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ORIGINAL PAPER

Are microbial symbionts involved in the speciation of the gall-inducing aphid, *Slavum wertheimae*?

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Abstract Microbial symbionts have come to be recognized as agents in the speciation of their eukaryote hosts. In this study, we asked if bacterial symbionts are, or were in the past, involved in the speciation of the gall-inducing aphid *Slavum wertheimae* (Hemiptera: Aphididae). This aphid is specific to the tree *Pistacia atlantica*, which has a fragmented distribution among mesic and xeric habitats, leading to corresponding fragmentation of the aphid population. Previous studies revealed genetic differentiation among populations of the gall-inducing aphid, suggesting cryptic allopatric speciation. *Pistacia atlantica* trees show no such variation. By means of diagnostic PCR, we screened several populations of *S. wertheimae* from mesic and xeric sites in Israel for the presence of nine known aphid symbionts: *Arsenophonus, Hamiltonella, Regiella, Rickettsia, Rickettsiella, Serratia, Spiroplasma, Wolbachia*, and X-type, as well as *Cardinium*, known to be a reproductive manipulator. Only one symbiont, *Wolbachia*, was detected in *S. wertheimae. Wolbachia* was found in all the aphids of the mesic populations, compared to 26% in the aphids from the xeric populations. Multilocus Sequence typing of *Wolbachia* revealed new haplotypes in the *fbpA* and *coxA* genes in both the mesic and xeric populations. Phylogenetic analysis showed that *Wolbachia* of *S. wertheimae* is closely related to *Wolbachia* strains from assorted hosts, mostly lepidopterans, but only distantly related to *Wolbachia* strains from other aphid species. We conclude that the cryptic speciation of mesic and xeric populations of *S. wertheimae* was likely driven by geographical isolation rather than by *Wolbachia*.

Keywords Bacterial symbionts · *Pistacia atlantica* · *Wolbachia*

Introduction

Many arthropods maintain symbiotic relationships with microorganisms that affect host development, reproduction, and survival. Some symbiont–host interactions are mutually obligate, with the symbiont producing essential nutrients lacking in the host's diet, while the host provides the symbiont with other nutrients which the microorganism cannot produce. Obligate symbionts (also termed "primary symbionts") are maternally transmitted, and consequently the phylogenies of the parties are typically congruent (Moran et al. 2008; Douglas 2015). Other, facultative symbionts (FS), are maternally transmitted but can also be transmitted horizontally among host lineages, therefore the phylogenies of hosts and FS are typically incongruent. While FS are generally not considered critical for host development and reproduction, multiple vital functions of FS revealed in recent years indicate important roles in the hosts' ecology and evolution. Some FS contribute directly to their host's fitness in various ways, such as conferring resistance to pathogens and natural enemies or enhancing fecundity, thereby indirectly promoting their own fitness (reviewed in Oliver and Martinez 2014; Douglas 2015; McLean et al. 2016).

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Fascinatingly, some FS use a different strategy to promote their fitness: they manipulate the reproduction of the host in ways that lead to increased proportions of female progeny, which will transmit the symbiont to the next generation, at the expense of males and/or females that do not carry the symbiont. These reproductive manipulations include parthenogenesis, male killing (male embryos die before hatching), feminization (genetic males develop as females), and cytoplasmic incompatibility (CI) (a cross between a symbiont-infected male and an uninfected female is incompatible, leading to a decrease in the proportion of uninfected individuals in the population) (Zchori-Fein and Bourtzis 2012).

To date, *Arsenophonus, Cardinium, Rickettsia, Spiroplasma*, and *Wolbachia* have been found to cause one or more of these reproductive manipulations. According to a recent analysis (Weinert et al. 2015), *Wolbachia*, which infects about 50% of arthropod species, is by far the most studied symbiont and the only one known to induce all four types of manipulations (Zug and Hammerstein 2015; Correa and Ballard 2016).

Theoretical studies corroborated by empirical evidence show that reproductive manipulations reduce gene flow between sympatric/parapatric populations, leading to pre- or post-zygotic reproductive isolation and accelerating speciation over time (reviewed in Engelstädter and Hurst 2009; Brucker and Bordenstein 2012; Vavre and Kremer 2014; Bennett and Moran 2015). Symbiont-induced parthenogenesis results in the production of asexual females alongside the original sexual population; with time, asexual females accumulate mutations in genes that are involved in sexual reproduction, leading to asexual speciation. Bidirectional CI is another possible speciation mechanism: individuals acquire different strains of the reproductive manipulator, resulting in reciprocal incompatibility in both cross directions (Bordenstein et al. 2001; Brucker and Bordenstein 2012).

In the current study, we focused on bacterial symbionts of the aphid *Slavum wertheimae* Hille Ris Lambers (Hemiptera: Aphididae: Eriosomatinae: Fordini), that induces galls in the Mt. Atlas mastic tree, *Pistacia atlantica* Desfontaines (Sapindales: Anacardiaceae). All aphid species harbor the obligate symbiont *Buchnera aphidicola*, which synthesizes amino acids lacking in the phloem diet (with the exception of several aphid species within the subfamily Cerataphidinae, in which *Buchnera* has been replaced by a yeast-like symbiont; Vogel and Moran 2013). Additionally, aphids are facultatively associated with an array of bacterial symbionts having diverse effects on the fitness of their aphid hosts, including a single example of male killing by *Spiroplasma* (Skaljac 2016; Simon et al. 2011; Zytynska and Weisser 2016).

Pistacia atlantica has a disjunctive Irano-Turanian distribution extending from central Asia through the Levant and North Africa as far as the Canary Islands. In the Pleistocene, when the climate was cooler, *P. atlantica* was more continuously distributed, but climatic changes in the region during the Pleistocene and Holocene left isolated populations in unconnected suitable habitats (Danin 1999). Consequently, the tree is fragmentally distributed in Israel from the mesic climate in the north to the xeric climate of the southern Negev desert highlands (~600 vs. <100 mm mean annual precipitation, respectively) (Fig. 1). The mesic and xeric populations of *P. atlantica*, although geographically separated and phenotypically distinct (Fig. 1), are genetically alike (Inbar and Kark 2007; Avrani et al. 2012). *Pistacia atlantica* is an obligate host to several species of gall-inducing aphids, including *S. wertheimae*, readily identified by the distinctive red, cauliflower-shaped galls on the lateral buds. A single tree can host numerous galls. Galls are induced in the spring, each gall by a single female; subsequently the aphids feed on the phloem sap within the gall and reproduce parthenogenetically for multiple generations until the fall, at which time, a winged aphid generation is released from the galls and disperses to other branches or nearby trees. The winged aphids then give birth to sexual aphids, which mate and lay eggs that will diapause throughout the winter and will hatch in the spring (Wool and Bogen 1999; Wool 2004). Since this aphid is specific to *P. atlantica*, the distributions of the two species are linked. However, unlike the host tree populations, the mesic and xeric aphid populations, although morphologically indistinguishable, differ genetically in their sequences of the mitochondrial genes COI and COII and their AFLP fingerprint profile, resulting in two distinct phylogenetic groups (genetic distances of 49 AFLP loci ranged between 0.09 and 0.141) and suggesting cryptic speciation. There were no such differences between aphids within each region (Avrani et al. 2012).

The presence and identity of bacterial symbionts in *S. wertheimae* have never been explored. Therefore, the goals of our research were (1) to study which facultative symbionts are hosted by *S. wertheimae*; (2) to study whether the mesic or xeric populations of *S. wertheimae* are differentially associated with reproductively manipulative bacterial symbionts. Such an association, if found, could suggest that symbionts play, or at least played in the past, a role in the cryptic speciation of the aphids, and/or have other adaptive effects on the aphid hosts.

Materials and methods

Gall aphid collections

Specimens of *S. wertheimae* were collected from 9 sites in Israel, including seven locations in the north (mesic) and

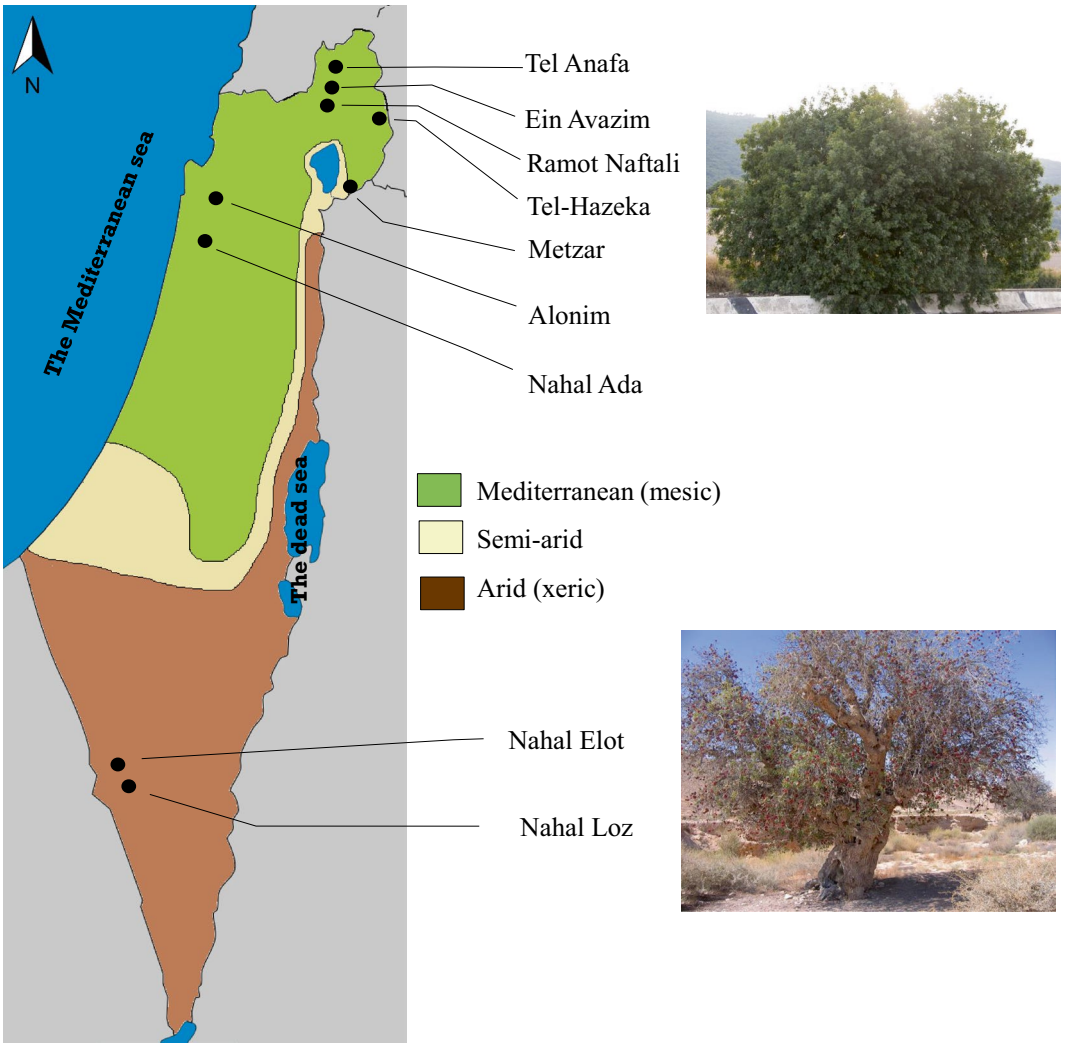


Fig. 1 A map showing the sampling sites' approximate locations (see supplementary Table S1 for coordinates), the climate type, and representative photo of a *P. atlantica* tree from mesic (*upper photo*) and xeric (*lower photo*) climates

two in the south (xeric) (Fig. 1). *Slavum wertheimae*, like most other aphid species, reproduces parthenogenetically; all individuals within a single gall are thus genetically identical. Therefore, all aphids within a gall were separated from the gall tissue and their DNA was extracted together, as a pool, using DNeasy Blood and Tissue Kit (Qiagen, GmbH). Hence the unit of replication in this study comprises DNA extracted from the aphids inhabiting a single gall. The numbers of trees and galls that were sampled at each site are detailed in Table S1.

PCR protocols

We screened the aphids' DNA for the presence of ten facultative bacterial symbionts using genus-specific primers, as detailed in Table 1. Nine of the symbionts are known from aphids, and five are known as reproductive manipulators in various hosts. PCR products were visualized in 1% agarose gels, and the identity of selected amplification products was verified by Sanger sequencing (McLab Laboratories, San Francisco, CA, USA). Each set of PCRs included a relevant positive control sample (an extraction of a symbiont-infected pea aphid, *Acyrthosiphon pisum*, supplied by Dr. Kerry Oliver, University of Georgia, Athens, GA, USA). Where no symbiont was detected, the presence and quality of DNA was re-verified by amplifying a fragment of the aphid's COI gene as described in Avrani et al. (2012).

The differences in symbiont frequencies in the mesic versus xeric populations were analyzed by the Pearson Chi-square test using SPSS 19.0 software.

Table 1 List of symbionts that were screened for, and primers used in the study

Symbiont	Known from aphids?	Known as reproductive manipulator?	Primers	Reference
Wolbachia pipientis	Yes	Yes	*Wol* 16 S	Heddi et al. (1999)
			MLST: *gat*B, *fts*Z, *cox*A *fbp*A, *hcp*A	http://pubmlst.org/Wolbachia/info/amp_seq_single.shtml
Cardinium hertigii	No	Yes	CAR-SP F/R	Nakamura et al. (2009)
Rickettsia spp.	Yes	Yes	16SA1/Rick 16SR	Tsuchida et al. (2002)
Spiroplasma	Yes	Yes	16SA1/TKSS	
Arsenophonus	Yes	Yes	A-infB F/R	Taylor et al. (2011)
Hamiltonella defensa	Yes	No	10F/419R	Russell et al. (2013)
Regiella insecticola	Yes	No	1279F/35R	
Rickettsiella	Yes	No	211F/470R	
X- type	Yes	No	10F/420R	Ferrari et al. (2012)
Serratia symbiotica	Yes	No	16SA1/PASScmp	Moran et al. (2005)

Wolbachia characterization

We used the multilocus sequence typing (MLST) protocol (http://pubmlst.org/Wolbachia) (Baldo et al. 2006) to characterize the strain/s of *Wolbachia* found in our samples. MLST is a robust classifying system that provides strain typing based on variation in five conserved housekeeping genes (*gatB, coxA, hcpA, ftsZ*, and *fbpA*), and is the current standard for *Wolbachia* identification. *Wolbachia* was sequenced from 16 samples from various mesic populations and from all ($n=6$) infected xeric samples, following the protocol specified in the MLST website. PCR reactions were performed in volumes of 25 µl, of which 5 µl was used to verify a single product in 1% agarose gels and the remaining 20 µl was used for direct Sanger sequencing (MCLAB Laboratories, San Francisco, CA, USA). The sequences' chromatograms were visualized, checked manually, and aligned using MEGA6 software; the consensus sequences obtained were then deposited in the MLST database.

To infer the phylogenetic relationships between *Wolbachia* from *S. wertheimae* and from other hosts, two phylogenetic analyses were performed on the MLST alleles sequence alignment (using MEGA7 software; Kumar et al. 2016).

1. Fifty-eight sequences from the MLST database were used for the first analysis, including all STs that have at least one allele in common with *S. wertheimae*'s *Wolbachia* ($n=35$), as well as representative STs from a variety of host taxa (Table S2). A single strain belonging to Supergroup A from a hemipteran host was included for rooting the trees. This analysis was performed on the concatenated sequences of the five MLST alleles (2073–2079 bp).
2. In the second analysis, we added sequences from two aphid species (Augustinos et al. 2011)—*Cinara cedri* and the gall-inducing aphid, *Baizongia pistaciae*—which were not deposited in the MLST database. The data of these sequences lacked the *ftsZ* allele in *C. cedri* and the *coxA* and *hcpA* alleles in *B. pistaciae* (Table S2). Due to the missing data, this analysis was done on concatenated sequences of the *gatB* and *fbpA* alleles (792–798 bp), which are the only two alleles with available sequences for all the hosts.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura–Nei model as implemented in the MEGA software (Tamura and Nei 1993). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and then selecting the topology with superior log likelihood value. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. Branch support was assessed by 1000 bootstrap replications.

Results

The only bacterial symbiont that was detected in our samples is *Wolbachia*, which was found in 100% of the mesic aphid samples ($n=25$ galls), compared to 26% of the xeric samples ($n=23$ galls) ($\chi^2_1=28.6$; $p<0.001$). The COI gene fragment was successfully amplified in all samples that

were negative for all symbionts, confirming that the results were not false negatives.

Characterization of *Wolbachia*

All *Wolbachia*-carrying individuals tested, from both mesic and xeric populations, carried a single and identical strain of *Wolbachia*. Comparison of our consensus sequences to the MLST database revealed new haplotypes in two genes—*fbpA* and *coxA*; the allelic profile of the *Wolbachia* isolate from *S. wertheimae* is therefore novel as well. The new sequences were deposited in the MLST database and assigned the sequence type (ST) number 460 (Table 2). Each of the other 3 alleles (i.e., those that were not new to the database-*gatB, hcpA*, and *ftsZ*) has been reported in the past from various hosts. The combination of these 3 alleles was found in a single additional ST in the database, from the lepidopteran *Jalmenus evagoras* (ST#154). The *hcpA* allele of *S. wertheimae* is shared by 34 other STs, of which 22 were isolated from 82 different lepidopteran hosts (Table S2). The *ftsZ* and *gatB* alleles of *S. wertheimae* are found in 8 other STs (seven Lepidopterans and one mite) and 4 other STs (two Lepidopterans, one Hemipteran, and one Dipteran), respectively. All the STs that had at least one allele in common with *S. wertheimae*'s *Wolbachia* belong to Supergroup B.

Maximum Likelihood phylogenetic analysis of the *gatB* and *fbpA* concatenated sequences (the only two alleles with available sequences for all the samples included in the analysis; see methods and materials section for details) revealed that the *Wolbachia* strain from *S. wertheimae* is clustered on a relatively recently evolved branch that includes many strains from Lepidopteran host species, as well as from two hemipterans and two dipterans (Fig. 2a). *Wolbachia* of the two other aphid species—*C. cedri* and *B. pistaciae*—clustered together as an out-group and were thus distantly related to *Wolbachia* from *S. wertheimae. Wolbachia* strains from other host species that belong to the suborder Sternorrhyncha (the suborder to which *S. wertheimae* belongs) are placed on various, earlier, branches of the tree (Fig. 2a). The Maximum Likelihood phylogenetic analysis that was done on the concatenated sequences of all five alleles (but without *C. cedri* and *B. pistaciae*) showed a similar pattern, with the closest relative being the butterfly *J. evagoras*, but here the specific branch of *S. wertheimae* included more hemipteran hosts, including one from the Sternorrhyncha, *Diaphorina citri* (Fig. 2b). It should be noted that some branches of the tree are poorly supported (<50% bootstrap support). Phylogenetic analyses of each allele by itself resulted in similar results, although in the analysis of the *coxA* allele, *Wolbachia* of *S. wertheimae* is located on more basal branches of the tree (data not shown).

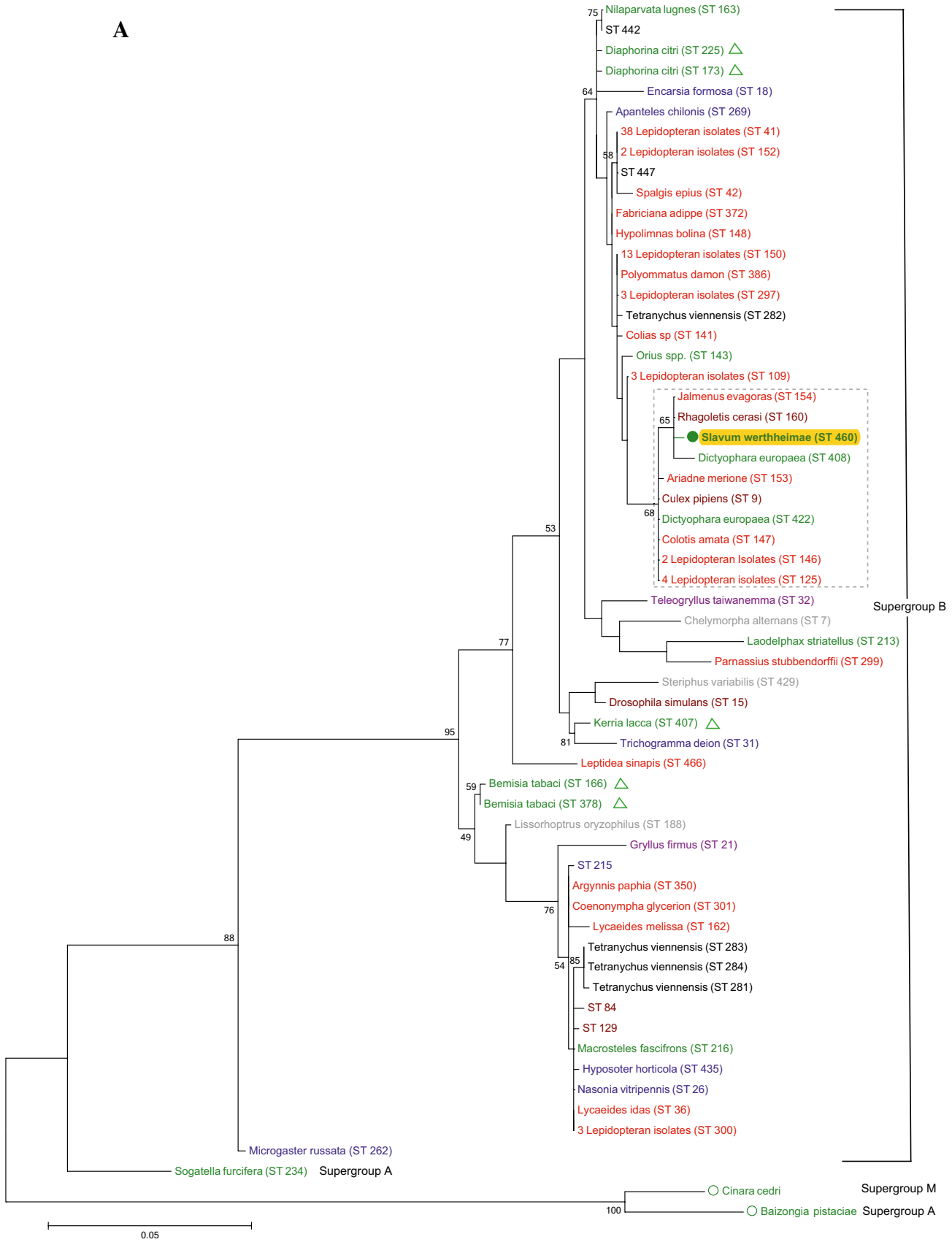
Table 2 *Wolbachia* alleles and their sequence type numbers in *S. wertheimae*. Numbers followed by * are alleles and profiles that were first described in this study

Allele	No. of allele in MLST database
gatB	101
coxA	228*
hcpA	40
ftsZ	73
fbpA	415*
Sequence type	460*

Discussion

The question of how new species evolve continues to intrigue evolutionary biologists and theoreticians. In the past, speciation was believed to occur mainly under conditions of allopatry; currently, it is widely accepted that speciation may take place sympatrically as well, as a consequence of differential adaptations among individuals in a given population (Bolnick and Fitzpatrick 2007; Butlin et al. 2008; Schluter 2009). In recent years, the ability of microbial symbionts, both reproductive manipulators and gut bacteria, to induce reproductive isolation through pre- and post-zygotic mechanisms has been demonstrated in several studies, and speciation is the inferred long-term consequence (e.g., Bordenstein et al. 2001; Brucker and Bordenstein 2012, 2013; Vavre and Kremer 2014; Gilbert et al. 2015).

The genetic profiles of populations of the gall-inducing aphid *S. wertheimae* along the Irano-Turanian distribution zone in Israel suggest allopatric cryptic speciation, as the aphids display two distinct phylogenetic groups, one distributed in the northern mesic region, and the second in the xeric southern zone (Avrani et al. 2012). Here, we found that all the mesic aphids carry *Wolbachia* compared to only 26% of the xeric ones. Is it possible, then, that *Wolbachia* played a role in the cryptic speciation of *S. wertheimae*? In light of our results we think this is unlikely because the genotype of *Wolbachia* in both regions is identical. If *Wolbachia* genotypes in the mesic and xeric populations were different, that could suggest the potential for bidirectional CI, but this is not the case here. Instead, an ecology-based speciation scenario seems more plausible. Current-fragmented populations of *S. wertheimae* represent the remains of a more continuous historical distribution of the host tree which was altered by climatic changes during the Pleistocene and Holocene, leading to the disjunction between northern and southern populations (Danin 1999). Thus, the most parsimonious explanation for the situation we see today is that all *S. wertheimae* populations originally harbored *Wolbachia*, until climate change caused a drastic shift in selection pressures that led to the loss of *Wolbachia*


A
Nilaparvata lugnes (ST 163)
ST 442
Diaphorina citri (ST 225)
Diaphorina citri (ST 173)
Encarsia formosa (ST 18)
Apanteles chilonis (ST 269)
38 Lepidopteran isolates (ST 41)
2 Lepidopteran isolates (ST 152)
ST 447
Spalgis epius (ST 42)
Fabriciana adippe (ST 372)
Hypolimnas bolina (ST 148)
13 Lepidopteran isolates (ST 150)
Polyommatus damon (ST 386)
3 Lepidopteran isolates (ST 297)
Tetranychus viennensis (ST 282)
Colias sp (ST 141)
Orius spp. (ST 143)
3 Lepidopteran isolates (ST 109)
Jalmenus evagoras (ST 154)
Rhagoletis cerasi (ST 160)
Slavum werthheimae (ST 460)
Dictyophara europaea (ST 408)
Ariadne merione (ST 153)
Culex pipiens (ST 9)
Dictyophara europaea (ST 422)
Colotis amata (ST 147)
2 Lepidopteran Isolates (ST 146)
4 Lepidopteran isolates (ST 125)
Teleogryllus taiwanemma (ST 32)
Chelymorpha alternans (ST 7)
Laodelphax striatellus (ST 213)
Parnassius stubbendorffii (ST 299)
Steriphus variabilis (ST 429)
Drosophila simulans (ST 15)
Kerria lacca (ST 407)
Trichogramma deion (ST 31)
Leptidea sinapis (ST 466)
Bemisia tabaci (ST 166)
Bemisia tabaci (ST 378)
Lissorhoptrus oryzophilus (ST 188)
Gryllus firmus (ST 21)
ST 215
Argynnis paphia (ST 350)
Coenonympha glycerion (ST 301)
Lycaeides melissa (ST 162)
Tetranychus viennensis (ST 283)
Tetranychus viennensis (ST 284)
Tetranychus viennensis (ST 281)
ST 84
ST 129
Macrosteles fascifrons (ST 216)
Hyposoter horticola (ST 435)
Nasonia vitripennis (ST 26)
Lycaeides idas (ST 36)
3 Lepidopteran isolates (ST 300)
Microgaster russata (ST 262)
Sogatella furcifera (ST 234)
Supergroup A
Supergroup B
Cinara cedri
Baizongia pistaciae
Supergroup M
Supergroup A
0.05

◂ **Fig. 2** Phylogenetic analyses of *Wolbachia*, using the Maximum Likelihood method. **a** A tree constructed from concatenated sequences of the *gatB* and *fbpA* alleles, which were available for all host species included in the analysis (i.e., 58 sequences from the MLST database + sequences of two aphid species, from Augustinos et al. 2011). There were a total of 792 positions in the final dataset. The tree with the highest log likelihood (−3492.9020) is shown. **b** A tree constructed from concatenated sequences of all the five alleles. This analysis involved 58 sequences (i.e., without the sequences of the two aphid species from Augustinos et al. 2011, because 1–2 allele sequences are lacking). There were a total of 2071 positions in the final dataset. The tree with the highest log likelihood (−8480.9079) is shown. In both trees, bootstrap support values are shown next to the branches (only values ≥50%). See text for further details. *Wolbachia* of *S. wertheimae*, obtained in this study, is highlighted in yellow and marked with a green dot (*dot*). The two other aphid species are marked with a green symbol (*open circle*); hosts from the suborder Sternorrhyncha are marked with a green triangle symbol (*open triangle*). Hosts' orders are color coded: Lepidoptera—red, Hemiptera—green, Diptera—maroon, Hymenoptera—blue, Orthoptera—purple, Coleoptera—gray, Acari—black (STs 442 and 447—order not specified in the database). Hosts' species, family, and order names, as well as the *Wolbachias*' MLST genes and Supergroups are detailed in Table S2

in the majority of the xeric aphids. If so, then probably the fitness cost of harboring *Wolbachia* is greater than the benefits, in the harsh xeric conditions. Indeed, *Wolbachia* are usually sensitive to heat, a fact that is often used to experimentally establish aposymbiotic insect populations (Li et al. 2014). In an alternative scenario, *Wolbachia* invaded the mesic and xeric aphid populations independently, after the populations became geographically isolated. This scenario seems less plausible, as the sequence type of *Wolbachia* is identical in mesic and xeric aphids. A third scenario is that *Wolbachia*-infected aphids disperse from the mesic to the xeric habitats, but this too is very unlikely because (1) mesic and xeric aphid populations differ genetically in both mitochondrial and nuclear genomes (this is the starting point of our study); (2) winged aphids have a very limited flight range (Wool and Bogen 1999), and although they may also disperse passively with winds, the probability of landing on one of very few host trees, at least 200 km away, is extremely low.

It will be technically difficult, perhaps even impossible, to test empirically whether *Wolbachia* manipulates the reproduction of *S. wertheimae*, since gall-inducing aphids are difficult to culture in the laboratory and complete only one sexual reproduction cycle every year. Nonetheless, comparing fitness parameters between *Wolbachia*-infected and *Wolbachia*-free *S. wertheimae* in xeric populations should be fairly doable and may help to clarify the role of this widespread symbiont in this aphid host.

Aphids often harbor a diverse array of facultative bacterial symbionts (Skaljac 2016; Zytynska and Weisser 2016), of which only *Wolbachia* was detected in *S. wertheimae* in our study. *Wolbachia* went unnoticed in aphids for many years; it was noted for the first time only in 2000 (Jeyaprakash and Hoy 2000). Since then, *Wolbachia* has been found in over a hundred species of aphids (Wang et al. 2009, 2014; Jones et al. 2011), including one gall-inducing aphid species, *B. pistaciae*, collected in Greece from *Pistacia terebinthus* (Augustinos et al. 2011). Unfortunately, MLST data of aphids' *Wolbachia* are limited to two species only (!) with 1–2 alleles missing in each profile (Augustinos et al. 2011). The allelic profile of *Wolbachia* from our samples is new to the MLST database, as the sequences of the *coxA* and *fbpA* genes differ from all previously known sequences. The *Wolbachia* of *S. wertheimae* is nested within a clade that includes various hosts, all from Supergroup B (Fig. 2); therefore we infer that *S. wertheimae*'s *Wolbachia* belongs to Supergroup B as well. In contrast, *Wolbachia* from the gall-inducing aphid *B. pistaciae* and the aphid *C. cedri* were found here to be only distantly related to *Wolbachia* of *S. wertheimae*, corresponding with their Supergroups affiliations (A and M, respectively, Augustinos et al. 2011). The tree that was constructed with all the five MLST alleles (Fig. 2b) likely reflects the phylogenetic relationships more reliably, since it is based on the whole MLST alleles set (~2000 bp). In this tree (Fig. 2b), *Wolbachia* from other Sternorrhynchan hosts is located in the same clade (*Diaphorina citri*) or in a sister clade (*Kerria lacca* and *Bemisia tabaci*) of the tree, but the closest relatives were found in the butterfly *J. evagoras* and the spider-mite *T. viennensis* (Fig. 2b). Overall, these findings corroborate the notion that *Wolbachia* is transferred horizontally between species and subsequently diversifies (Correa and Ballard 2016), although it is hard to explain how (i.e., both the evolutionary and the ecological mechanisms) *Wolbachia* strains from distantly related hosts, like aphids and butterflies, are more similar than closely related hosts. Our findings coincide with the high divergence of *Wolbachia* in aphids found by Augustinos et al (2011), but in our system, although the aphids are subject to different selection pressures and *Wolbachia*'s infection rates are subject to drift in the xeric populations, *Wolbachia* has not diverged genetically (yet?) between the mesic and xeric populations. Obtaining MLST data from more aphid

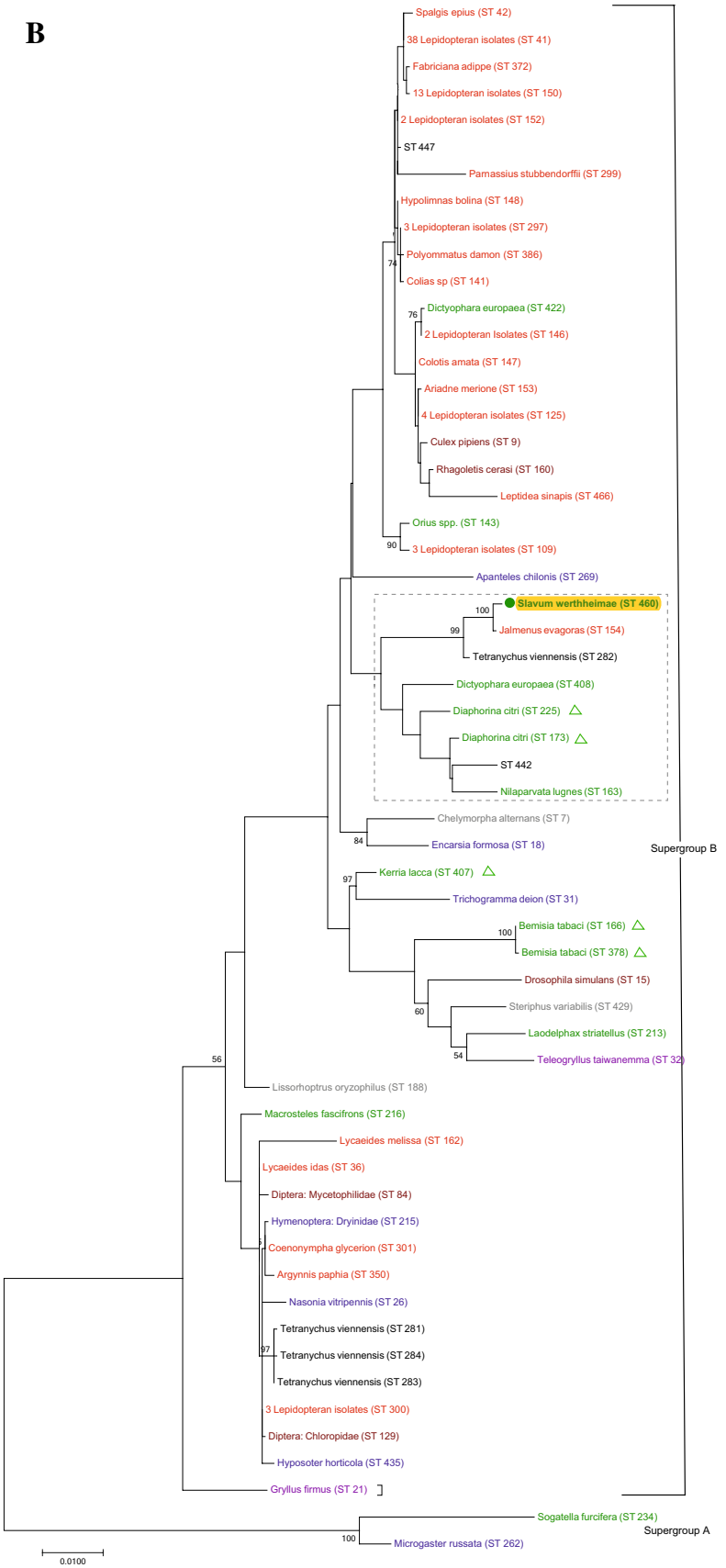


Fig. 2 (continued)

species will clarify the evolutionary history of *Wolbachia* in this important insect family.

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