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**Biocomplexity and Selective Modulation of Whitefly
Symbiotic Composition**

J.K. Brown, E. Zchori-Fein, N. Katzir

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Project Title: Biocomplexity and Selective Modulation of Whitefly Symbiotic Composition

Investigators

Principal Investigator (PI): Judith K. Brown

Co-Principal Investigator (Co-PI): Einat Zehori-Fein

Collaborating Investigators: Nurit Katzir

Institutions

The University of Arizona
Tucson, USA

Agricultural Research
Organization Neve Ya'ar Research
Center, Israel

Agricultural Research Organization
Neve ya'ar Research Center, Israel

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Abbreviations commonly used in the report, in alphabetical order: DGGE (denaturizing gradient gel electrophoresis); FISH (fluorescent *in-situ* hybridization); PCR (polymerase chain reaction); 16S rDNA (16S ribosomal DNA); CI (cytoplasmic incompatibility).

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Signature
Principal Investigator

- Alice C. Langen, Director
Signature Research Compliance
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Description Cooperation:

The Israeli team has determined the complete symbiotic complex of *B. tabaci* populations from both Israeli and the ones sent from Arizona. Based on the results, *Rickettsia*-specific primers were designed and made available to the US team. All together, the synergism between the groups provided a strong platform without which none of the groups could have achieved its goals.

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Abstract

Whiteflies are sap-sucking insects that harbor obligatory symbiotic bacteria to fulfill their dietary needs, as well as a facultative microbial community with diverse bacterial species. The sweetpotato whitefly *Bemisia tabaci* (Gennadius) is a severe agricultural pest in many parts of the world. This species consists of several biotypes that have been distinguished largely on the basis of biochemical or molecular diagnostics, but whose biological significance is still unclear. The original objectives of the project were (i) to identify the specific complement of prokaryotic endosymbionts associated with select, well-studied, biologically and phylogeographically representative biotypes of *B. tabaci*, and (ii) to attempt to 'cure' select biotypes of certain symbionts to permit assessment of the affect of curing on whitefly fitness, gene flow, host plant preference, and virus transmission competency. To identify the diversity of bacterial community associated with a suite of phylogeographically-diverse *B. tabaci*, a total of 107 populations were screened using general Bacteria primers for the 16S rRNA encoding gene in a PCR. Sequence comparisons with the available databases revealed the presence of bacteria classified in the: Proteobacteria (66%), Firmicutes (25.70%), Actinobacteria (3.7%), Chlamydiae (2.75%) and Bacteroidetes (<1%). Among previously identified bacteria, such as the primary symbiont *Portiera aleyrodidarum*, and the secondary symbionts *Hamiltonella*, *Cardinium* and *Wolbachia*, a *Rickettsia* sp. was detected for the first time in this insect family. The distribution, transmission, and localization of the *Rickettsia* were studied using PCR and fluorescence *in situ* hybridization (FISH). *Rickettsia* was found in all 20 Israeli *B. tabaci* populations screened as well as some populations screened in the Arizona laboratory, but not in all individuals within each population. FISH analysis of *B. tabaci* eggs, nymphs and adults, revealed a unique concentration of *Rickettsia* around the gut and follicle cells as well as its random distribution in the haemolymph, but absence from the primary symbiont housing cells, the bacteriocytes. *Rickettsia* vertical transmission on the one hand and its partial within-population infection on the other suggest a phenotype that is advantageous under certain conditions but may be deleterious enough to prevent fixation under others. To test for the possible involvement of *Wolbachia* and *Cardinium* in the reproductive isolation of different *B. tabaci* biotypes, reciprocal crosses were performed among populations of the *Cardinium*-infected, *Wolbachia*-infected and uninfected populations. The crosses results demonstrated that phylogeographically divergent *B. tabaci* are reproductively competent and that cytoplasmic incompatibility inducer-bacteria (*Wolbachia* and *Cardinium*) both interfered with, and/or rescued CI induced by one another, effectively facilitating bidirectional female offspring production in the latter scenario. This knowledge has implications to multitrophic interactions, gene flow, speciation, fitness, natural enemy interactions, and possibly, host preference and virus transmission.

Although extensive and creative attempts undertaken in both laboratories to cure whiteflies of non-primary symbionts have failed, our finding of naturally uninfected individuals have permitted the establishment of *Rickettsia*-, *Wolbachia*- and *Cardinium*-free *B. tabaci* lines, which are been employed to address various biological questions, including determining the role of these bacteria in whitefly host biology.

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Achievements

Significance of main scientific achievements or innovations

Collectively, the cryptic whitefly *Bemisia tabaci* (Genn.) (Sternorrhyncha: Aleyrodidae) species complex and plant-infecting viruses it transmits pose a major constraint on vegetable and fiber crop production worldwide. Insects, in general and members of the order Homoptera in particular, are known to harbor a diverse assemblage of bacteria, which may influence various aspects of their host's biology. To assess that diversity in a large number of *B. tabaci* populations both DGGE (denaturing gradient gel electrophoresis) and PCR-based analyses were applied. The extensive PCR survey of *B. tabaci* populations found in the AZ laboratory revealed the presence of large numbers of bacteria species (Tables 1&2, Appendix).

Collectively, the bacterial community associated with *B. tabaci* constitutes a large number of bacteria previously reported as arthropod symbionts, free-living bacteria and plant endophytes. Some of these bacteria (such as the endophytes and the free living) may have originated from the gut content of the whitefly, while others may represent yet undiscovered symbiotic associations. Of particular interest are the putative vertebrate pathogens discovered, such as is *Orientia tsutsugamushi*, the causative agent of scarab typhus, which was found in a sample from India. This is the first time such a wide variety of potentially human pathogenic bacteria have been detected in a phloem-feeding insect.

Using degenerate gradient gel electrophoresis (DGGE) analysis, we were also able to show that the bacterial community of whiteflies sampled in Israel and the US is composed of at least 3 facultative bacteria, namely, *Cardinium*, *Hamiltonella* and *Rickettsia* (See Gottlieb et al., 2006, attached).

***Rickettsia* studies.** Because *Rickettsia* was detected for the first time in that insect family, the Israeli laboratory focused on *Rickettsia* for a large portion of this project. To establish the identity of the whitefly bacterium, and further characterize its phylogenetic affiliation, the guidelines of Fournier et al. (2003) were followed, and the results showed the *Rickettsia* found clusters with the pea aphid and the two-spotted spidermite *Rickettsia* (See Perlman et al., 2006, attached). Whole-mount fluorescence *in situ* hybridizations (FISH) of various developmental stages established, for the first time, the long-assumed specific-localization of the *B. tabaci* primary symbiont *Portiera*, in the bacteriocytes. This technique also shows a random and uneven localization of *Rickettsia* in most of the *B. tabaci* eggs, nymphal stages and adults tested. In various adult and nymph specimens, the bacteria are seen aggregating along the gut structure (See Gottlieb et al., 2006, attached). Our data suggest that

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the *Rickettsia* is vertically transmitted in the whitefly through the bacteriocytes. The effect of *Rickettsia* on *B. tabaci* is yet to be resolved; however, since it is highly prevalent in Israeli and US *B. tabaci* populations, it can be speculated that its phenotype is advantageous under certain conditions but may be deleterious enough under others to prevent fixation.

Most *B. tabaci* populations tested for presence of *Rickettsia* exhibited variation in infection-status; therefore it was possible to establish a *Rickettsia*-free line. Preliminary screening of various *B. tabaci* populations also revealed *Hamiltonella*-free individuals, from which we are now establishing a colony.

***Cardinium* studies.** The suspected role of *Wolbachia* and *Cardinium* in cytoplasmic incompatibility (CI) or other reproductive aberrations in *B. tabaci* has not been demonstrated until now. The current screening of the bacteria associated with *B. tabaci* populations identified B biotype populations with a widely varied complement of bacteria, and allowed the US team to study the respective involvement of two of them in gene flow in the whitefly host. The biotype colonies used in the AZ laboratory for biological and molecular studies are shown in Table 3, together with geographic origin, host range, and endosymbiont infection.

When reciprocal crosses were carried out among the AZ-B, AZ-A, and Jat biotypes, results revealed the unidirectional production of female offspring in AZ-B x Jat, or AZ-B x AZ-A crosses, whereas, Jat x AZ-A crosses yielded bidirectional production of females. The unidirectional incompatibility of Jat and AZ-A with the AZ-B biotype is consistent with our discovery that AZ-A and Jat are infected with CI-causing bacteria, *Cardinium* and *Wolbachia*, respectively. The fact that female offspring are produced bidirectionally from Jat x AZ-A reciprocal crosses, suggests that each of these two divergent bacterial species is capable of rescuing CI induced by the other. Further reciprocal Jat x AZ-A crosses employing *Cardinium*-infected (AZ-A) as the mother, produced three times more female offspring than when the *Wolbachia*-infected (Jat) biotype was the mother. A novel, rescue phenomenon such as this, involving interactions between two phylogenetically divergent bacteria, has not been previously reported.

To date it has not been possible to rid *B. tabaci* of suspect CI-bacteria, making it likewise impossible to prove CI-causality over CI-association. Taking advantage of the recently discovered *Cardinium* association with *B. tabaci*-FLB1 (Table 3) we have observed unidirectional production of females in AZ-B and FLB1 crosses, and bidirectional compatibility for FLB1 x AZ-A crosses.

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In summary, although the differential curing has failed (see appendix), we now have a tool for establishing whitefly lines, which carry specific symbiotic complex and can be used for studying the various effects each symbiont has on its *B. tabaci* host. Thus, the BARD objectives were met nearly in full.

Agricultural and/or economic impacts of the findings

Our combined results provide a coherent picture of bacterial assembly in diverse *B. tabaci* populations, and serve the required basis for further studies on whitefly-bacteria interactions. The finding of *Rickettsia*, *Cardinium*, and *Wolbachia* in a number of populations has enlightened the observed biology regarding rearing methods of that pest for natural enemies' production, and Bio-Bee Biological Systems in Sde-Eliyahu (Israel) are further investigating the relationships. The information acquired has implications to multi-trophic interactions, gene flow, speciation, fitness, natural enemy interactions, and possibly, host preference and virus transmission competency.

Details of cooperation

The Israeli team has determined the complete symbiotic complex of *B. tabaci* populations from both Israeli and the ones sent from Arizona. Based on the results, *Rickettsia*-specific primers were designed and made available to the US team. All together, the synergism between the groups provided a strong platform without which none of the groups could have achieved its goals.

List of Publications (in preparation, submitted, accepted, in press, or published)

- Brown, J.K. and Rogan, D. 2006. A diverse bacterial community comprising mutualistic, facultative, free-living and possibly phloem-associated species is associated with *Bemisia tabaci*. (in preparation).
- Caballero, R., Bird, J., Rogan, D., and Brown, J.K. 2006. *Wolbachia* and *Cardinium* spp. cause cytoplasmic incompatibility in biotypes of the whitefly *Bemisia tabaci*, and the detection of a novel partial CI-rescue (in preparation).
- Gottlieb, Y. M. Ghanim, E. Belausov, D. Gerling, V. Portnoy, E. Chiel, G. Tzuri, A. R. Horowitz, S. Kontsedalov, S. Steinberg, N. Mozes-Daube, S. Gal, N. Katzir and E. Zchori-Fein. 2006. Identification and localization of *Rickettsia* in *Bemisia tabaci*. *Appl. Env. Microbiol.* 72: 3646-3652.
- Perlman, S. J., M.S. Hunter and E. Zchori-Fein. 2006. The emerging diversity of non-pathogenic *Rickettsia*. *Proc. Royal Soc. L.* DOI: 10.1098/rspb.2006.3541

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Appendix

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Material and Methods

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Unpublished data

Table 1: Composition of major groups of microorganisms (%) detected in 109 *Bemisia tabaci* individuals from 17 geographical locations, worldwide.

Organism	# Detected	% Detected
Proteobacteria	72/109	66.00%
Firmicutes	28/109	25.70%
Actinobacteria	4/109	3.70%
Chlamydiae	3/109	2.75%
Bacteroidetes	1/109	0.90%
Uncultured	1/109	0.90%
Total	109	99.95%

Table 2. Composition of prokaryotes identified to lower order classification levels in 107 *B. tabaci* populations.

Organism		# Detected	% Detected
Alphaproteobacteria (22)			
	Rickettsiales	19/22	86.40%
	Rhizobiales	3/22	13.60%
Betaproteobacteria (6)			
	Burkholderiales	6/6	100%
Gammaproteobacteria (44)			
	Enterobacteriales	23/44	52.30%
	Oceanospirillales	14/44	38.60%
	Pseudomonadales	2/44	4.50%
	Aeromonadales	2/44	4.50%
Bacillales (4)			
	Staphylococcus	1/4	25%
	Paenibacillaceae	1/4	25%

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	Bacillaceae	2/4	50%
Lactobacillales (24)			
	Streptococcaceae	24/24	100%
Actinobacteridae (4)			
	Actinomycetales	4/4	100%
Chlamydiales (3)			
	Simkaniaceae	3/3	100%

Table 3. Characteristics of whitefly colonies used for the reciprocal crosses.

Biotype	Geographic Origin	Host Plant	Year	Host Range	CI-inducing bacteria
AZ-A	Brawlee, CA	Cotton	1987	Moderate	<i>Cardinium</i>
Jatropha	Puerto Rico	<i>Jatropha</i>	1990	Monophagous	<i>Wolbachia</i>
AZ-B	Tucson, AZ	Poinsettia	1988	Broad	None detected
FLB1	Florida	Tomato	2004	Broad	<i>Cardinium</i>

Attempts to selectively remove facultative bacteria from selected *B. tabaci* populations were made in both the US and Israel throughout the course of the project.

In Arizona - Three different approaches were tested to eliminate *Cardinium* and *Wolbachia* infections in AZ-A, FLB1, and Jat biotypes, respectively. First, the antibiotic Oxytetracycline at 0.001% and 0.005% was used to water cotton or Jatropha plants on which whiteflies were reared. Whiteflies were serially transferred to clean plants approximately every six-eight weeks, and plants were watered routinely with the antibiotic solutions for ten generations, or until the entire colony died. Second, adult whiteflies were subjected to a heat treatment at 33°C or 39°C for 45 min and colonies were initiated using all surviving whiteflies.

Subsequently, 2-3 day old adults were removed from the colony and subjected to the same heat regiment as the parental generations, followed by transfer of survivors to clean plants to initiate a new colony. Heat treatments were continued for each successive generation through ten generations or until the colony died. Third, a combination of a 45 min heat treatment at 39°C and colony initiation with adults followed by continuous treatment of plants on which whiteflies were reared with 0.005% oxytetracycline was carried out. For all three approaches, adult whiteflies were removed periodically from the colony and tested by PCR for the presence of the respective bacterial species as described below.

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After exhaustive repetitions using different treatments (antibiotics, heat, or both) over a ~3 year period of time and multiple generations (3-8) for some treatments it was not possible to 'cure' *B. tabaci* of *Wolbachia* (Jat) or *Cardinium* (AZ-A). Each treatment or set of treatments resulted in a gradual decline of the whitefly population, and when adults did survive for a period of time, the respective bacterium was always detected by PCR. Repeated analysis using PCR and primers noted below for the respective bacterial species, including those for the primary endosymbiont were carried out. The primary symbiont and CI-inducer persisted in living whiteflies.

In Israel - Two different approaches were tested to eliminate *Rickettsia* and *Hamiltonella*, microinjections and antibiotic treatments. For the microinjections, a 'Nanoliter 2000' (WPI Inc. USA) microinjector equipped with a pooled glass pipette filled with either water or food coloring was used to inject various developmental stages of whiteflies under a stereoscope. As a control, eggs (<2h old) were collected from cotton leaves and placed on 1% agar plate, where they were let hatching and then transferred as crawlers to a new plant. 90% of the eggs hatched and 50% of transferred crawlers had developed into adults. However, it turned out that injected eggs (either on agar plates or on a leaf) with the finest needle were killed immediately due to the hydrostatic pressure. Attempts to dechorionate the eggs (a procedure done for *Drosophila* eggs microinjections) using bleach or manual removal failed numerous times. Injections directly into nymphs resulted in the same effect. Injections into adults' abdomen (a technique used in aphids) resulted in the survival of very few individuals, and mortality of all surviving adults soon after.

For the antibiotic treatments, Ampicilin in decimal concentrations ranging from 1% to 0.0001% in PBS was added to vials containing one stem of a cotton plant kept in a closed glass vials. After 48h, 20 adults were placed in the vials and after 48h they were collected and taken for PCR analysis. The antibiotic solution was replaced with water, and plants were kept until F₁ emergence. Using specific primers for *Portiera*, *Hamiltonella* and *Rickettsia*, one and five individuals of parents and offspring from each treatment were tested in two replicates. In the 1% ampicilin treatment the plant died after 3 days in antibiotics. In all other treatments PCR results showed that there is no elimination of the specified bacteria across generations.

Material and Methods (of non-published data)

Whitefly biotypes colonies-AZ. Three biotypes of *Bemisia tabaci* complex were available for this study. The Arizona A (AZ-A) is an indigenous population collected in

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Phoenix, Arizona in 1983 and has been maintained by serial transfer as a laboratory colony on cotton (*Gossypium hirsutum*) or pumpkin (*Cucurbita maxima*) since that time. The Arizona B (AZ-B) is the invasive Old World biotype introduced in the USA mainland in approximately 1986 and collected in Arizona in 1988 and maintained in lab culture on cotton or pumpkin. The *Jatropha* (Jat) biotype is a nearly monophagous population native to Puerto Rico. It was collected from the island in 1985 from native, infested *Jatropha gossypifolia* (Euphorbiaceae) and established in a colony and maintained thereafter on *J. gossypifolia*. The colony was lost from laboratory culture in 2001 when it was displaced in culture by the AZ-B biotype.

Polymerase chain reaction and DNA sequencing of bacterial 16S rDNA. In addition to a number of whitefly colonies that maintained in the AZ laboratory (above) (and shared with the Israeli Co-PI), there is a large collection of *B. tabaci* populations available in the AZ laboratory in ethanol/-80°C freezer. Our whitefly colonies and representative worldwide populations were used for analyses described herein. Total nucleic acids were extracted grinding adult whiteflies or whitefly eggs on a Petri dish covered with aluminum foil and parafilm using the tip of a 0.4 ml micro-centrifuge tube. Freshly made lysis buffer was used each time, containing 5mM Tris-HCl (pH 8.0), 0.5 mM EDTA (pH 8.0), 0.5% Nonidet NP-40, and 1mg/ml of Proteinase K (Sigma®), and kept in ice. Total extracts were briefly centrifuged, incubated at 65°C for 15 min, and 95°C for 10 min, and held on ice as described in Frohlich et al. (1999). All PCR reactions were performed in a total volume of 25 µl per reaction, containing 2.5 µl of 10x reaction buffer, 0.15 mM dNTPs, 2.5 mM MgCl₂, 3.75 U of *Taq* polymerase, 15 pM of each primer, and 5 µl of DNA template. PCR products were fractionated on agarose (1%), stained with ethidium bromide, and visualized under ultraviolet light.

CI-bacteria. PCR amplification of *Cardinium* or *Wolbachia* 16S rDNA in *B. tabaci* adults was carried out to detect bacterial presence. The DNA sequence was obtained for PCR products to confirm bacterial identity. PCR was carried out for bacterial detection in adult whiteflies used in mating studies, in the resultant offspring, and from the eggs collected from females from whitefly colonies or archived female adults preserved in ethanol at -80°C when the colony was no longer viable. Whitefly adults of parents and offspring were ground individually in lysis buffer as described above. To obtain eggs from whiteflies in colonies, females were allowed to oviposit overnight on clean plants and the eggs were viewed under a microscope, detached from the leaf using a sharp scalpel, and transferred to lysis buffer. To obtain eggs from females no longer maintained in colonies, adults that had been archived at -

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80°C in 95% ethanol were dissected under a microscope and fully developed eggs were removed from the ovaries. Three replicates and at least 5 eggs/replicated were examined for each biotype. Five eggs from each replicate were ground in 20 µl of lysis buffer, and used for PCR and DNA sequencing.

General 16S rDNA. Semi-nested PCR was carried out to amplify a ~1500 bp fragment of the 16S rDNA gene using the primers F27 (5'-AGAGTTTGATCMTGGCTCAG) and R1513 (5'-ACGGYTACCTTGTACGACTT). From this reaction, 1 µl of the PCR product was diluted with 99 µl of H₂O and 5 µl of the template was used for the second round of amplification. PCR conditions for the initial round of PCR were 4 min at 94° (hot start), followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C, and a final extension of 20 min at 72°C.

Wolbachia 16S rDNA. To specifically amplify the 16S rDNA sequence for *Wolbachia* the PCR primers (F) V1 (5'-TTGTAGCCTGCTATGGTATAACT) and (R) V6 (5'-GAATAGGTATGATTTTCATGT) were employed to obtain a PCR product of 900 bp. PCR was performed using a hot start at 94° for 4 min, followed by 35 cycles of 1 min each at 94°C, 50°C, and 72°C, with a final extension for 20 min at 72°C.

Cardinium 16S rDNA. To obtain a PCR product for phylogenetic analysis, the PCR primers (F) 5'-GCGGTGTAAAATGAGCGTG) and (R2) 5'-TGTGTACAAGGTCCGAGAACG were used to amplify a region of ~1100 bp. PCR employed a hot start at 94°C for 4 minutes, followed by 35 cycles at 94°C for 40 sec, 57°C for 40 sec and 72°C for 45 sec, and a final extension of 20 min at 72°C (Weeks *et al.* 2003). A nested pair of primers FOR- 5'-GCGGTGTAAAATGAGCGTG and REV 5'-ACCTMTTCTTAAGCCT was employed for diagnostic assays to confirm presence of *Cardinium* in parents and offspring for mating studies (per below).

DNA sequencing. PCR products were sequenced in both directions either before or after cloning. When the DNA sequence was determine directly for PCR products the primers used for PCR were the sequencing primers. For those for which cloning was necessary, PCR products were inserted into the plasmid vector using the p-GEM-Easy vector system, according to manufacturer's etiquette (Promega®, Madison, WI). Clones of two-three amplicons from each sample were sequenced in both directions using internal T7 and Sp6 priming sites. DNA sequencing was carried out using a capillary sequencer available in the Genomics Analysis Technology Core facility (The University of Arizona, Tucson).

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Sequence analysis. DNA sequences were subjected to BLAST analysis to identify the closest match or matches. Those and other relevant sequences including closest relatives and appropriate outgroups, were obtained from the NCBI database and formatted for DNASTAR. Sequences were then aligned in relevant combinations using Clustal W in the MEGALIGN software program (Lasergene, Madison, WI). Pairwise percentage nucleotide distances were calculated using MEGALIGN. Phylogenetic trees are reconstructed using by maximum parsimony (MP) and maximum-likelihood (ML) analysis using PAUP (Phylogenetic Analysis Using Parsimony) software version 3.1.1 (Swofford 2002). Under parsimony, heuristic searches, with stepwise addition, and tree-bisection-reconnection (TBR) random branch-swapping options. For maximum likelihood trees, bootstrapping for 1000 replicates, using the heuristic search option was performed. Reference sequences for previously studied bacteria including *Wolbachia* or *Cardinium* and selected outgroup sequences were obtained from the Genbank database (trees not shown).

Mating studies. Reciprocal crossing experiments for the AZ-A, AZ-B, and Jat biotypes were conducted using either single male-female pairs, or groups of twenty males and females, all previously unmated. Single crosses were most desirable because they enabled us to document precisely interactions between parents and to identify specific parents and their offspring. Group mating were carried out to ensure that results were not affected by mate-finding barriers that might possibly occur in single pair experiments, such as unknown behavioral cues or pheromone attractants.

Cotton plants were grown in insect-free growth chamber under artificial lights and *J. gossypifolia* plants were grown in insect-free cages in an insect-free greenhouse or growth room. Crosses between the AZ-A and AZ-B biotypes were carried out using cotton plants. Because of the monophagous behavior of the Jat biotype, which feeds and reproduces mainly on *J. gossypifolia*, crosses between the Jat and AZ-A were carried out using *J. gossypifolia*, the host on which females were born and reared. For the AZ-B and Jat crosses, a colony of the AZ-B biotype was pre-adapted to *J. gossypifolia* plants for one-two generations. Experiments were conducted in cages with 30 cm of radius and height of 60 cm in a growth chamber (1.75 m²) at 27°C, with a 12:12 hour photoperiod and 80% relative humidity.

To establish the crosses, leaves infested with fourth instar *B. tabaci* were detached from plants and placed in covered petri dishes, under ambient light at room temperature. Every 4-8 hrs petri dishes were checked and emerged adults were permitted to pass through the teneral period. Typically no attempts to pair or mate were observed during these stages. When

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adults appeared fully adjusted, at about 8-16 hrs post emergence, they were collected, sexed, and transferred to cages in groups of twenty males and females each, on cotton plants at the 5-leaf stage, or when Jat females were the mothers, whiteflies were transferred to *J. gossypifolia* seedlings established from cuttings. Controls consisted of establishing crosses between virgin females and males of each biotype. Observations were recorded at least once daily to document male and female number, presence, location, and quantity on leaves, and if pairing was observed. Adults were allowed to mate and oviposit for 10 days before they were collected live and frozen for molecular analysis.

Experiments were terminated after collecting each F₁ generation adults, approximately 3 weeks after removal of the parents, and after the last oviposited egg batch was known to hatch and nymphs to develop to the adult stage. Live F₁ generation adults were frozen, sexed, and kept frozen at -80°C until PCR analysis. Sex ratio was determined in each replicate and cross type. Some of the F₁ females were backcrossed in mating with males from their respective colony to determine if viable offspring could be produced. The F₂ adults (males and females) likewise were collected live, frozen, and pedigree was established using molecular sequence analysis. It was not possible to collect F₂ offspring for Jat x B crosses because the *J. gossypifolia* plants on which whiteflies were reared did not survive long enough to permit their collection.

Whitefly pedigree analysis. The mitochondria cytochrome oxidase I (*mtCOI*) fragment (780 bp) (Brown, 2000; Frohlich et al. 1999) and an intron sequence for the *B. tabaci* parasodium channel (*Kdr*) (Morin et al., 2004) were used to verify colony purity and to confirm the pedigree of parents, and the F₁ and F₂ generations produced in the mating experiments. The *mtCOI* sequence was amplified using primers F-5'-TTGATTTTTTGGTCAT CCAGA AGT (MTD 10) and (R) 5'-TCCAATGCACTAATCTGCCATATTA (MT 12) to yield an expected size fragment of ~850 bp. PCR conditions were 30 cycles for 1 min at 95°C, 52°C and 72°C, with a final extension of 20 min at 72°C. Sequences were edited to achieve a 780 base fragment. PCR primers used to amplify the domain IIS4-6 region of the parasodium channel gene were (F) 5'-GCCAAATCCTGGCCAACT (*Kdr*-1) and (R) 5'-GCCAGGAAAGCGATCAAC (*Kdr*-3) to obtain an expected size fragment of ~900 bp in size. PCR parameters were an initial hot start of 3 min at 95°, followed for 5 cycles of 1 min at 95°C, 1 min each at 67 and 72°C; and then 30 cycles of 1 min each at 95, 65, and 1 min at 72°C, with a final extension of 20 min at 72°C.