Screening of spider mites (Acari: Tetranychidae) for reproductive endosymbionts reveals links between co-infection and evolutionary history

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Reproductive endosymbionts have been shown to have wide-ranging effects on many aspects of their hosts' biology. A first step to understanding how these endosymbionts interact with their hosts is to determine their incidences. Here, we screened for four reproductive endosymbionts (Wolbachia, Cardinium, Spiroplasma and Rickettsia) in 28 populations of spider mites (Acari: Tetranychidae) representing 12 species. Each of the four endosymbionts were identified in at least some of the tested specimens, and their infection patterns showed variations at the species-level and population-level, suggesting their distributions can be correlated with both the phylogeny and ecology of the hosts. Co-infections of unrelated bacteria, especially double infections of Wolbachia and Cardinium within the same individuals were common. Spiroplasma and Rickettsia infections were specific to particular host species, respectively. Further, the evolutionary histories of these endosymbionts were inferred by comparing the phylogenies of them and their hosts. These findings can help to clarify the interactions between endosymbionts and arthropods.

Symbiotic bacteria are ubiquitous and have profound impacts on their host’s biology1–4. The interest in the field has come largely from the discovery of Wolbachia, a bacterium that manipulates hosts’ reproduction through cytoplasmic incompatibility (CI), parthenogenesis, male-killing, feminization5 and oogenesis6. Within the last decade, a second symbiotic bacterium, Cardinium, has been found to have reproductive effects, including cytoplasmic incompatibility7,8, parthenogenesis9 and feminization10. Other reproductive endosymbionts have been recorded in the genera Spiroplasma and Rickettsia. Spiroplasma induces male-killing in Drosophila11, butterflies12, ladybird beetles13 and planthoppers14. Rickettsia has been shown to manipulate the reproductive biology of wasps15 and beetles16,17. In addition, many of them also may provide direct fitness benefits to infected individuals, such as protection from pathogens18 or increasing fecundity19 under certain circumstances. These endosymbionts should thus be recognized as important components of arthropod biology.

Wolbachia is widespread in arthropods20 and its distribution is related to host ecology and host biology21,22. Some lineages of Wolbachia (termed supergroups A and B) have spread ubiquitously, while others (e.g., supergroups C and D) are taxon-specific. Cardinium infections are rarer than Wolbachia, and are restricted to Hymenoptera, Hemiptera, Diptera and Acari23–25. Double infections of Wolbachia and Cardinium within the same host species have been found26–27. The distributions of Spiroplasma and Rickettsia and other reproductive bacteria have been widely investigated in some arthropods28–30.

In view of the wide distribution of endosymbionts in arthropods and their potential influences on hosts, much remains to be learned about host-bacteria interactions. Spider mites (Acari: Tetranychidae) represent a distinctive evolutionary group that is comprised of about 1200 species, including many closely related species31. They are so named because some species utilize silk in constructing webbing on leaves or pads for oviposition and also for dispersal via ballooning much in the manner of some spiders. Spider mites have two reproductive
strategies (bisexual and parthenogenetic). Many species of them have a wide host range, whereas others are highly host-specific. For example, *Tetranychus urticae*, *Tetranychus truncatus*, *Tetranychus kanzawai* and *Panonychus citri* are polyphagous and are serious pests of agricultural and horticultural crops. However, these genera also include oligophagous species, such as *Tetranychus bambusae* and *Oligonychus orthii* which inhabit only Poaceae plants. Previous studies have revealed that *Wolbachia* is widespread in spider mites. For example, *Wolbachia* has been detected in the genus *Tetranychus*23,33, *Oligonychus*33, *Panonychus*33, *Schizotetranychus*33, Bryobia34 and *Amphitetranychus*35. Furthermore, it was found associated with CI in phenotypes in species4,26,27,32,33,36. *Cardinium* was present in 15 species of family Tetranychidae, and induced CI in *Tetranychus piercei*, *Tetranychus phaseolus*, *T. truncatus*27 and *Eutetranychus suganamensis*37. Unlike the widespread distribution of *Wolbachia* and *Cardinium*, *Rickettsia* and *Spiroplasma* were less common, they were only found in *T. urticae*38,39. As yet, comparative studies that focus on these endosymbionts in a group of spider mites species are very limited.

Here, we surveyed for the first time incidences of the four endosymbionts in economically important species of spider mites. Double infections of more than one endosymbiont were frequent within the same species, we then evaluated the levels of co-infection. We further clarified the phylogenetic relationships of these detected endosymbionts to infer their evolutionary histories. Our data provide insights into the evolution and distribution of endosymbions in spider mites and may thus be regarded as a basis for future studies on spider mites and endosymbionts interactions.

**Results**

**Incidence of tested endosymbionts in spider mites.** Of the 12 spider mite species examined, *Wolbachia* was found to infect 8 species with prevalence ranging from 16.7 to 100%. *Cardinium* was found to infect 7 species and their infection frequencies ranging from 4.3 to 100% (Table 1). Among them, *Cardinium* infections in *T. kanzawai* and *Amphitetranychus viennensis* are new reports. *Wolbachia* infections were more frequent in *T. truncatus* than in *A. viennensis* (80.4% vs 36.5%, P < 0.05), while *Cardinium* infections showed no difference between them (58.9% vs 53.1%, P = 0.18). Other endosymbionts infections showed some host species-specificity, as *Spiroplasma* was found in *T. truncatus* and *Rickettsia* was detected in *T. urticae* G (Table 1, Fig. 1).

**Correlated infections with multiple endosymbionts.** Of note, co-infections of unrelated endosymbionts were observed in several species. For instance, *Wolbachia* and *Cardinium* usually co-infect *T. truncatus*, *T. kanzawai*, *T. phaseolus*, *T. piercei*, *A. viennensis* and *Petriobia harri*. Similarly, *T. urticae* G was infected by *Wolbachia* and *Rickettsia*, and *T. truncatus* showed triple infections (Table 1, Fig. 1). Furthermore, Spearman correlation analyses of the presence/absence of each endosymbiont within spider mite individuals against the presence/absence of other endosymbionts revealed that infections with *Wolbachia* and *Cardinium* were significantly correlated to each other (r = 0.4344, P < 0.01). Similarly, infections with *Wolbachia* and *Spiroplasma* were significantly correlated to each other (r = 0.4737, P < 0.01) in *T. truncatus*.

**Phylogeny of spider mites hosts and detected endosymbionts.** Bayesian and maximum likelihood phylogenies of spider mites based on COI, 18S rRNA and 28S rRNA were identical. Species of the genus *Tetranychus* appeared to be monophyletic with strong support of posterior probabilities (>0.9) and moderate support of maximum likelihood bootstrap values (>50), other branches were not well resolved (Fig. 1).

Analyses of the wsp gene sequences in the seven positive species revealed nine *Wolbachia* strains, which were designated as wPhar, wAvie, wTph, wTurt, wTipe, wTkan, wTpie, wTtru1 and wTtru5 (Table 1). These strains, except for wTpie, were characterized by MLST. *Wolbachia* phylogenies based on MLST genes were largely identical for both Bayesian and maximum likelihood analyses, and most splits were highly supported. All of the *Wolbachia* strains from spider mites were assigned to supergroup B. *Wolbachia* strains from *T. truncatus*, *T. kanzawai*, *T. urticae* and *T. paeraniciola* showed little divergence, and formed a monophyletic group (Fig. 2). While the *Wolbachia* strains obtained from *T. phaseolus*, *A. viennensis* and *P. harri* respectively exhibited a distinct node in the phylogenies (Fig. 2).

Based on the 16S rRNA sequences, a total of seven *Cardinium* strains (cTpie, cPhar, cAvie, cTph, cTurt, cTpie, cTTru1 and cTTru5) were found. Owing to the 16S rRNA’s low discriminating ability, we performed phylogenetic analyses using the gyrB sequences. The gyrB gene of cTpie was not successfully sequenced, which was therefore not represented in the *Cardinium* phylogeny. The remaining strains all belonged to group A-clade, and strains derived from *A. viennensis*, *T. kanzawai*, *T. phaseolus* and *T. truncatus* plus with *Cardinium* from other spider mite species formed a monophyletic group (Fig. 3).

Sequencing of the *Spiroplasma*’s 16S rRNA, rpoB gene and the *Rickettsia’s gltA* gene identified one *Spiroplasma* strain from *T. truncatus* and one *Rickettsia* strain from *T. urticae* G, respectively (Table 1, Table S2). Phylogenetic tree based on the rpoB gene demonstrated that the *Spiroplasma* of *T. truncatus* fell into the ixodetis group, which includes *Spiroplasma ixodetis* and *Spiroplasma* infecting tick, planthopper, moth and flies (Fig. 4). The *Rickettsia* detected from *T. urticae* G fell within the bellii group (Fig. 5).

**Correlation between Wolbachia, Cardinium and hosts genetic distances.** The pattern of association and genetic divergence between *Wolbachia*, *Cardinium* and hosts were examined. Pairwise genetic distances of hosts and associated *Wolbachia* were significantly correlated (r = 0.4828, P = 0.005). While there was no significant correlation between *Cardinium* and hosts genetic distances (r = 0.1939, P = 0.29).

**Discussion**

Studies of endosymbiont incidences in a wide variety of arthropods suggest that *Wolbachia* is the most common bacterial symbiont20. Acari is assumed to be a hotspot for *Wolbachia* infections25,29. The finding of about 67% *Wolbachia*-positive species in our study is in line with estimations of a general *Wolbachia* prevalence among arthropods (40–60%). *Cardinium* infections were identified in 7 out 12 species (58.3%), suggesting that spider
| Population code | Species                | Host plant   | Location          | Collection date | Collection source | Wolbachia % infected | Cardinium % infected | Spiroplasma % infected | Rickettsia % infected |
|-----------------|------------------------|--------------|-------------------|-----------------|-------------------|----------------------|----------------------|------------------------|----------------------|
| 1               | Tetranychus truncatus  | Cotton       | Harbin, Heilongjiang | Aug-2011        | Field collected   | 100 (24/24) wTtru5   | 100 (24/24) cTtru   | 83.3 (20/24) Spiroplasma sp. | –                    |
| 2               | Eggplant               | Changshun, Jilin | Aug-2011          | Field collected  | –                 | 100 (12/12) cTtru   | –                    | –                      | –                    |
| 3               | Japan Caryatia         | Yanji, Jilin | Aug-2011          | Field collected  | –                 | 12.5 (3/24) cTtru   | –                    | –                      | –                    |
| 4               | Mung bean              | Shenyang, Liaoning | Aug-2011       | Field collected  | –                 | 4.3 (1/23) cTtru     | –                    | –                      | –                    |
| 5               | Bean                   | Hohhot, Inner Mongolia | Aug-2014      | Field collected  | –                 | 100 (12/12) cTtru   | –                    | 82.6 (19/23) Spiroplasma sp. | –                    |
| 6               | Eggplant               | Jiuquans, Gansu | Sep-2012         | Field collected  | –                 | 75 (9/12) wTtru5     | –                    | –                      | –                    |
| 7               | Snake gourd            | Cangzhou, Hebei | Aug-2014         | Field collected  | –                 | 100 (24/24) cTtru   | –                    | 100 (24/24) Spiroplasma sp. | –                    |
| 8               | Eggplant               | Changzhi, Shaxixi | Aug-2014        | Field collected  | –                 | 100 (20/20) wTtru1   | –                    | 100 (20/20) Spiroplasma sp. | –                    |
| 9               | Corn                   | Chuzhou, Anhui | Sep-2010         | Field collected  | –                 | 16.7 (12/24) wTtru1  | –                    | –                      | –                    |
| 10              | Tetranychus kanzawai  | Chinese rose | Qingdao, Shandong | Jun-2011        | Field collected   | 41.7 (5/12) wTkan    | 33.3 (4/12) cTkan   | –                      | –                    |
| 11              | Tetranychus urticae(Green form) | Apple | Taian, Shandong | Aug-2014        | Lab reared        | 100 (12/12) wTurt    | –                    | 75 (9/12) Rickettsia sp. | –                    |
| 12              | Tetranychus phaeus     | Bean         | Quanzhou, Fujian  | Jun-2014        | Field collected   | 100 (12/12) wTphe    | 66.7 (8/12) cTphe   | –                      | –                    |
| 13              | Tetranychus malayesiensis | Lingshui, Hainan | Jun-2014      | Field collected  | –                 | –                    | –                    | –                      | –                    |
| 14              | Tetranychus piercei    | Kidney bean  | Mayang, Hunan    | Jul-2014        | Field collected   | 75 (9/12) wTpie      | 58.3 (7/12) cTpie   | –                      | –                    |
| 15              | Tetranychus ludeni     | Melon        | Shantou, Guangdong | Jul-2014        | Field collected   | –                    | –                    | –                      | –                    |
| 16              | Amphotetanychus viennensis | Plum      | Daqing, Heilongjiang | Aug-2012        | Field collected   | –                    | –                    | –                      | –                    |
| 17              | Purple yam             | Yonggiu, Guangxi | Aug-2014     | Field collected  | –                 | 66.7 (8/12) wTpsy    | –                    | –                      | –                    |
| 18              | Tetranychus phaeus     | Bean         | Quanzhou, Fujian  | Jun-2014        | Field collected   | 100 (12/12) wTphe    | 66.7 (8/12) cTphe   | –                      | –                    |
| 19              | Tetranychus phaeus     | Lingshui, Hainan | Jun-2014      | Field collected  | –                 | –                    | –                    | –                      | –                    |
| 20              | Tetranychus piercei    | Kidney bean  | Mayang, Hunan    | Jul-2014        | Field collected   | 75 (9/12) wTpie      | 58.3 (7/12) cTpie   | –                      | –                    |
| 21              | Tetranychus lutei      | Melon        | Shantou, Guangdong | Jul-2014        | Field collected   | –                    | –                    | –                      | –                    |
| 22              | Amphotetanychus viennensis | Plum      | Daqing, Heilongjiang | Aug-2012        | Field collected   | –                    | –                    | –                      | –                    |
| 23              | Purple-leaf plum       | Zhengzhou, Henan | Jul-2012      | Field collected  | –                 | 16.7 (4/24) wAvie    | –                    | –                      | –                    |
| 24              | Purple-leaf plum       | Sannenci, Henan | Jun-2012     | Field collected  | –                 | 83.3 (20/24) cAvie   | –                    | –                      | –                    |
| 25              | Peach                  | Nanjing, Jiangsu | Aug-2014     | Field collected  | –                 | 70.8 (17/24) wAvie   | 62.5 (15/24) cAvie   | –                      | –                    |
| 26              | Petrosia hartii        | Clover       | Nanjing, Jiangsu | Oct-2010        | Field collected   | 75 (9/12) wPhar      | 75 (9/12) cPhar     | –                      | –                    |
| 27              | Panonychus citri       | Citrus       | Suzhou, Jiangsu  | Sep-2010        | Field collected   | –                    | –                    | –                      | –                    |

Table 1. Prevalence of investigated endosymbionts in different lines of spider mites. *The infection rate of each endosymbiont was presented. Data in the bracket indicates the number of infected individuals and the number of test individuals, respectively. – indicates endosymbionts were not detected.

mites are prone to be infected with Cardinium, which is consistent with previous estimates. Wolbachia and Rickettsia showed host species-specificity, they were only detected in T. truncatus and T. urticae, respectively. Ecological traits of host can affect the infection dynamics of endosymbionts in them. Although the infection frequencies of these endosymbionts varied among geographical populations, our survey data did not detect a clear correlation between their distribution and host ecological traits (Table 1). It is worth noting that three lab reared lines of T. urticae G were completely infected with Wolbachia, indicating fixation of infection has been reached in rearing. In addition, three species T. malayesiensis, T. ludei, P. citri did not carry any of the tested endosymbionts, raising the possibility that there have been repeated losses of infection during post-speciation from an infected ancestor or due to limited samples. Wolbachia infections were more frequent in T. truncatus than...
in *A. viennensis*. The two species are phylogenetically divergent and have different habitats, thus it remains possible that host phylogeny combined with host ecology shapes the distribution of endosymbionts in spider mites.
Manipulating reproduction and providing fitness advantages in their hosts are thought to be two important determinants of endosymbionts infection frequencies. The high infection frequencies of \textit{Wolbachia} and \textit{Cardinium} in spider mites may be due to their reproductive manipulations or fitness advantages. Nine \textit{Wolbachia} strains were detected from the positive specimens, and several of these strains have previously been studied in detail. For example, there is evidence that \textit{Wolbachia} induces CI in several spider mites, including \textit{T. urticae}, \textit{T. phaselus}, \textit{T. truncatus}, \textit{T. piercei} and \textit{A. viennensis}. Regarding \textit{Cardinium}, it was found to induce CI in \textit{T. piercei}, \textit{T. phaselus} and \textit{T. truncatus}. Furthermore, Weinert et al. have speculated that high \textit{Cardinium} incidences in spider mites might reflect evolutionary changes in arthropod immunity, as spider mites lack components of the immune deficiency (IMD) pathway, and IMD is activated by diaminopimelic acid-type (DAP-type) peptidoglycan, which is produced by \textit{Cardinium}.

Spider mites showed co-infections with more than one endosymbiont. Statistical analyses revealed that infections with \textit{Wolbachia} and \textit{Cardinium}, \textit{Wolbachia} and \textit{Spiroplasma} were significantly correlated to each other within the same individuals. There are a number of possible mechanisms that can facilitate such endosymbiont co-infections. For example, the co-infecting endosymbionts may additively or synergistically confer fitness advantages.

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Phylogenetic analyses of \textit{Cardinium} based on the \textit{gyrB} gene sequences from this study (highlighted) and others downloaded from GenBank. \textit{Cardinium} group names are used in accordance to the reference. Bayesian posterior (left numbers) and ML bootstrap values (right numbers, values $>50\%$ are indicated) are given in the trees.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Phylogenetic analyses of \textit{Spiroplama} based on the \textit{rpoB} gene sequences. \textit{Spiroplama} infecting \textit{T. truncatus} is indicated in bold letters. Bayesian posterior (left numbers) and ML bootstrap values (right numbers, values $>50\%$ are indicated) are given in the trees.}
\end{figure}
advantages on their host. Another mechanism is that when one of the co-infecting endosymbionts causes a reproductive manipulation, the manipulation may facilitate not only its own prevalence but also spread of another co-infecting endosymbiont via a hitchhiking effect. As mentioned previously, both Wolbachia and Cardinium can induce CI in doubly-infected spider mites T. piercei, T. phaselus, T. truncatus and A. viennensis, which would increase the prevalence of both endosymbionts. Also, the CI phenotypes induced by Wolbachia in T. truncatus and T. urticae would theoretically facilitate the spread of co-infecting Spiroplasma and Rickettsia, respectively.

Spiroplasma and Rickettsia act as reproductive mediators in some arthropods. They are much less common in spider mites than Wolbachia and Cardinium. Here, Spiroplasma and Rickettsia were identified only in T. truncatus and T. urticae G, respectively. Phylogenetic analyses confirmed that the two endosymbionts have horizontally transferred among different hosts, and there is experimental evidence for horizontal transmission of these symbionts via host plants or host invertebrates, thus Spiroplasma and Rickettsia occurrences in spider mites may be the result of an individual horizontal transmission event, respectively. Furthermore, Spiroplasma of T. truncatus fell into the ixodetis group, which includes Spiroplasma ixodetis and Spiroplasma infecting tick, planthopper, moth and flies. Unlike the male-killing Spiroplasmas infecting the small brown planthopper, Laodelphax striatellus, the Spiroplasma strain found in T. truncatus is not a male killer because it was found in both males and females. While it seemed increase its host development speed (unpublished data), and we are currently testing the underlying reasons. Rickettsia in T. urticae clustered within the bellii group, in which there are Rickettsia strains found in sap sucking arthropods and predatory insect hosts. Previously, Hoy and Jeyaprakash also found that four North American populations of T. urticae were infected with Rickettsia, as well as Wolbachia and Caulobacter. However, what role the Rickettsia play in the biology of spider mites is unknown.

Theory suggested that maternally inherited symbionts’ strict mutualistic associations with their hosts will result in co-cladogenesis phylogenetic patterns. Multiple strains of Wolbachia and Cardinium were detected in this study, and their infection histories could be inferred. Mapping the Wolbachia phylogeny to spider mites' phylogeny revealed some degree of congruence, as similar strains are found in closely related hosts. Meanwhile, pairwise genetic distances of hosts and associated Wolbachia were significantly correlated. Two scenarios might explain this finding. First, the common ancestor of spider mite hosts could have originally harbored Wolbachia and that the host and Wolbachia have co-speciated. Second, horizontal transmission can explain the sharing of Wolbachia strains among different hosts. Contrastingly, there was no significant association between the phylogeny of Cardinium and its host, and the Mantel test confirmed this result. Whilst we cannot completely exclude the possibility that there has been repeated loss of infection during post-speciation from an infected ancestor, this difference between the two phylogenies indicated that Cardinium was not solely acquired vertically and points to the likelihood of horizontal transmission. In addition, the phylogeny based on a single gene will fail to reflect the accurate phylogeny of Cardinium, thus more rapidly evolving and phylogenetically informative Cardinium genes are required in further study.

Figure 5. ML inference of Rickettsia strains from T. urticae and other arthropod hosts based on the gltA gene sequences. Rickettsia strain obtained from this study is indicated in bold letters. Rickettsia group names are used in accordance to the reference. Bayesian posterior (left numbers) and ML bootstrap values (right numbers, values >50% are indicated) are given in the trees.
In summary, four endosymbionts were identified in the tested specimens, and their distributions were found to be shaped by both host phylogeny and host ecology. The levels of co-infections within the same individuals were significantly higher than would be expected by chance. Comparison between the phylogenies of hosts and associated endosymbionts allowed us to explore the evolutionary histories of these endosymbionts’ infections. Together with these endosymbionts’ reproductive effects on spider mites, these findings are helpful for understanding the interaction between endosymbionts and spider mites.

Methods

All experimental protocols were approved by Chinese Academy of Sciences and Nanjing Agricultural University. Methods were carried out in accordance with relevant guidelines and regulations.

Spider mites samples. This study was based on specimens from 28 populations of spider mites representing 12 species that were collected from field or lab reared lines (Table 1). All samples were stored in 100% ethanol and frozen at −20 °C until DNA extraction.

PCR screening and sequencing. Spider mite DNA was extracted as previously described52. The DNA quality was tested by amplifying a fragment of the cytochrome oxidase, subunit I (COI) gene of spider mites53. Then, the presence of Wolbachia, Cardinium, Spiroplasma and Rickettsia was assessed by PCR amplification using specific primers and annealing temperatures listed in Table S1. PCRs were carried out on a Veriti machine (ABI Biosystems, USA) in 25 μl volume containing 12.5 μl 2 × Taq Master Mix (Vazyme Biotech, China), 0.5 μl primer (20 μM each), 1 μl of DNA extract. Positive and negative controls were included in PCR reactions. PCR products (5 μl) were visualized on a 1.5% agarose gel stained with ethidium bromide54. The positive products were purified using AxyPrep DNA Gel Extraction kit (AxyGEN, USA) and then directly sequenced (Majorbio Company, Shanghai, China). For Wolbachia, single-infection status was confirmed during wsp sequencing. Then, the MLST gene sequences of single infected Wolbachia from different individuals were amplified using standard primers and PCR protocols (http://www.pubmlst.org/wolbachia/). The COI, 18SrRNA and 28SrRNA sequences of spider mites were amplified and sequenced to construct host phylogeny. The obtained sequences have been deposited in GenBank (Table S2).

Phylogenetic analysis. All sequences were aligned and manually corrected using BioEdit55. Prior to phylogenetic analysis, the best-fitting nucleotide models were determined by jModeltest version 2.15. Wolbachia phylogeny was determined by reconstructing Bayesian Inference (BI) and Maximum-Likelihood (ML) trees of the concatenated data set of MLST genes from this study and PubMLST database (http://www.pubmlst.org/wolbachia/). Bayesian analyses were performed in MrBayes 3.157 under the GTR + I + G model. Four Markov chains were run for 15,000,000 generations with sampling every 100 generations, and the first 37,500 generations were discarded. Convergence of runs was assumed when split frequencies reached <0.01. ML analysis was also conducted for the concatenated data set of MLST genes in MEGA 5.058 under the GTR + I + G model, bootstrap pseudoreplicates were calculated 1,000 times.

The phylogenetic analyses of Cardinium, Spiroplasma and Rickettsia were performed using Bayesian Inference (BI) and Maximum-Likelihood (ML) estimation for the sequences of gyrb gene, rpoB gene and gltA gene, respectively. The evolutionary models used were as follows: gyrb- GTR + I + G, rpoB- GTR + I + G, and gltA-HKY + G. For each Bayesian analysis, four Markov chains were run 1,000,000 generations with sampling every 100 generations, and the first 25% of samples were discarded as burn-in. Convergence of runs was assumed when split frequencies reached <0.01. ML analyses were conducted in MEGA 5.058, bootstrap pseudoreplicates were calculated 1,000 times.

In addition, Bayesian and ML phylogenetic trees of spider mite were constructed using a supermatrix that consisting of the COI, 18SrRNA and 28SrRNA sequences. Analyses were performed in MrBayes 3.1 and MEGA 5.0 under the GTR + I + G model, respectively.

Statistical analysis. Endosymbiont infection patterns were first compared on the level of genera and species of spider mites. Two extensively sampled species, T. truncatus and A. viennensis were selected to test the differences of Wolbachia and Cardinium infections. Because co-infection was common in spider mites, co-infection levels were evaluated with a Spearman correlation analysis of the presence/absence of each endosymbiont within spider mite individuals against the presence/absence of other endosymbionts. The analysis was performed with Sata 11.069. In addition, we tested for correlation between host genetic distance and the corresponding Wolbachia, Cardinium strains genetic divergence to infer their infection patterns. Correlation analyses were performed using a Mantel test in Arlequin 3.146 with 1000 permutations. Genetic distance matrices of the concatenated MLST dataset for Wolbachia, gyrb genes for Cardinium and the concatenated nuclear and mitochondrial loci for spider mites were calculated with the Kimura 2-parameter model in MEGA 5.0.

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Conceived and designed the experiments: Y.-K.Z., G.-X.Q. and X.-Y.H. Performed the experiments: Y.-K.Z., K.Y. and Y.-T.C. Wrote the paper: Y.-K.Z., G.-X.Q. and X.-Y.H.

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