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INVERTEBRATE MICROBIOLOGY

Dynamics of the Endosymbiont *Rickettsia* in an Insect Pest

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Abstract A new heritable bacterial association can bring a fresh set of molecular capabilities, providing an insect host with an almost instantaneous genome extension. Increasingly acknowledged as agents of rapid evolution, inherited microbes remain underappreciated players in pest management programs. A *Rickettsia* bacterium was tracked sweeping through populations of an invasive whitefly provisionally described as the "B" or "MEAM1" of the *Bemisia tabaci* species complex, in the southwestern USA. In this population, *Rickettsia* provides strong fitness benefits and distorts whitefly sex ratios under laboratory conditions. In contrast, whiteflies in Israel show few apparent fitness benefits from *Rickettsia* under laboratory conditions, only slightly decreasing development time. A survey of *B. tabaci* B samples revealed the distribution of *Rickettsia* across the cotton-growing regions of Israel and the USA. Thirteen sites from Israel and 22 sites from the USA were sampled. Across the USA, *Rickettsia* frequencies were heterogeneous among regions, but were generally very high, whereas in Israel, the infection rates were lower and declining. The distinct outcomes of *Rickettsia* infection in these two countries conform to previously reported phenotypic differences. Intermediate frequencies in some areas in both countries may indicate a cost to infection in certain environments or that the frequencies are in flux. This suggests underlying geographic differences in the interactions between bacterial symbionts and this serious agricultural pest.

Keywords Whitefly · *Bemisia tabaci* · Middle East-Asia Minor 1 (MEAM1) · *Bemisia argentifolii* · B biotype · Diagnostic PCR

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Introduction

Many insects form intimate associations with microbial symbionts that reside within host cells and can have large effects on host biology by providing essential nutrients, increasing fitness, or manipulating reproduction [22, 62, 95]. Insects dominate animal diversity, provide valuable ecosystem services and include some of the most costly agricultural and medical pest species. A new symbiont in an insect population brings new genes, with the potential to affect host invasiveness and ecology [50, 51]. In spite of theory and indirect evidence of symbiont fluctuations in host populations [42, 46, 72, 85], observations of dynamic change in symbiont infections are scarce. Among the few direct examples [23, 47, 53, 84] is the rapid spread of a *Rickettsia* bacterium in populations of the sweet potato whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) in the southwestern USA [38].

Bemisia tabaci is highly polyphagous and has been ranked among the world's worst invasive species [56]. It causes hundreds of millions of dollars of crop damage annually by phloem feeding, vectoring of over one hundred plant viruses and contaminating plants with sticky honeydew [6, 37, 48, 65]. *Bemisia tabaci* was recently suggested to be a complex of distinct cryptic species, formerly described as biotypes [5, 17, 81]. Two of the most invasive putative species are the cosmopolitan "B", also referred to as Middle East-Asia Minor 1 (MEAM 1) or *B. argentifolii*, and the "Q" or Mediterranean (MED), which may be the true *B. tabaci* [81]. Both of these species are serious field pests of cotton and other crops in Israel [43], while in the USA, *B. tabaci* B became established in the 1980s [6, 71] and remains the most important pest of cotton in the southwest [63]. The Q has been only detected in greenhouses in the USA since 2004 [20, 59]. In Israel, it was sampled in both open field and protected crops; however, since 2009, almost no Q has been detected in Israeli cotton fields [44].

Rickettsia is a common insect symbiont [69] and arthropod-vectored vertebrate pathogen [1]. In other insects, it has been associated with male-killing [54, 89], parthenogenesis induction [29, 34], and leafhopper-vectored papaya bunchy top disease [15]. Whitefly *Rickettsia* is in the *bellii* clade, most closely related to other *Rickettsia* occurring in phytophagous insects [30]. *Rickettsia* has also been reported in *B. tabaci* Asia II 3 (or ZHJ1), Asia II 7, Indian Ocean (or MS), and China 1 [3, 4, 33]. The *B. tabaci* species complex can house diverse intracellular bacterial symbionts in addition to *Rickettsia*, including the essential nutritional symbiont *Portiera aleyrodidarum* [78] and up to six other genera of secondary symbionts [4, 24, 64, 88, 94]. The secondary symbionts have mostly unknown effects on the host and are found at different frequencies and combinations within *B. tabaci* populations [33]. The *B. tabaci* B appears to house just *Rickettsia* and be fixed for *Hamiltonella* in both Israel and the USA [10, 31, 33, 64, 94] as well as in other countries [3, 13, 33]. However, *Wolbachia*, *Arsenophonus*, and *Cardinium* have also been reported from surveys in other populations around the world [14, 64].

Whitefly-*Rickettsia* phenotypic interactions have recently been studied in Israel and the USA, generating an increasing body of conflicting evidence concerning the nature of the symbiosis. In Israel, *Rickettsia* can be transmitted among feeding adult whiteflies through phloem [8], and no reproductive manipulations have been observed. *Rickettsia* is associated with few apparent fitness effects in Israel, with the exception of slightly faster development time [11], greater susceptibility to insecticides [52], and resistance to heat shock [7]. In the USA, by contrast, there is little evidence of *Rickettsia* horizontal transmission through phloem [38]. However, laboratory experiments detected strong fitness benefits of infection, including increased fecundity and developmental survivorship, as well as female-biased sex ratio distortion.

Given the conflicting phenotypic observations of *B. tabaci* B *Rickettsia* in Israel and the USA, we hypothesized underlying differences in the host-symbiont interaction and predicted dissimilar symbiont dynamics in the two locations. In the southwestern USA, *Rickettsia* spread from 1 % infection frequency in 2000, to 51 % in 2003, to 97 % in 2006, and remained high in 2008 and 2009 [38]. The distribution of *Rickettsia* throughout the USA beyond the southwest had not been documented until the current study. We expected that the strong fitness benefits demonstrated under laboratory conditions in the USA would have driven the spread of *Rickettsia* throughout the country. *Rickettsia* was first sampled in *B. tabaci* in Israel at intermediate frequencies in the mid-2000s but had not been documented until the current study [10, 30, 32]. In Israel, predictions of *Rickettsia* frequencies were more difficult to make, given the complexity of potential horizontal transmission of this symbiont and the costs and benefits of *Rickettsia* under different environmental stresses.

Here, we describe a survey that reveals the distribution of *Rickettsia* in *B. tabaci* populations across the cotton-growing regions of Israel and the USA. In addition, we performed an empirical assessment of the sensitivity of the different *Rickettsia* diagnostic PCR detection methods used in both laboratories, in a simple reproducible protocol that allows more direct comparison of results from different surveys.

Methods

Rickettsia Frequencies, USA

B. tabaci adults were collected from cotton fields, where they may reach high numbers in the summer [63]. Twenty-two sites were sampled in 2011 (Online Resource 1), which covered

four regions spanning the major cotton-growing belt in the USA. Where *B. tabaci* could not be recovered from cotton fields, collections were made from other plants that may seed the summer cotton populations: greenhouse sweet potato, greenhouse cotton, and mixed field plants (including cotton), in LA, MS1, and FL, respectively. Six additional collection sites (not shown) were excluded from the analysis because fewer than ten individuals were found. Collection sites were marked with GPS (GPSMAP® 60CSx and eTrex Venture® HC, GARMIN). Two of the USA sites (GA1 and CA4) were re-sampled in 2012 by cooperators.

A standardized collection protocol was implemented for the main sampling effort in 2011. Adult whiteflies were collected individually from leaves into new vials with a customized aspirator to avoid damage to the insect. One whitefly per leaf was collected to minimize the chances of selecting many newly emerged siblings. In high-density fields, five whiteflies were collected per plant, with at least 3 m between each plant. In low-density fields, many plants were searched to find each whitefly. Where possible, more than 100 adults were collected per site and a subset of approximately 50 per site was screened for *Rickettsia* (range: 48–53, Online Resource 1). The aspirated whiteflies were immediately chilled on ice, and cold 100 % ethanol was added. *Bemisia tabaci* were sexed and separated from other species under a stereomicroscope while still suspended in cold 100 % ethanol. The whiteflies were kept in 100 % ethanol at –20 °C until DNA extraction.

Total DNA was extracted from individual adult females using a Chelex protocol as described previously [12], then screened with 529F/1044R *Rickettsia*-specific primers targeting the *16S rRNA* gene as described previously [12]. Using the same PCR conditions, microsatellite primers Bem23F/R, which give a band at 220 bp for the B putative species and a band at 410 bp for the Q putative species, were used to determine whitefly identity and also as a positive control for DNA quality [18]. PCR products were visualized in 1 % agarose with TAE buffer using SYBR Safe for microsatellite products (where assessing band size is critical) and the more sensitive SYBR Green for detection of *Rickettsia* products. Negative PCRs were repeated once to either confirm as uninfected or exclude as poor quality DNA.

Rickettsia frequencies were analyzed by logistic regression in R [73] with collection region as an explanatory variable, to test for heterogeneity in frequencies among regions. As the sites were sampled in collecting trip blocks across the 2011 season, there was also the potential that the frequencies in the different regions were influenced by changes in *Rickettsia* frequency across the season. Therefore, collection date (day number since first collection was made in 2011) was also included as a covariate to exclude it as a confounding variable. A quasibinomial model was used when the residual deviance was greater than the degrees of freedom. Fisher's exact test was used to determine whether infection frequencies differed between 2011 and 2012 in each of the two sites that were sampled in both years, with infected and uninfected whiteflies for 2011 and 2012 in a 2×2 contingency table in JMP version 8.0.2.

Rickettsia Frequencies, Israel

As part of an ongoing pesticide resistance-monitoring program, *B. tabaci* B was collected from 14 cotton field sites in Israel from 2000–2001 and 2009–2012. No *B. tabaci* B (only *B. tabaci* Q) were found in 2002–2008. The samples were aggregated into three main geographical regions. The "Northern" region extended from the northern border of Israel to the city of Haifa in the south, the "Central" region extended from Haifa south to the city of Revadim, and all more southerly sites were placed in the "Southern" region (Online Resource 1).

Total DNA was extracted from individual male and female adults using a lysis protocol and screened with RbF/RbR *Rickettsia*-specific primers targeting the *16S rRNA* gene as described previously [30]. In Israel as in the USA, B and Q whitefly putative species were distinguished by the size of a PCR product amplified by primers Bem23F/R [18]. Any sample that was negative for the *Rickettsia* PCR was then tested with primers amplifying the host mitochondrial cytochrome oxidase I (*COI*) gene [27], present in all whiteflies regardless of *Rickettsia* status. The purpose of the COI PCR was to determine if the sample was truly negative for *Rickettsia* or should be excluded because the DNA extract was insufficient quality for PCR. This control for DNA quality has been used in other studies [for example, 3, 4, 30, 34, 38, 51]. PCR products were visualized in 1 % agarose with ethidium bromide. Approximately, 50 whiteflies were screened per site (range 13–217, Online Resource 1). *Rickettsia* frequencies for each year were analyzed by logistic regression in R [73] with year and region as variables.

Rickettsia Genetic Diversity, USA and Israel Comparison

A subset of *Rickettsia*-specific *16S rRNA* PCR products were sent for cleanup, quantification, and sequencing at the University of Arizona Genetics Core to confirm the specificity of the primers for field-collected whiteflies. PCR products from 111 individuals were sent, as listed in Online Resource 1. Sequences were analyzed in Geneious version 5.0.4 and compared to previously published whitefly *Rickettsia* sequences and to the NCBI nr database using Megablast (available from http://www.ncbi.nlm.nih.gov/).

To assess genetic diversity among *Rickettsia* populations in Israel and the USA, two *Rickettsia* DNA regions were sequenced from individual females, using the methods described above for the 528F/1044R primers. A portion of the citrate synthase gene, *gltA*, was sequenced from three females

from a laboratory culture collected in Israel, two females from each of two laboratory cultures ("MAC1" and "MAC2") collected in site AZ2, 11 females collected from field sites in the USA, and 12 individuals collected from field sites in Israel (see Online Resource 1), using the primers rcit133F/1197R (57 °C annealing temperature, 1-min extension time) [74]. An intergenic spacer, *rpmE-tRNAfMet*, was also sequenced from one female from the laboratory culture in Israel, eight females from the laboratory cultures collected in site AZ2, 12 females collected from field sites in the USA, and 14 individuals collected from field sites in Israel (see Online Resource 1), using the primers rpmEF/R (55 °C annealing temperature) [26]. The sequences from this study were aligned with other selected sequences available in GenBank, using MAFFT version 7 [49]. Phylogenetic analyses were carried out using maximum likelihood in RAxML version 7.0.4 [79] with the GTRCAT model and 1000 rapid bootstrap replicates, and Bayesian inference (BI) using MrBayes 3.2 [80]. Two parallel runs of four simultaneous Monte Carlo Markov chains were run for two million generations, and trees sampled every 1000 generations. Convergence of the separate runs was checked using the average deviation of split frequencies diagnostic (≤0.01). The burnin value was set at 25 % of sampled topologies, and post-burnin trees were summarized as a 50 % majority rule consensus tree.

Limits of Detection, USA and Israel Comparison

To assess the likelihood of false negative results for *Rickettsia*-positive whiteflies in the laboratories in the USA and Israel, we conducted an assay to determine the limits of detecting the bacterium using PCR. In both laboratories, adult whiteflies from laboratory cultures were processed using the standard DNA extraction protocol in each laboratory as described above. A tenfold dilution series was performed on each DNA extraction, and the diluted DNA at each concentration was screened for *Rickettsia* using the standardized protocol for DNA amplification and visualization used in each laboratory. DNA was diluted until no gel band was visible, up to 1/100,000. An example gel photo is provided in Online Resource 2. A total of 80 individuals were tested.

To confirm that the limits of detection analysis performed on laboratory culture whiteflies was comparable to the limits of detection expected for field-collected whiteflies, the titer of *Rickettsia* in field and laboratory-line females in the USA was further compared with quantitative PCR. The quantitative PCR was performed on a BIORAD CFX Connect Real-Time System with methods as described previously [38] using the $2^{-\Delta Ct}$ method [55]. Twelve laboratory individuals were tested, along with field collected individuals from AZ2 (n=9) and MS2 (n=8; Online Resource 1). The mean normalized expression values of the *Rickettsia gltA* gene relative to the whitefly β-*actin* for each site were compared by ANOVA with collection site as the explanatory variable and $2^{-\Delta Ct}$ values as the response variable in JMP version 8.0.2.

Results

Rickettsia Frequencies, USA

The infection frequency of *Rickettsia* in *B. tabaci* was high across the 22 sites sampled in the USA in 2011 (84 % across all sites, n=1103, Fig. 1). The frequency of *Rickettsia* infection was significantly heterogeneous among the four geographic regions sampled ($F_{3,18}$=5.89, P=0.008). The Southwest (Region 2) had the highest average infection frequency at 93 % (n=353). The average *Rickettsia* infection frequency was lower in the San Joaquin Valley of California (Region 1, 71 %, n=150), South Texas (Region 3, 80 %, n=148), and the Southeast (Region 4, 83 %, n=452). Several individual sites showed a greater than 95 % infection frequency (CA1, AZ2, NM, TX4, TX3, and MS2), and the lowest infection frequency (58 %) was found in CA3. The two sites that were resampled in 2012 had similar *Rickettsia* frequencies to the previous year: 76 % (2011) and 74 % (2012, n=100, p=1.00) in CA4, and 76 % (2011) and 84 % (2012, n=100, p=0.45) in GA1. All USA field-collected *B. tabaci* were confirmed as the B putative species. Collection date within the 2011 season was not a significant influence on *Rickettsia* frequencies ($F_{1,17}$=0.001, P=0.97), nor was there a statistically significant interaction between region and collection date ($F_{3,14}$=3.07, P=0.06). Collection date was therefore removed from the final model, in which only region was a significant explanatory variable.

Rickettsia Frequencies, Israel

The *Rickettsia* frequency in the *B. tabaci* B putative species in the cotton growing regions of Israel was significantly influenced by collection year ($F_{1,10}$=10.26, p=0.019) but neither region ($F_{2,8}$=0.06, p=0.95) nor the interaction of year and region ($F_{2,6}$=0.18, p=0.84) were significant factors. There were intermediate–high frequencies of *Rickettsia* in the early 2000s declining to low frequencies in 2012 (Fig. 2). The average infection frequency in 2000–2001 was 61 % (n=87), and in 2011–2012, the average had declined to 12 % (n=433). The highest *Rickettsia* infection frequency at a single site was found in Nahshon (Ayalon Valley) in the Central region (95 %, n=20, 88 %, n=34 for the region as a whole) in 2001. In contrast, no *Rickettsia* was detected in Amiad in 2011 (n=19) nor in Revadim in 2012 (n=38).

Rickettsia Genetic Diversity, USA and Israel Comparison

The specificity of the *16S rRNA* diagnostic PCR primers used in the USA was confirmed for a subset of PCR products. No

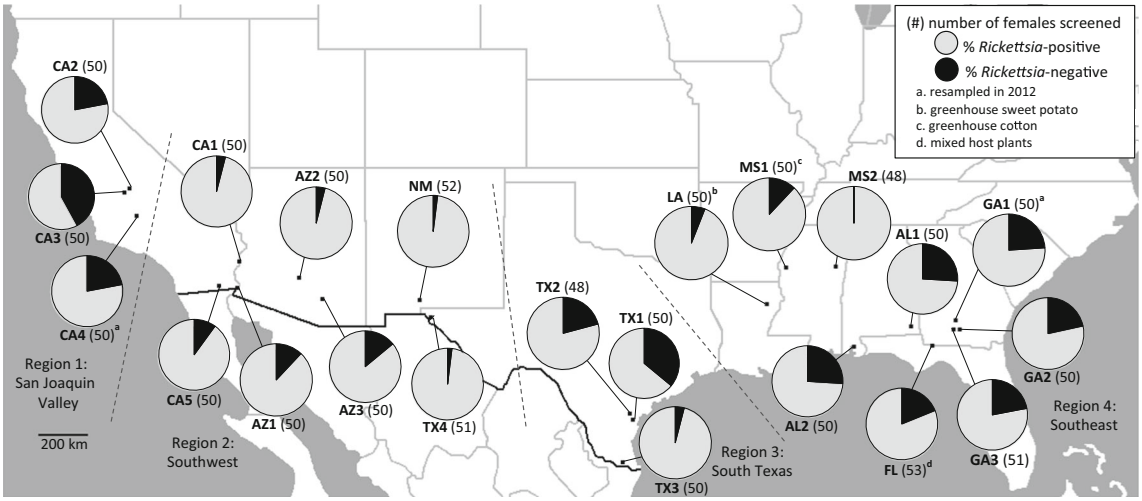

Fig. 1 Survey of *Rickettsia* infection frequency in *B. tabaci* in the cotton-growing regions of the USA from 22 sites from four major regions in 2011, based on diagnostic PCR screening of individual females. In some sites, samples were collected from the greenhouse or other field crops thought to seed field populations in the region

genetic variation was found among the 518 bp of *16S rDNA Rickettsia* PCR products sequenced from individuals at each field site in the USA (n=111). This sequence for these field-collected samples was an exact match (100 % similarity) to the *Rickettsia 16S rRNA* gene sequence from *B. tabaci* B collected in Israel [30] (GenBank accession DQ077707), which had previously been shown to be identical to *Rickettsia 16S rRNA* gene sequence from *B. tabaci* B collected in the USA in site AZ2 [38].

A minimum of 953 bp (maximum 986 bp) of quality sequence was recovered for the *gltA* gene of the 30 tested samples from field- and laboratory-collected *B. tabaci* from Israel and the USA. The final alignment including sequences retrieved from GenBank consisted of 67 taxa and 1314 characters. All of the sequences from our study were identical. They also shared 100 % similarity with five of the other previously sequenced *Rickettsia gltA* sequences collected from *B. tabaci* B, Q, Reunion4, Reunion3 and BurkinaD3 (GenBank accessions EU760764-5 and FJ766352-4, respectively). The ML and BI phylogenetic analyses resulted in trees with nearly identical topology that placed the *gltA* sequences from this study in the *bellii* clade, clustered with other *Rickettsia* from *B. tabaci* (Online Resource 3). The 327 bp *rpmE-tRNAfMet* intergenic spacer region was also identical (100 % similarity) among all of the 35 tested samples from field- and laboratory-collected *B. tabaci* from Israel and the USA but shared only 58 % pairwise identity with the same region in the *Rickettsia bellii* genome (GenBank accession DQ852359). The ML and BI phylogenetic analyses resulted in trees that were not well resolved for the known *Rickettsia* groups (Online Resource 3). As the sequences were identical, representative sequences from each collection region were uploaded to the GenBank database with the accession numbers KM023649 (*gltA*) and KM023650 (*rpmE-tRNAfMet*) for the laboratory lines and as listed in Online Resource 1 for field locations.

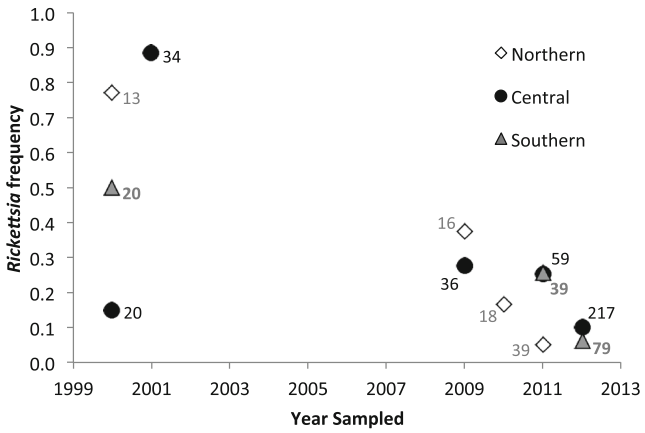

Fig. 2 Survey of *Rickettsia* infection frequency in *B. tabaci* B in the cotton-growing regions of Israel sampled in 2000–2001 and 2009–2012. Frequencies were determined using diagnostic PCR screening of individual adults. *Numbers next to the symbols* refer to the number of whiteflies sampled per region (diamond = Northern, circle = Central, triangle = Southern). These were pooled into regions from the individual sites sampled within regions, as listed in Online Resource 1

Limits of Detection, USA and Israel Comparison

The different diagnostic PCR protocols for *Rickettsia* detection in the USA and Israel were able to detect *Rickettsia* from individual whiteflies at a 1/100 dilution for all but one male sample (Fig. 3). In both countries, the limit of detection was higher overall for female samples than for male samples. The limit of detection was also higher overall for USA samples compared to Israel samples. The mean maximum DNA dilution at which *Rickettsia* could be detected was 1/25600 for females (n=30) and 1/3624 for males (n=25) using the USA

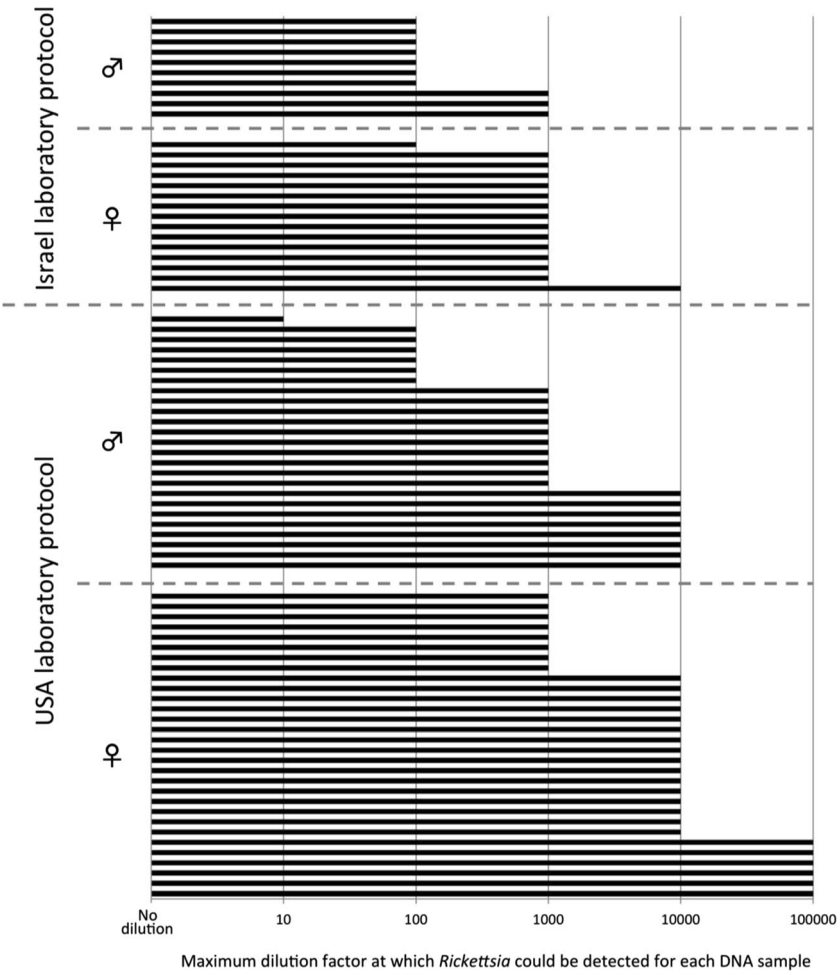

Fig. 3 Limits of detection analysis for the standard *Rickettsia* diagnostic PCR protocols used in the laboratories screening whiteflies collected in Israel and the USA. *Each bar* indicates the maximum fold dilution factor at which a band was observed in a gel for a PCR run on DNA extracted from an individual adult whitefly. *Dotted lines* separate data from male and female whiteflies and from the two laboratories

laboratory protocol and 1/1540 for females (n=15) and 1/ 370 for males (n=10) using the Israeli laboratory protocol.

There was no significant difference among the relative density estimates for *Rickettsia* in the field and laboratory, as tested with quantitative PCR (analysis of variance, $F_{2,26}$=0.1107, p= 0.90). The mean normalized expression values of the *Rickettsia gltA* gene relative to β-*actin* were 32.7±1.9 (SEM) for the MS2 field site, 32.3±1.8 (SEM) for the AZ2 field site, and 34.1±2.5 (SEM) for the laboratory lines originally collected in site AZ2. The similar densities of *Rickettsia* in the laboratory and field samples indicate that the limits of detection analysis conducted with laboratory whiteflies is relevant for the field-collected samples, as they all have similar levels of the bacterium.

Discussion

Dynamics of *Rickettsia*

Across the USA, *Rickettsia* was found at high frequencies in *B. tabaci* populations in 2011 (mean 84 %), building on previous observations of a rapid increase in *Rickettsia* frequencies in the southwestern USA since 2000 [38]. The pattern in Israel was very different; *Rickettsia* frequencies were never as consistently high as observed in the USA and had declined to a mean frequency of 12 % in 2011–2012. The distinct dynamics of *Rickettsia* in these two countries are consistent with observations of a much greater fitness benefit and female bias from *Rickettsia* association in whiteflies in the southwestern USA [38] than in whiteflies in Israel [9, 11]. These results support the hypothesis that there are underlying differences in the symbiosis in these two populations.

The frequencies were significantly heterogeneous across the USA in 2011. Frequencies were especially high in the southwestern cotton-growing region that includes the Imperial Valley, CA, Arizona, New Mexico, and West Texas, where *Rickettsia* has been at high frequencies since 2006 [38]. Intermediate frequencies were observed in the San Joaquin Valley and parts of the Southeast and Southern Texas. The similarity in *Rickettsia* frequencies between 2011 and 2012 in each of these two regions provides no support for

the hypothesis that *Rickettsia* is sweeping into these areas. In the Southwest, frequencies increased from 53 to 97 % in three years [38] so we might expect that this type of sweep repeated elsewhere would reveal a significant increase even over a 1-year interval. Regional fitness differences of *Rickettsia*-infected whiteflies could be due to external abiotic or biotic factors. Other symbiont systems are influenced by temperature and pesticide use [7, 28, 52, 75], parasitoid natural enemies [66, 67, 87, 91–93], insect pathogens [35, 47, 57, 68, 76, 82, 86], or the suite of plants on which the arthropod hosts feed [2, 25]. For example, if there was differential selection on *Rickettsia*-infected whiteflies on different host plants, the *Rickettsia* frequencies might vary when the whiteflies are migrating among different weed hosts and crops. In addition, regional differences in agricultural management practices including conservation of natural enemies, pesticide use, and planting dates [19, 45, 58, 60, 63] may obscure the effects of symbionts on the overall impact of agricultural pests.

The very low infection frequency found in Israeli *B. tabaci* B since 2009 is remarkably different from other countries where *Rickettsia* has been reported at very high frequencies in *B. tabaci* B including 100 % (n=40) in a laboratory population collected in China in 2008 [3], 70.2 % (n=373) in field sites in China from 2005–2009 [14], 90 % (n=9) in Tunisia in 2005, 83 % (n=18) in Antilles in 2004, and 95 % in laboratory populations in Reunion in 2005 [34]. The declining frequencies observed in Israel are consistent with a previous survey that found a 72 % (n=205) *Rickettsia* frequency in *B. tabaci* B sampled from a variety of plants in 2005 [10] and suggest conditional fitness effects associated with *Rickettsia* infection. The decline in frequencies over recent years suggests that at present, the costs of *Rickettsia* infection outweigh the benefits. It is not clear, however, what is driving the change of frequencies in this system. Occasional horizontal transmission may influence the infection dynamics in Israel [8]. Future work may investigate whether the sympatric distribution of Q in the field in Israel might affect the maintenance or recent decline of *Rickettsia* frequencies in this area. Whether the presence of Q, also frequently infected with *Rickettsia*, could be involved in either the earlier maintenance or recent decline of *Rickettsia* frequencies in Israel is unclear.

The presence of different *Rickettsia* genetic variants could underlie the mixed phenotypic reports and different infection frequencies in the USA and Israel. However, we found no sequence differences in several *Rickettsia* genes, including an intergenic spacer region known to be highly variable in Rickettsiaceae [74]. As an example, the sequence from *Rickettsia* in this study had only 58 % pairwise identity to the same region in the type species of the *bellii* clade (GenBank accession number DQ852359). Further sequencing of more of the *Rickettsia* genome may uncover genetic variation. Alternatively, whitefly nuclear genetic structure may be different among regions. An invasion bottleneck followed by explosive growth could underlie the different *Rickettsia* interactions in the USA compared to those in their origin in the Middle East [16, 27]. This is especially likely in the southwestern USA [21, 63], where the highest *Rickettsia* frequencies have been detected.

Intracellular insect symbionts can have variable host relationships affected by biotic and abiotic environmental factors acting on symbionts directly, or indirectly through their influences on hosts. Parameter estimates based on uniform laboratory conditions may not accurately model population dynamics across the geographical distribution of the symbiosis [41, 70, 85]. For example, Hoffman [39, 40] observed a stable cline in *Wolbachia* frequencies in Australian *Drosophila melanogaster* populations; the symbiont *Serratia* protects aphids against heat shock [75] and is associated with arid regions of aphid distribution [36]; the prevalence of *Cardinium* in *Culicoides* is associated with land surface temperature in the Mediterranean region [61]; and *Regiella* infection affects host plant specialization in *Acyrthosiphon pisum* in Japan [83]. In this study, the differences in *Rickettsia* dynamics observed between countries, and the heterogeneity uncovered within the USA provide further insight into the ecological context of the symbiotic relationships in the *B. tabaci* species complex.

Limits of Detection

In the current study, the standard protocols to detect *Rickettsia* were different between the two laboratories and establishing identical protocols would have jeopardized the consistency among sample years within countries. Either protocol could have produced false positive and false negative results but at different rates. A false positive due to unspecific amplification was safeguarded against by sequencing representative samples, but false negatives could occur if the sample had an infection titer lower than could be detected with the DNA extraction, PCR, and electrophoresis methods used. We determined how comparable the survey results were between the two laboratories using serial dilutions on test samples. Both protocols were sufficient to detect *Rickettsia* in extractions of single whiteflies: all but one test sample could be diluted at least 100-fold and still be reliably detected. These results indicate that false negatives are unlikely to be common in this study, and that the observations of differences in frequencies observed between the two countries are true rather than artifacts that could be attributed to the diagnostic PCR methods used.

Rickettsia detection was less sensitive in males, which argues for focusing on females for whitefly symbiont surveys. Bing et al. [4] also reported lower levels of the symbiont *Hemipteriphilus* in male whiteflies. This could be because symbiont transmission depends upon replication in ovarian

tissue, and is less often under selection in males. The protocols also varied in detection limit between laboratories. This could reflect differences in protocol sensitivity or in *Rickettsia* titer among populations.

Protocols for diagnostic PCR in surveys of secondary symbionts of arthropods vary widely, for example, in DNA extraction (chelex, lysis buffer, phenol-chloroform, commercial kit) and PCR product visualization methods (ethidium bromide, SYBR Green, SYBR Safe, Gel Red, Gel Green), making it difficult to compare the relative sensitivity of diagnostic PCR results among published surveys. False negatives may be a common factor in surveys of secondary symbionts generally for low titer infections of arthropods [77, 90]. Performing diagnostic PCR on a serial dilution of the samples is straightforward, does not require any additional equipment, and gives a qualitative indication of the detection limits of the overall protocol used. We suggest this simple dilution series method as a fast way to assess the likelihood of false negatives as a simple step toward a more standardized method for symbiont screening. Reporting the limits of detection in survey papers would provide an objective measure of the rate at which infections may be underreported.

Conclusions

Here, we report the frequencies of a symbiont in field populations of an invasive agricultural pest. *Rickettsia* was shown to be near 100 % infection frequency in some regions of the USA but was at low and declining frequencies across Israel. These distribution patterns are important, as this symbiont has previously been shown to have dramatic effects on host biology. The distinct outcomes of *Rickettsia* infection in these two countries point to mechanistic differences in the interaction and argue for tracking symbiont dynamics in more hosts and locations. Ongoing projects comparing the genomes of *Rickettsia* from different whiteflies will help to uncover any genetic differences between regions and may point to the mechanistic role of *Rickettsia* in the whitefly host. Further investigation of the environmental or genetic differences underlying selection for different *Rickettsia* frequencies in whitefly populations will help to understand the basis of symbiont dynamics and predict the impact of *Rickettsia* frequencies on the pest status of *B. tabaci* in agricultural systems.

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