extracted adult females and males. Here, entire mites were placed directly in the PCR tube, subsequently frozen at − 20 °C during 15 min then briefly centrifuged before adding the PCR mix. The PCR mix were optimized for using entire mites (Table 2), whereas amplification conditions and gel electrophoresis were performed as previously described for PCR using DNA extracts. We tested four adult *T. urticae* females and males that were either uninfected, infected with *Wolbachia* only, or coinfected by *Rickettsia* and *Wolbachia*.

Application to detect contamination in spider-mite laboratory populations

We applied both multiplex PCRs to detect potential contaminations by spider-mite individuals (i.e., from a different species and/or with a different infection status) across 29 different mite populations in the laboratory. From each population, we created a pool of 100 females and extracted DNA as previously described. For each pool, we performed both 'species identification' and 'endosymbiont detection' multiplex PCRs.

Results

Multiplex PCR to detect spider-mite species and symbionts

Overall, our method allowed the unambiguous identification of the three target spider-mite species of this study: *T. urticae*, *T. ludeni* and *T. evansi* (Fig. 2), and of the three symbiont species found in *T. urticae* (Fig. 3). Further, using several sequences from GenBank (Supplementary Table S2), we could establish that the generalist primer designed in this study (TspG_F) should anneal with the partial 5.8S rDNA sequences from 18 different *Tetranychus* species. However, whereas both *T. ludeni*-specific primer (TlS_R) and *T.*

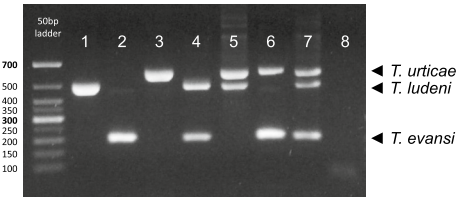

Fig. 2 Amplification profile of the multiplex PCR allowing the identification of *Tetranychus urticae*, *T. ludeni* and *T. evansi*. The size fragment of the spider-mite partial 5.8S rDNA, ITS2 and 28S rDNA rDNA amplified by each specific primer pair is given in Table 1. The position of amplicons generated from each spider-mite species are shown on the right part of the picture. Lane 1: *T. ludeni*; lane 2: *T. evansi*; lane 3: *T. urticae*, lane 4: co-occurrence *T. ludeni*-*T. evansi*; lane 5: co-occurrence *T. urticae*-*T. ludeni*; lane 6: co-occurrence *T. urticae*-*T. evansi*; lane 7: co-occurrence of the three species; lane 8: negative control without DNA template

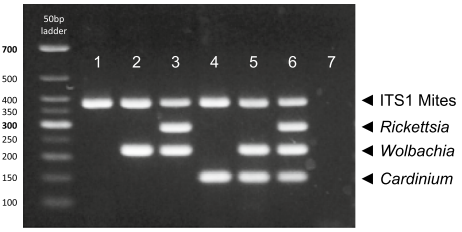

Fig. 3 Amplification profile of the multiplex PCR allowing the simultaneous detection of *Wolbachia, Cardinium* and *Rickettsia* in spider mites. The size of the amplified fragment for each specific primer pair is given in Table 3. The position of amplicons generated from each symbiont are shown on the right part of the picture. DNA quality was controlled by amplifying a fragment of the spider-mite partial ITS1 rDNA. The presence of several bands indicates coinfections. Lane 1: uninfected mite; lane 2: *Wolbachia* single infection; lane 3: *Rickettsia–Wolbachia* coinfection, lane 4: *Cardinium* single infection; lane 5: *Wolbachia-Cardinium* coinfection; lane 6: triple infection; lane 7: negative control without DNA template

evansi-specific primer (TeS_R) are highly specific to these two species (i.e., they should bind only to the ITS2 sequences from *T. ludeni* and *T. evansi*, respectively), the *T. urticae*-specific primer (TuS_R) might bind to the 28S rDNA gene sequences of few other species, but not to that of many others, including *T. evansi* and *T. ludeni* (Supplementary Table S2). At the annealing temperature used in our protocol ($T_m = 65$ °C), the *Tetranychus*-generalist primers (ITS2G _F and ITS2G_R), designed for the 'endosymbiont detection' multiplex PCR, should bind to the ITS1 sequences of several *Tetranychus* species (i.e., despite the occurrence of some SNPs between the primers sequence and the target site of these primers due to the high intraspecific variability of the ITS1 region, which makes it unsuitable for species discrimination; Navajas et al. 1999; Hurtado et al. 2008), and the bacterial-specific primers blast to an extensive number of sequences of their respective target symbionts obtained from many different arthropod host species, including *T. urticae* and several other spider-mite species (Primer-Blast results; data not shown).

Sensitivity tests

For species identification (Fig. 4) and endosymbiont detection (Fig. 5), we could detect contaminations by a single mite for all sizes of pools, although the strength of the signal decreases with increasing pool size in the multiplex PCR for species identification (i.e. the relative abundance of contaminant DNA decreases with increasing pool size: 1/10, 1/25, 1/50 and 1/100).

Application to non-DNA extracted individuals

We obtained 100% success at detecting single infections by *Wolbachia* and coinfections *Wolbachia–Rickettsia*, and no unspecific amplifications in uninfected individuals for both females (Fig. 6a) and males (Fig. 6b).

Application to detect contamination in spider-mite laboratory populations

Amplifications of DNA from pooled individuals form laboratory populations was successful, and, for 27 of 29 of them, the obtained profiles were those expected based

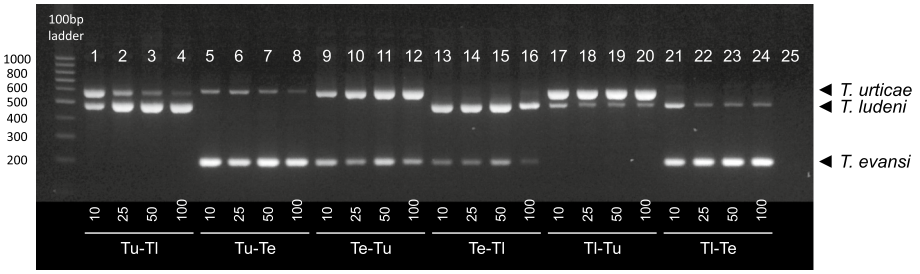

Fig. 4 Multiplex sensitivity to detect contaminations by mites from a different species in different pool sizes. The position of amplicons generated from each spider-mite species are shown on the right part of the picture. Pool sizes (10, 25, 50 and 100 females) and compositions are given under the lanes. Tu–Tl (lanes 1–4): one *Tetranychus urticae* (Tu) female in pools of *T. ludeni* (Tl) females; Tu–Te (lanes 5–8): one Tu female in pools of *T. evansi* (Te) females; Te–Tu (lanes 9–12): one Te female in pools of Tu females; Te–Tl (lanes 13–16): one Te female in pools of Tl females; Tl–Tu (lanes 17–20): one Tl female in pools of Tu females; Tl-Te (lanes 21–24): one Tl female in pools of Te females; negative control: no DNA template (lane 25)

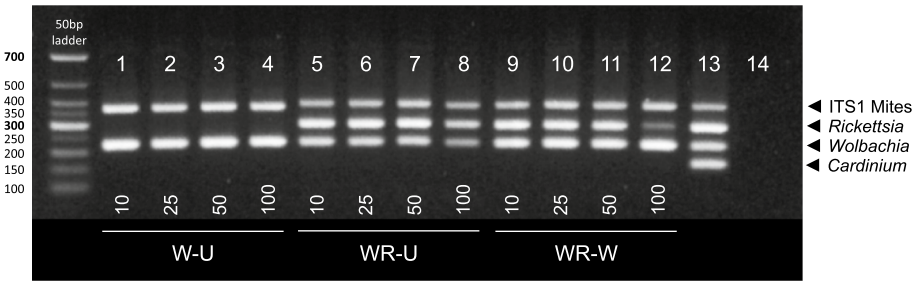

Fig. 5 Multiplex sensitivity to detect contaminations by mites with different infection types within different pool sizes. The position of amplicons generated from each symbiont, along with a fragment of the spider-mite ITS1 rDNA, are shown on the right part of the picture. Pool sizes (10, 25, 50 and 100 females) and compositions are given under the lanes. W–U (lanes 1–4): one *Wolbachia*-infected (W) female in pools of uninfected (U) females; WR–U (lanes 5–8): one *Wolbachia–Rickettsia* coinfected (WR) female in pools of U females; WR–W (lanes 9–12): one WR female in pools of W females; Positive control: DNA template with *Rickettsia–Wolbachia–Cardinium* coinfection (lane 13); Negative control: no DNA template (lane 14)

on the known species and endosymbiont composition (Fig. 7). However, for the two remaining populations ('Tl CVM' and 'Tu FR'), which were expected to be symbiont-free based on earlier tests (cf. Methods), we detected infections by *Wolbachia* (Fig. 7a). In the PCR profile for species identification (Fig. 7b), the population 'Tl CVM' showed a double amplification, for *T. ludeni* and for *T. urticae*, whereas 'Tu FR' showed only one band for *T. urticae*. The combination of both results suggests that both populations have been contaminated by *Wolbachia*-infected *T. urticae* mites from another population (note that this result matches expectations since only *T. urticae* populations were infected with *Wolbachia* in our laboratory at the time of this study). Although these contaminations might have occurred during DNA extraction, or during PCR, subsequent tests (new DNA extractions followed by a standard PCR of the *Wolbachia wsp* gene; data not shown) confirmed that the source of both contaminations was *Wolbachia*-infected *T. urticae* mites during laboratory rearing.

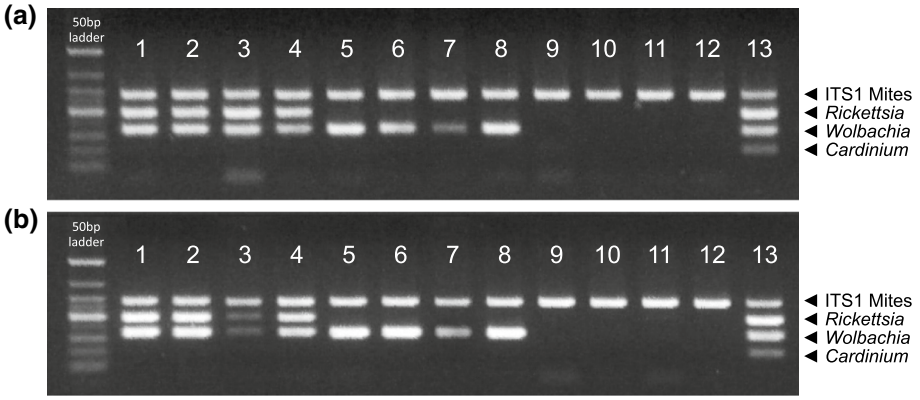

Fig. 6 Simultaneous detection of *Wolbachia, Cardinium* and *Rickettsia* in non-extracted DNA from adult females (**a**) and males (**b**) of *Tetranychus urticae*. The position of amplicons generated from each symbiont, along with a fragment of the spider-mite ITS1 rDNA, are shown on the right part of the picture. Lane 1–4, *Rickettsia–Wolbachia* coinfection; lane 5–8, *Wolbachia* single infection; lane 9–12, uninfected mite; lane 13, positive control (DNA template with *Rickettsia–Wolbachia–Cardinium* coinfection)

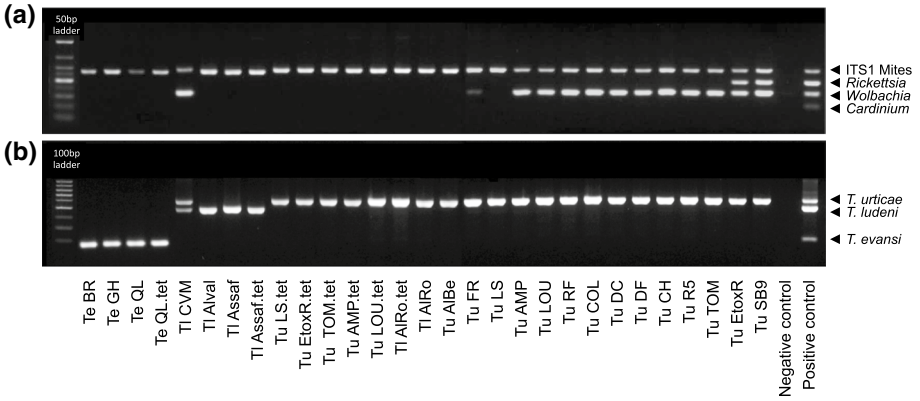

Fig. 7 Application of both multiplex PCRs to detect contamination within laboratory populations of *Tetranychus* mites. **a** symbiont infection status, and **b** species identification for 29 different populations of *T. evansi* (Te; n = 4), *T. ludeni* (Tl; n = 4), or *T. urticae* (Tu; n = 21). The name of each laboratory population is given on the bottom of the picture. The position of amplicons generated from each symbiont, along with a fragment of the spider-mite ITS2 gene (**a**), or from each spider-mite species (**b**) are shown on the right part of the picture. The infection status of each population was known prior to the PCR, only nine populations of *T. urticae* being infected by *Wolbachia* (from Tu AMP to Tu TOM), and two coinfected by *Wolbachia* and *Rickettsia* (Tu EtoxR and Tu SB9)

Discussion

This study reports the development of multiplex PCR methods for rapid identification of *T. urticae*, *T. evansi* and *T. ludeni* (Fig. 2), and detection of their endosymbionts, namely *Wolbachia*, *Cardinium* and *Rickettsia* (Fig. 3). Given the difficulties in relying on morphological characters, the identification of spider mites at the species level is often a difficult task

(Matsuda et al. 2013; Skoracka et al. 2015). Moreover, the efficient detection of these endosymbionts, which often cause reproductive abnormalities in their host arthropods (Werren et al. 2008; Engelstadter and Hurst 2009; Ma et al. 2014) provides important information concerning the populations sampled. In mass diagnosis, the introduction of multiplex PCRs reduces considerably the need of DNA template when DNA is limited or precious, and the cost of labor time and reagents. Consequently, multiplex PCR systems are increasingly used in biological studies as they allow simultaneous amplification of several DNA fragments within one reaction.

In multiplex PCR, short amplicon lengths and high annealing temperatures are crucial for enhanced detection efficiency. Competition between different primers, due to differences in GC content, degree of mismatch between primer and priming site, secondary structure or interference with each other, can also influence the sensitivity and efficiency of amplification of the target molecules (Henegariu et al. 1997; Markoulatos et al. 2002; Sint et al. 2012). The primers presented in this study were designed to overcome these problems, to have high melting temperatures (T_m), and produce DNA fragments of distinct, but short size, allowing easy discrimination of the three spider mites species and of their endosymbiont by agarose gel electrophoresis. In addition, the successful multiplex PCR amplification of the diagnostic products is also dependent on the ratio of the primer pairs and the extending time (Sint et al. 2012). Series of optimization have thus been conducted to provide a protocol that improves the detection success and ensures an even amplification of all targeted DNA fragments. These optimizations, however, have been conducted using Qiagen materials, and we cannot ensure the same results using different materials without performing novel series of optimizations.

Besides being time- and cost-effective, one of the main advantages of the multiplex PCRs developed in this study concerns its utilisation on DNA extracted from pooled spider mites. The sensitivity tests performed in this study shows the technique to be highly sensitive and allows detecting up to one individual mite in a pool of 100 mites belonging to a different species, or having a different infection status (Figs. 4, 5). This can be particularly useful, for instance, when founding new laboratory populations from field-collected individuals, and to regularly test for contaminations between laboratory populations belonging to different species or carrying different endosymbionts. Both multiplex PCRs presented in this study are already routinely used in our own laboratory since their initial development in 2014: pools of 100 adult females are collected from each of our populations every 6 months, their DNA extracted and both multiplex PCRs systematically performed on each sample (Fig. 7). This procedure is also carried out on each population before its use in any experiment performed.

Another important feature of these multiplex PCRs is the possibility to use them, at the individual level, on non-extracted spider mites for both laboratory and field studies. Indeed, they are easily applicable, for instance, to estimate species prevalence while studying interspecific competition or performing an invasion experiment (e.g., Sarmento et al. 2011; Sato et al. 2014; Orsucci et al. 2017), or to determine endosymbiont establishment and invasion in laboratory populations (e.g., Xi et al. 2005). Moreover, the use of these multiplex PCR on non-extracted single individuals can be particularly useful for quick and cost-effective prevalence assessment on field-collected populations. Indeed, the multiplex PCR developed in this study to detect endosymbionts presents the main advantage of including an internal control for failed PCR at the individual level (i.e., the inclusion of primers for host DNA amplification; Fig. 3). However, very low within-host densities of the target symbionts in field-collected mites might be difficult to detect using multiplex PCR without prior DNA extraction. In particular, we optimized

the PCR mix to increase the specificity of amplification by increasing the volume of Q-solution (QIAGEN Multiplex PCR materials), but this can also result in a decreased sensitivity of the PCR. However, problems at detecting very low within-host densities of various parasite species also hold true for standard PCR methods (Gomez-Diaz et al. 2010; Lachish et al. 2012; Zélé et al. 2014). The sensitivity tests performed here, nevertheless, proved the technique to be highly sensitive and allows detection of a very low density of symbionts in pools of mites (Fig. 5), and both non-extracted individual females and males were successfully tested (Fig. 6), which confirmed the feasibility and reliability of this method.

A major issue with the application of these methods to detect endosymbionts is the use of a single primer pair. Despite the fact that each pair of primers, to detect *Wolbachia*, *Cardinium* and *Rickettsia*, is generalist and blasts to an extensive number of sequences of their respective target symbionts, past studies have revealed that using a combination of primer pairs might be necessary to detect novel or rare symbiont strains (Simões et al. 2011). The method developed here is thus particularly useful for the detection of expected symbionts, but does not guarantee detection of novel strains. In line with this, standard PCR methods and sequencing should be used following the detection of symbionts in newly collected field populations to confirm the infection and detect potential false positives for these bacteria (Duron et al. 2008). While using non-extracted DNA, another issue is the impossibility to perform several tests on each individual (the entire body is placed in the PCR mix). This can be particularly problematic when having a limited number of individuals. In this case, we would rather advise to perform a standard DNA extraction prior to the multiplex PCR. Another solution, however, to determine the species and endosymbiont infection from a single female individual, would be to allow a female to oviposit in the laboratory before performing the multiplex PCR for endosymbiont detection without DNA extraction, and to keep its offspring, to determine a posteriori its species by performing the multiplex PCR for species determination on the offspring.

Here, we developed a multiplex PCR to detect, and discriminate between, *T. urticae*, *T. evansi* and *T. ludeni*. We made this choice because these are the species that were present in the samples we have been collecting in the field in the Iberian Peninsula and Southern France (Zélé et al. 2018). Using several sequences from GenBank, we could establish that our reverse primers allowing the identification of *T. evansi* and *T. ludeni* are highly specific, but the one allowing the identification of *T. urticae* may also allow the detection of other spider mites species (Supplementary Table S2), thus hampering their discrimination. In the case of Southern Europe, the main concern is *T. turkestani*, which has been found in both Spain and Portugal (Migeon and Dorkeld 2006–2017). However, we have failed to find this species in the several field surveys performed in the Peninsula since 2009. In any case, this multiplex PCR should be extended for the detection and discrimination of many other spider-mite species. For instance, multiplex PCR assays have also been developed to rapidly identify, within a single-step reaction, nine different species of the click beetle larvae within the genus *Agriotes* (Staudacher et al. 2011). In particular, the suitability of the species-specific primers developed by Shim et al. (2016) for *T. urticae*, *T. kanzawai*, *T. phaselus* and *T. truncates* for multiplex PCR could be tested, as they produce different size product and have relatively similar melting temperature. Similarly, the multiplex PCR developed here for endosymbiont detection could be extended to detect other endosymbionts such as *Spiroplasma*, that also occasionally infects spider mites (Zhang et al. 2016; Staudacher et al. 2017), and the host generalist primers could be designed in more conserved regions such as the 18S (Monzó et al. 2010). For instance, Kurata et al.

(2016) developed a multiplex PCR to identify up to seven endosymbionts species in whiteflies (within two different multiplex PCR assays) but it does not allow the detection of *Spiroplasma*.

Finally, the use of multiplex PCRs without DNA extraction could be adapted to other arthropod species, since non-extracted DNA from first instar larvae of some insect species are already being used in simplex PCR (e.g., for the mosquito *Culex pipiens*; Milesi et al. 2017); as well as in non-arthropods samples, as shown for non-extracted genital swabs for HSV diagnostic (Pandori et al. 2006; Miari et al. 2015).

In conclusion, the two multiplex PCR methods developed here for the identification of *T. urticae*, *T. evansi* and *T. ludeni*, and for the detection of *Wolbachia*, *Cardinium* and *Rickettsia* in spider mites, respectively, are rapid, reliable, specific, sensitive and cost-effective. The assays are versatile since they can also be used directly on non-extracted spider mites individuals. Consequently, this method is suitable for a wide variety of applications for reliable species identification and assessment of infection status. Although this study focused only on three spider-mite species and on the detection of three endosymbionts in various spider-mite species, these multiplex PCR methods could be widely developed for the detection of several host species and arthropods endosymbionts, by redesigning sequence-specific primers.

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References

Arimoto M, Satoh M, Uesugi R, Osakabe M (2013) PCR-RFLP analysis for identification of *Tetranychus* spider mite species (Acari: Tetranychidae). J Econ Entomol 106:661–668. https://doi.org/10.1603/ec12440

Ballard JWO, Melvin RG (2007) Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in Drosophila. Insect Mol Biol 16:799–802

Ben-David T, Melamed S, Gerson U, Morin S (2007) ITS2 sequences as barcodes for identifying and analyzing spider mites (Acari: Tetranychidae). Exp Appl Acarol 41:169–181. https://doi.org/10.1007/s10493-007-9058-1

Breeuwer JAJ (1997) Wolbachia and cytoplasmic incompatibility in the spider mites *Tetranychus urticae* and *T*. turkestani. Heredity 79:41–47. https://doi.org/10.1038/hdy.1997.121

Chen XL, Xie RR, Li GQ, Hong XY (2009) Simultaneous detection of endosymbionts *Wolbachia* and *Cardinium* in spider mites (Acari: Tetranychidae) by multiplex-PCR. Int J Acarol 35:397–403. https://doi.org/10.1080/01647950903292756

Clemente SH, Rodrigues LR, Ponce R, Varela SAM, Magalhães S (2016) Incomplete species recognition entails few costs in spider mites, despite first-male precedence. Behav Ecol Sociobiol 70:1161–1170. https://doi.org/10.1007/s00265-016-2124-0

de Mendonça RS, Navia D, Diniz IR, Auger P, Navajas M (2011) A critical review on some closely related species of *Tetranychus* sensu stricto (Acari: Tetranychidae) in the public DNA sequences databases. Exp Appl Acarol 55:1–23. https://doi.org/10.1007/s10493-011-9453-5

Duron O, Bouchon D, Boutin S, Bellamy L, Zhou LQ, Engelstadter J, Hurst GD (2008) The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol 6:27. https://doi.org/10.1186/1741-7007-6-27

Engelstadter J, Hurst GDD (2009) The ecology and evolution of microbes that manipulate host reproduction. Ann Rev Ecol Evol Syst 40:127–149. https://doi.org/10.1146/annurev.ecolsys.110308.120206

Ferragut F, Garzon-Luque E, Pekas A (2013) The invasive spider mite *Tetranychus evansi* (Acari: Tetranychidae) alters community composition and host-plant use of native relatives. Exp Appl Acarol 60:321–341. https://doi.org/10.1007/s10493-012-9645-7

Gomez-Diaz E, Doherty PF, Duneau D, McCoy KD (2010) Cryptic vector divergence masks vector-specific patterns of infection: an example from the marine cycle of Lyme borreliosis. Evol Appl 3:391–401. https://doi.org/10.1111/j.1752-4571.2010.00127.x

Gotoh T, Noda H, Hong XY (2003) *Wolbachia* distribution and cytoplasmic incompatibility based on a survey of 42 spider mite species (Acari: Tetranychidae) in Japan. Heredity 91:208–216. https://doi.org/10.1038/sj.hdy.6800329

Gotoh T, Noda H, Ito S (2007a) *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity 98:13–20. https://doi.org/10.1038/sj.hdy.6800881

Gotoh T, Sugasawa J, Noda H, Kitashima Y (2007b) *Wolbachia*-induced cytoplasmic incompatibility in Japanese populations of *Tetranychus urticae* (Acari: Tetranychidae). Exp Appl Acarol 42:1–16. https://doi.org/10.1007/s10493-007-9072-3

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH (1997) Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques 23:504–511

Hosseini R, Keller MA, Schmidt O, Framenau VW (2007) Molecular identification of wolf spiders (Araneae: Lycosidae) by multiplex polymerase chain reaction. Biol Control 40:128–135. https://doi.org/10.1016/j.biocontrol.2006.10.007

Hoy MA, Jeyaprakash A (2005) Microbial diversity in the predatory mite *Metaseiulus occidentalis* (Acari: Phytoseiidae) and its prey, *Tetranychus urticae* (Acari: Tetranychidae). Biol Control 32:427–441. https://doi.org/10.1016/j.biocontrol.2004.12.012

Hurtado MA, Ansaloni T, Cros-Arteil S, Jacas JA, Navajas M (2008) Sequence analysis of the ribosomal internal transcribed spacers region in spider mites (Prostigmata: Tetranychidae) occurring in citrus orchards in Eastern Spain: use for species discrimination. Ann Appl Biol 153:167–174. https://doi.org/10.1111/j.1744-7348.2008.00250.x

Kurata A, Fujiwara A, Haruyama N, Tsuchida T (2016) Multiplex PCR method for rapid identification of genetic group and symbiont infection status in *Bemisia tabaci* (Hemiptera: Aleyrodidae). Appl Entomol Zool 51:167–172. https://doi.org/10.1007/s13355-015-0378-z

Lachish S, Gopalaswamy AM, Knowles SCL, Sheldon BC (2012) Site-occupancy modelling as a novel framework for assessing test sensitivity and estimating wildlife disease prevalence from imperfect diagnostic tests. Methods Ecol Evol 3:339–348. https://doi.org/10.1111/j.2041-210X.2011.00156.x

Li DM, Fan QH, Waite DW, Gunawardana D, George S, Kumarasinghe L (2015) Development and validation of a real-time PCR assay for rapid detection of two-spotted spider mite, *Tetranychus urticae* (Acari: Tetranychidae). PLoS ONE 10:e0131887. https://doi.org/10.1371/journal.pone.0131887

Liu Y, Miao H, Hong XY (2006) Distribution of the endosymbiotic bacterium *Cardinium* in Chinese populations of the carmine spider mite *Tetranychus cinnabarinus* (Acari: Tetranychidae). J Appl Entomol 130:523–529. https://doi.org/10.1111/j.1439-0418.2006.01112.x

Ma WJ, Vavre F, Beukeboom LW (2014) Manipulation of arthropod sex determination by endosymbionts: diversity and molecular mechanisms. Sex Dev 8:59–73. https://doi.org/10.1159/000357024

Markoulatos P, Siafakas N, Moncany M (2002) Multiplex polymerase chain reaction: a practical approach. J Clin Lab Anal 16:47–51. https://doi.org/10.1002/jcla.2058

Matsuda T, Fukumoto C, Hinomoto N, Gotoh T (2013) DNA-based identification of spider mites: molecular evidence for cryptic species of the genus *Tetranychus* (Acari: Tetranychidae). J Econ Entomol 106:463–472. https://doi.org/10.1603/ec12328

Miari VF, Wall GR, Clark DA (2015) Evaluation of non-extracted genital swabs for real-time HSV PCR. J Med Virol 87:125–129. https://doi.org/10.1002/jmv.23967

Migeon A, Dorkeld F (2006–2017) Spider Mites Web: a comprehensive database for the Tetranychidae. http://www.montpellier.inra.fr/CBGP/spmweb/

Milesi P, Weill M, Lenormand T, Labbe P (2017) Heterogeneous gene duplications can be adaptive because they permanently associate overdominant alleles. Evol Lett 1:169–180. https://doi.org/10.1002/evl3.17

Monzó C, Sabater-Munoz B, Urbaneja A, Castanera P (2010) Tracking medfly predation by the wolf spider, *Pardosa cribata* Simon, in citrus orchards using PCR-based gut-content analysis. Bull Entomol Res 100:145–152. https://doi.org/10.1017/s0007485309006920

Navajas M, Lagnel J, Fauvel G, De Moraes G (1999) Sequence variation of ribosomal internal transcribed spacers (ITS) in commercially important phytoseiidae mites. Exp Appl Acarol 23:851–859. https://doi.org/10.1023/a:1006251220052

Orsucci M, Navajas M, Fellous S (2017) Genotype-specific interactions between parasitic arthropods. Heredity 118:260–265. https://doi.org/10.1038/hdy.2016.90

O'Shea KL, Singh ND (2015) Tetracycline-exposed *Drosophila melanogaster* males produce fewer offspring but a relative excess of sons. Ecol Evol 5:3130–3139. https://doi.org/10.1002/ece3.1535
Pandori MW, Lei J, Wong EH, Klausner J, Liska S (2006) Real-time PCR for detection of herpes simplex virus without nucleic acid extraction. BMC Infect Dis 6:104. https://doi.org/10.1186/1471-2334-6-104
Perez-Sayas C, Pina T, Gomez-Martinez MA, Camanes G, Ibanez-Gual MV, Jaques JA, Hurtado MA (2015) Disentangling mite predator-prey relationships by multiplex PCR. Mol Ecol Res 15:1330–1345. https://doi.org/10.1111/1755-0998.12409
Perrot-Minnot MJ, Cheval B, Migeon A, Navajas M (2002) Contrasting effects of *Wolbachia* on cytoplasmic incompatibility and fecundity in the haplodiploid mite *Tetranychus urticae*. J Evol Biol 15:808–817. https://doi.org/10.1046/j.1420-9101.2002.00446.x
Ros VID, Breeuwer JAJ (2009) The effects of, and interactions between, *Cardinium* and *Wolbachia* in the doubly infected spider mite *Bryobia sarothamni*. Heredity 102:413–422. https://doi.org/10.1038/hdy.2009.4
Ros VID, Fleming VM, Feil EJ, Breeuwer JAJ (2012) Diversity and recombination in *Wolbachia* and *Cardinium* from *Bryobia* spider mites. BMC Microbiol 12:S13. https://doi.org/10.1186/1471-2180-12-s1-s13
Sarmento RA, Lemos F, Dias CR, Kikuchi WT, Rodrigues JCP, Pallini A, Sabelis MW, Janssen A (2011) A herbivorous mite down-regulates plant defence and produces web to exclude competitors. PLoS ONE 6:e23757. https://doi.org/10.1371/journal.pone.0023757
Sato Y, Alba JM, Sabelis MW (2014) Testing for reproductive interference in the population dynamics of two congeneric species of herbivorous mites. Heredity 113:495–502. https://doi.org/10.1038/hdy.2014.53
Shim JK, Khaing TM, Seo HE, Ahn JY, Jung DO, Lee JH, Lee KY (2016) Development of species-specific primers for rapid diagnosis of *Tetranychus urticae*, *T. kanzawai*, *T. phaselus* and *T. truncatus* (Acari: Tetranychidae). Entomol Res 46:162–169. https://doi.org/10.1111/1748-5967.12154
Simões PM, Mialdea G, Reiss D, Sagot MF, Charlat S (2011) *Wolbachia* detection: an assessment of standard PCR Protocols. Mol Ecol Res 11:567–572. https://doi.org/10.1111/j.1755-0998.2010.02955.x
Sint D, Raso L, Traugott M (2012) Advances in multiplex PCR: balancing primer efficiencies and improving detection success. Methods Ecol Evol 3:898–905. https://doi.org/10.1111/j.2041-210X.2012.00215.x
Skoracka A, Magalhães S, Rector BG, Kuczynski L (2015) Cryptic speciation in the Acari: A function of species lifestyles or our ability to separate species? Exp Appl Acarol 67:165–182. https://doi.org/10.1007/s10493-015-9954-8
Staudacher K, Pitterl P, Furlan L, Cate PC, Traugott M (2011) PCR-based species identification of *Agriotes* larvae. Bull Entomol Res 101:201–210. https://doi.org/10.1017/s0007485310000337
Staudacher H, Schimmel BCJ, Lamers MM, Wybouw N, Groot AT, Kant MR (2017) Independent effects of a herbivore's bacterial symbionts on its performance and induced plant defences. Int J Mol Sci 18:182. https://doi.org/10.3390/ijms18010182
Suh E, Sim C, Park J-J, Cho K (2015) Inter-population variation for *Wolbachia* induced reproductive incompatibility in the haplodiploid mite *Tetranychus urticae*. Exp Appl Acarol 65:55–71. https://doi.org/10.1007/s10493-014-9846-3
Vala F, Breeuwer JAJ, Sabelis MW (2000) *Wolbachia*-induced 'hybrid breakdown' in the two-spotted spider mite *Tetranychus urticae* Koch. Proc R Soc B Biol Sci 267:1931–1937
Vala F, Weeks A, Claessen D, Breeuwer JAJ, Sabelis MW (2002) Within- and between-population variation for *Wolbachia*-induced reproductive incompatibility in a haplodiploid mite. Evolution 56:1331–1339
Weeks AR, Breeuwer JAJ (2001) *Wolbachia*-induced parthenogenesis in a genus of phytophagous mites. Proc R Soc Lond B Bio 268:2245–2251. https://doi.org/10.1098/rspb.2001.1797
Weeks AR, Marec F, Breeuwer JAJ (2001) A mite species that consists entirely of haploid females. Science 292:2479–2482. https://doi.org/10.1126/science.1060411
Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ (2015) The incidence of bacterial endosymbionts in terrestrial arthropods. Proc R Soc Lond 282:20150249. https://doi.org/10.1098/rspb.2015.0249
Werren JH, Baldo L, Clark ME (2008) *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–751. https://doi.org/10.1038/nrmicro1969
Xi ZY, Khoo CCH, Dobson SL (2005) *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. Science 310:326–328. https://doi.org/10.1126/science.1117607
Xie RR, Chen XL, Hong XY (2011) Variable fitness and reproductive effects of Wolbachia infection in populations of the two-spotted spider mite *Tetranychus urticae* Koch in China. Appl Entomol Zool 46:95–102. https://doi.org/10.1007/s13355-010-0014-x
Xie RR, Sun JT, Xue XF, Hong XY (2016) Cytoplasmic incompatibility and fitness benefits in the two-spotted spider mite *Tetranychus urticae* (red form) doubly infected with *Wolbachia* and *Cardinium*. Syst Appl Acarol 21:1161–1173. https://doi.org/10.11158/saa.21.9.1

Yu MZ, Zhang KJ, Xue XF, Hong XY (2011) Effects of *Wolbachia* on mtDNA variation and evolution in natural populations of *Tetranychus urticae* Koch. Insect Mol Biol 20:311–321. https://doi.org/10.1111/j.1365-2583.2010.01066.x

Zeh JA, Bonilla MM, Adrian AJ, Mesfin S, Zeh DW (2012) From father to son: transgenerational effect of tetracycline on sperm viability. Sci Rep 2:375. https://doi.org/10.1038/srep00375

Zélé F, Vézilier J, L'Ambert G, Nicot A, Gandon S, Rivero A, Duron O (2014) Dynamics of prevalence and diversity of avian malaria infections in wild *Culex pipiens* mosquitoes: the effects of *Wolbachia*, filarial nematodes and insecticide resistance. Parasite Vector 7:437. https://doi.org/10.1186/1756-3305-7-437

Zélé F, Santos I, Olivieri I, Weill M, Duron O, Magalhães S (2018) Endosymbiont diversity and prevalence in herbivorous spider mite populations in South-Western Europe. FEMS Microbiol Ecol. https://doi.org/10.1093/femsec/fiy015 **(in press)**

Zhang YK, Chen YT, Yang K, Qiao GX, Hong XY (2016) Screening of spider mites (Acari: Tetranychidae) for reproductive endosymbionts reveals links between co-infection and evolutionary history. Sci Rep 6:27900. https://doi.org/10.1038/srep27900

Zhao DX, Chen DS, Ge C, Gotoh T, Hong XY (2013a) Multiple Infections with *Cardinium* and two strains of *Wolbachia* in the spider mite *Tetranychus phaselus* Ehara: Revealing new forces driving the spread of *Wolbachia*. PLoS ONE 8:e54964. https://doi.org/10.1371/journal.pone.0054964

Zhao DX, Zhang XF, Hong XY (2013b) Host-symbiont interactions in spider mite *Tetranychus truncates* doubly infected with *Wolbachia* and *Cardinium*. Environ Entomol 42:445–452. https://doi.org/10.1603/en12354

Zhu LY, Zhang KJ, Zhang YK, Ge C, Gotoh T, Hong XY (2012) *Wolbachia* strengthens *Cardinium*-induced cytoplasmic incompatibility in the spider mite *Tetranychus piercei* McGregor. Curr Microbiol 65:516–523. https://doi.org/10.1007/s00284-012-0190-8