SYSTEMATICS OF THE *Bemisia tabaci* COMPLEX AND THE ROLE OF ENDOSYMBIONTS IN REPRODUCTIVE COMPATIBILITY

by

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DEDICATION
This work is dedicated to my mother, Imelda; and to the memory of my father, Rufino (1920-1994), and my brother, Andres (1940-2005). Both q. e. p. d.
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ABSTRACT

Reciprocal and homologous crosses were carried out using pairs or groups of twenty males and females for three biotypes of the whitefly *Bemisia tabaci* complex. Crosses were undertaken for the A biotype-Arizona (AzA), the B biotype-Africa (AzB), and the monophagous, Jatropha (Jat) biotype-Puerto Rico. The maternal haplotype pedigree of parents and selected offspring (F₁, F₂) was determined using the mitochondria cytochrome oxidase I sequence. All reciprocal crosses yielded viable female offspring, indicating reproductively compatibility, except for AzB ♀ x AzA ♂, or AzB ♀ x Jat ♂ crosses, which yielded females unidirectionally. As an unidirectional pattern was reminiscent of cytoplasmic-mediated incompatibility (CI), the possibility was investigated that the phenotype might be caused by CI-bacteria, instead of a germ line barrier. Using the 16S rRNA sequence three prospective CI-bacterial species were identified in whitefly colonies. A *Cardinium* spp. (Bacteroidetes) was present in the A biotype (isolates AzA, CulA, RivA, SalA), whereas the B biotype (isolates AzB, FlB1, FlB2) was infected with *Rickettsia bellii* (Proteobacteria), and a *Wolbachia* spp. (Proteobacteria) was associated with the Jat biotype. The unidirectional incompatible phenotypes were consistent with CI-bacterial infection of AzA (*Cardinium*) and Jat (*Wolbachia*), but no such association was apparent for B biotype-*Rickettsia* infections. The bidirectional compatibility for Jat x AzA suggested a CI-bacteria-mediated reciprocal rescue. However, that *Wolbachia*-infected Jat♀ and *Cardinium*-infected AzA♂ crosses yielded fewer females, compared to AzA-*Cardinium*♀ and Jat-*Wolbachia*♂ crosses, suggested that *Cardinium* could better counter *Wolbachia*-induced female mortality than *Wolbachia*. This suggested the
possibility that these phylogenetically divergent bacteria might utilize similar CI-mechanisms. In this study, the suspect CI-bacteria were strongly associated with complete or partial obstruction of gene flow in certain crosses, and with sex bias in the AzA x Jat crosses. This is the first evidence that female offspring can be produced between phylogeographically divergent, and polyphagous and monophagous \textit{B. tabaci}, for which gene flow barriers are widely reported, suggesting that hybridization is utilized as a means of diversification in \textit{B. tabaci}. The inability to rid colonies of CI-bacteria has necessitated introgression experiments to investigate direct CI-causality over CI-association.
INTRODUCTION

1. Whitefly taxonomy

Whiteflies are placed in the family Aleyrodidae and in the order Hemiptera (von Dohlen and Moran 1995). There are approximately 1500 described species (Martin 2004), however, it is believed the number will increase with time. The family is divided into two subfamilies, Aleurodicinae and Aleyrodinae (Mound and Halsey 1978). The name assigned to a third subfamily, Udamoselinae, is considered a nomen dubium because there are no extant specimens. The subfamily Aleurodicinae is more primitive and has fewer named species. Its members have more generalized wing venation e.g., more veins, and are commonly larger in size than members of Aleyrodinae. They are primarily restricted to South America. The subfamily Aleyrodinae is considered more evolved. Its members have reduced wing venation and are, for the most part smaller. They have a more cosmopolitan distribution and include the species of greatest economic importance (Gill 1990). Historically, the taxonomy of Aleyrodidae has been based on the morphology of the last (fourth) nymphal instar, the latter portion of which is often incorrectly identified as the pupal stage (Chapman 1998). Other taxonomic characters used to separate species and genera include body shape, lingula, setae, the operculum, papillae, pores, and vasiform orifice (Gill 1990). Taxonomic terminology was introduced by Maskell (1895), and improved upon by Quaintance and Baker (1913, 1914) and Russell (1943, 1948).

The first identified species of Aleyrodidae was *Phalaena (=Tinea) proletella,*
described by Linnaeus (1758) (Systema Naturae, 10th edition, p. 537). In 1836, it was mistakenly identified as a member of Lepidoptera (Douglas et al. 1877-1878). According to Mound and Halsey (1978), several species have been placed in Dictyoptera, Neuroptera, and Homoptera (especially Aphididae and Psyllidae). The most well known species, *Aleurodes (=Bemisia) tabaci*, was described by Gennadius (1889), after it was discovered as an upsurgent pest for the first time in tobacco *Nicotiana* spp. (L.) fields in Greece. The genus *Bemisia* was named by Quaintance and Baker (1913) in honor to Florence E. Bemis (Mound and Halsey 1978; Quaintance and Baker 1913). *B. tabaci* is known by several common names, e.g., cotton whitefly, sweet potato whitefly, and the tobacco whitefly, and the B biotype has been referred to by some as the ‘silverleaf whitefly’.

2. Biology

Whiteflies undergo a modified paurometabolus metamorphosis with the following stadia: egg, four nymphal instars, and adult (Chapman 1998). Whiteflies are oviparous with eggs usually being pyriform; although they can be ovoid, elongate-oval, or reniform. Eggs are usually smooth, but they can have a sculptured surface (Byrne and Bellows 1991; Gill 1990; Quaintance and Baker 1913). This stadium is usually accompanied by a short pedicel (at or near the base) but some have an extended form (Byrne and Bellows 1991; Paulson and Beardsley 1985). This pedicle is known to be a conduit for water moving from the plant (Byrne et al. 1990). Eggs are usually oviposited on the underside of leaves. They can be oviposited singly, scattered, grouped, in a semi-circular pattern, in
a complete circle, or laid in a spiral fashion (Dowell et al. 1981; Hamon 1981). The number of eggs oviposited varies within the family and species. In *B. tabaci* the number of eggs is influenced by temperature and season, and has been reported to be more than 300 eggs under certain circumstances (Azab et al. 1971; Dittrich et al. 1985).

The four nymphal instars are unique. The first instar (crawler stage) is elliptical in shape and is initially flattened dorsal/ventrally. It becomes more convex dorsally with feeding. It has marginal setae and microsetae. They also have functional walking legs, short antennae, and conspicuous eyes. It is generally considered the only truly mobile immature stage (Eichelkraut and Cardona 1989; Gill 1990; Summers et al. 1996). The next three instars are, for the part, sessile. They have functional legs, which are used to shift position while reinserting the mouthparts (D. N. Byrne, personal communication). The second, third, and fourth instars are often oval to elongate-oval (some may be circular or heart-shaped) (Byrne and Bellows 1991; Gill 1990). The body can be covered by wax secretions, which take several forms of different colors and shapes (Gill 1990). The fourth instar is sometimes erroneously called the ‘pupal stage’. When first entering the fourth instar the insects feed through inserted mouthparts. The latter portion of the fourth nymphal instar has withdrawn mouthparts and is non-feeding (Gelman et al. 2002). It is opaque and eventually the pharate adult can be seen through the cuticular test (Byrne and Bellows 1991; Gill 1990; Nechols and Tauber 1977).

Whiteflies have several other interesting characteristics. Among them is the presence of a dorsal anus, which consists of the vasiform orifice, lingula and operculum. These are located on the last abdominal segment (Gill 1990; Hodges and Evans 2005).
Dorsal anuses are also present in the sister family Psyllidae, but this is a circumstance of enlarged genitalia (Mound 1983). Honeydew exits the anus and fills the orifice; the lingula is lifted, and the honeydew is released. It is then propelled as a droplet by the tongue-like lingula (Byrne 1990).

Adults have piercing/sucking mouthparts, compound eyes, and ocelli, 2 to 7-segmented antennae and functional wings and legs. Adults of Aleyrodinae are approximately 1-2 mm long. One study reported an average length of *B. tabaci* as 0.85 mm for males and 0.91 mm for females (Byrne and Bellows 1991) while Caballero (1992) reported 0.98 mm and 1.18 mm for males and females, respectively. These differences may be due to environmental factors such as temperature and host nutritional value. Members of Aleurodicinae such as *Aleurodicus dugesi* Cockerell, the giant whitefly and *Bakerius phrygilanthi* Bondar are often much larger (up to 5 mm) (Caballero 1992; Gill 1990, 1992a; Polaszek 2002). Some of the primitive species are 2-5 mm long, e.g. *Aleurodicus dugesii* Cockerell (Gill 1992). Wings are usually white and covered by wax secretion; some wings have colored spots or bands (Gill 1990). Eichelkraut and Cardona (1989) reported a sex ratio of 1:1 in an indigenous population of *B. tabaci* found in Colombia. Another investigation indicated a sex ratio 1:2 in Brazil (Gondim and Sales 1983). A sex ratio of 1:1 has also been reported for *Siphoninus phillyreae* (Haliday) (Leddy et al. 1995). *Trialeurodes ricini* is reported to have an average of sex ratio of 1:1.5 reared in five different hosts (Shishehbor and Brennan 1996). The sex ratio (male: female) may change with season.

Whiteflies have an arrhenotokous system of reproduction, where fertilized eggs
develop into diploid females (XX) and unfertilized ones into haploid males (XO) (Byrne and Bellows 1991; White 1973). Earlier populations of the greenhouse whitefly, *T. vaporariorum*, were reported as being thelytokous, i.e. reproduction consisting solely of females (Hargreaves 1915; Williams 1917). Population designated the American race was arrhenotokous and the one called the English race was thelytokous (Schrader 1920). Material examined by Russell (1948) of the two collections revealed they did not differ morphologically and no thelytokous examples have been found in recent years (Ahman and Ekbom 1981). The only apparently thelytokous whitefly is *Parabemisia myricae*, populations consist exclusively of females (Byrne and Bellows 1991; Hamon *et al.* 1990).

The karyotype of *B. tabaci* is 10, whereas, *T. vaporariorum* and *Aleurotulus nephrolepidis* have 13 chromosomes, and *Aleyrodes proletella* has 14 chromosomes (Blackman and Cahill 1998; Thomsen 1927).

3. Biological strains, races, or types of *Bemisia tabaci* (Gennadius)

The terms biotype, strain, or race are used to differentiate between morphological similar forms of organisms. These are often distinguished based on host range, behavior, or physiological traits (Mayr and Ashlock 1990).

The term ‘host race’ was first applied to *B. tabaci* by Bird (1957) to describe the observed monophagous feeding behavior of a whitefly population associated with *Jatropha gossypifolia* (L.) (Euphorbiaceae), the ‘Jatropha race’. He observed that another population referred to as the ‘*Sida* race’ (after *Sida cordifolia*) had a much broader host range comprising species within over eight plant families (Bird and
Maramorosch 1975, 1978; Bird and Sanchez 1971). In addition to differences in host range, these two populations also differed in their capacity to transmit begomoviruses found on the island in native hosts and cultivated bean plants (Bird 1957).

A second example of *B. tabaci* ‘race’ differentiation involved a population that was unable to breed on cassava, *Manihot esculenta* L., in Brazil (the plant’s native range), whereas, *B. tabaci* had long been reported to colonize cassava in Africa (Costa and Russell 1975). After the introduction of this crop plant as a staple into Africa by the Europeans, cassava was found readily colonized by *B. tabaci* there (Storey 1936).

A well known case history of upsurgence of *B. tabaci* occurred in the southwestern U.S. in the 1980s, by what later became known as the ‘A’ biotype (Costa and Brown 1990). Here, the term ‘biological type’ was substituted for ‘race’ but the intent of the term did not fundamentally change the concept that was evolving to refer to phenotypic differences observed widespread in this species. In 1981 high population levels of the A biotype of *B. tabaci* were experienced in southeastern California and southwestern Arizona, resulting in widespread infection by whitefly-transmitted viruses in cotton and vegetable crops (Brown and Nelson 1984; Duffus and Flock 1982). The outbreak is thought to have been associated with the development of insecticide resistance in the local population that was exposed to compounds intended to control primary pests in cotton. This was particularly significant because it marked the beginning of *B. tabaci* becoming an economically important virus vector in the southwestern U.S., owing to its ability to transmit previously unidentified plant viruses, among which were *Lettuce infectious yellows* (genus, *Crinivirus*) and *Squash leaf curl*
(genus, \textit{Begomovirus}) (Brown and Nelson 1986). Thus, whitefly-transmitted viruses reached epidemic proportions in Arizona and California vegetable and cotton crops for the first time during this era.

In 1986 an unprecedented outbreak of \textit{B. tabaci} were reported on ornamental plants in Florida, especially poinsettia, \textit{Euphorbia pulcherrima} Willd. (Price \textit{et al.} 1987). \textit{B. tabaci} was reported on the same plant host in Arizona in 1988 (Byrne and Miller 1990), and it was subsequently shown to comprise an exotic biotype introduced from the Old World, referred to for the first time as the ‘B’ biotype (Costa and Brown 1990, 1991; Frohlich \textit{et al.} 1999). This marked the first differentiation of the locally occurring ‘A’ biotype from the ‘B’ biotype, which was based on different host range/preferences, polymorphic general esterase patterns, and the ability of the ‘B’ (but not the ‘A’) biotype to induce silvering in leaves of pumpkin plants (Costa and Brown 1991). This latter discovery in Arizona was followed by the corroboration that the Florida infestation was also caused by the B biotype (initially, it had not been identified as an invasive biotype), and of the subsequent dispersal of the B biotype throughout the Americas and Caribbean region in less than four years time (Brown \textit{et al.} 1995a; Costa \textit{et al.} 1993a).

Subsequently, the B biotype was shown to cause phytotoxic disorders in a number of plant species that it colonized, including silvering of leaves in \textit{Cucurbita} sp. (Brown \textit{et al.} 1991; Costa and Brown 1991; Yokomi \textit{et al.} 1990; reviewed in Brown 2001), irregular ripening of tomato (Schuster \textit{et al.} 2002), and stem whitening or blanching in cole crops and pale coloration in lettuce (Brown \textit{et al.} 1991; Costa \textit{et al.} 1993a). The silvering phenotype is considered a reliable primary diagnostic for the B biotype in the
Americas, however, at least one population from Uganda and another from Reunion Island do not appear closely related to the B biotype, and yet they induce silvering in *Cucurbita* species. Thus, it is not clear at this time how widespread the ‘silvering phenotype’ for Old World *B. tabaci*.

Finally, the discovery that the B biotype had an origin in the Old World, or Eastern Hemisphere, (probably the Middle East or Africa) (Brown 2001; Brown *et al.* 2000; Frohlich *et al.* 1999) facilitated the identification of natural enemies with which it appeared to have co-evolved that were introduced into the U.S. as biological control agents (Kirk *et al.* 2000). A number of phenotypic differences were identified for the A and B biotypes including increased oviposition, greater ingestion of sap and excreting of honeydew, broader host range, differential esterase activities, and increased competency in virus transmission (Bedford *et al.* 1994; Bethke *et al.* 1991; Byrne and Miller 1990; Coats *et al.* 1994; Costa and Brown 1991). The growing recognition that this whitefly exhibits genetic and phenotypic polymorphisms led to the proposal that it is best described as a complex (Brown *et al.* 1995b) or group, while systematic and taxonomic clarifications are sought.

4. Economic Importance

*B. tabaci* is the most economically important whitefly species worldwide (Gerling 1990). *B. tabaci* has piercing/sucking mouthparts which facilitate feeding on phloem sap. This feeding results in two types of damage: direct damage that is the result of the extraction of large amounts of sap from host plants and indirect, primarily resulting from
excess excreta (honeydew) that serves as a substrate for the growth of a complex of sooty mold fungi. This sticky excrement also affects cotton lint quality (Henneberry et al. 1996). Additionally, B. tabaci is important owing to its ability to serve as the vector for a large number of plant viruses that cause yield-limiting diseases of vegetable and fiber crops (Brown and Czosnek 2002). The most important and widespread group of viruses transmitted by B. tabaci is the genus Begomovirus (family Geminiviridae) (Brown 1990, 1992, 1994, 2000, 2001; Brown and Bird 1992; Cohen 1990; Cohen et al. 1988).

5. Endosymbionts of B. tabaci

A. Aleyrodid symbiont morphotypes

Whiteflies feed on plant phloem sap (Borror et al. 1989; Chapman 1998). Phloem sap is known to be deficient in a number of essential nutrients, especially amino acids (Buchner 1965). Symbionts have coevolved with these insects benefiting them, in part, by synthesizing essential amino acids absent in plant sap. Symbionts can be transmitted transovarially from whitefly parents to offspring (Buchner 1965).

Among the 20 rather well characterized biotypes reported for the B. tabaci complex (Brown et al. 2000, 1995b) and in some cases they have been shown to possess different complements of symbionts. Among these biotypes are the Arizona B (AzB), Arizona A (AzA) and Jatropha (Jat). Costa et al. (1995) described three types of symbionts associated with the latter B. tabaci biotype colonies maintained in the Arizona laboratory. The pleomorphic and the Coccoid Type I were present in all three whitefly biotypes. A third type (the Coccoid Type II) was found only in AzA and Jat, and was not
observed in the B biotype (Table 1.1). The pleomorphic endosymbiont has been proposed to serve as the primary symbiont, and was named *Porteira aleyrodidarum* (GenBank AY268082) (Thao and Baumann 2004a). It is most closely related to pseudomonads and not to *E. coli*, as are the primary symbionts of aphids.

According to Costa et al. (1995), the pleomorphic endosymbiont has no visible cell wall, whereas the Coccoid Type I has a Gram-negative cell wall along with inner and outer cell membranes. Coccoid Type I measured 1.5 x 1 µm with a maximum thickness of 22 nm. Additionally, they possessed no filament-like structures in their cytoplasm. Large numbers of osmiophilic granular bodies were scattered or aligned adjacent to the internal membrane of bacteriocytes for all three biotypes examined (Costa et al. 1995).

Coccoid Type II measured 0.66-0.78 x 0.70-2.5 µm with a maximum wall thickness of 30 nm and in the case of the AzA biotype had a distinctive filament-like structure in the cytoplasm, which is reminiscent of the recently described *Cardinium* sp (Zchori-Fein et al. 2004). Coccoid Type II bacteria were found in large clusters in AzA ovary tissues in greater numbers than the Coccoid Type I. In the Jat biotype, a Coccoid Type II symbiont was scattered in groups in numbers equal to or less than the Coccoid

<table>
<thead>
<tr>
<th>Biotype</th>
<th>Pleomorphic</th>
<th>Coccoid, Type I</th>
<th>Coccoid, Type II</th>
</tr>
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<tbody>
<tr>
<td>Arizona A</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arizona B</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Jatropha</td>
<td>+</td>
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</tr>
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*Table 1.1.* Numbers and shapes or ‘morphotypes’ of endosymbionts housed in bacteriosomes in three biotypes of *B. tabaci.*
Type I. It is suspected by some that this morphotype may be *Wolbachia* spp.

Thus, the Coccoid Type I observed in the AzA, AzB, and Jat biotypes is thought possibly to be one of two types of secondary symbionts identified to date (Brown *et al.* in preparation; Thao *et al.* 2003; Zchori-Fein and Brown 2002) and may correspond to the T-type secondary symbiont also found in other whitefly species (Szklarzewicz and Moskal 2001). It may also represent an A-type symbiont, *Arsenophonus*, which has been identified in Old World *B. tabaci* (Zchori-Fein and Brown 2002) (see section on Secondary Symbionts).

In contrast, Thao *et al.* (2003) have suggested that the large numbers of osmiophilic granular bodies (Costa *et al.* 1995), together with the Coccoid Type II symbiont correspond to two stages of the life cycle of the *Chlamydia*-like bacterium. Based on 16S-23S rDNA analysis Thao *et al.* (2003) designated this bacterium species as *Fritschea bemisiae* (Chlamydiales: Simkaniaceae). Members of the order Chlamydiales are obligate, intracellular, prokaryotes that exist in two morphologically distinct stages, the reticulate body and the elementary body. A ‘reticulated body’ has been observed that is proposed to represent the reproductive stage and morphologically resembles other Gram-negative bacteria, whereas, a second, ’elementary body’, is the dissemination stage that is described as being metabolically inert (Everett *et al.* 1999).

We also have detected *Chlamydia* spp. in several but not all isolates of *B. tabaci*, including the Jat biotype (Zchori-Fein and Brown 2002). Consequently, it is also possible that coccoid forms observed in former studies represent two or more different organisms amongst the biotypes examined. Direct labeling experiments are needed to
resolve the question of endosymbiont identity, and as well, it is essential that workers define the particular biotype and/or *B. tabaci* colony with which they are working. Thus, it will be interesting to learn the precise identities of the morphotypes observed some time ago by Costa *et al.* (1995), in relation to contemporary molecular sequence, TEM observations, and biological consequences, for these well-studied biotypes of *B. tabaci*.

B. Primary Symbiont of *B. tabaci*.

The pleomorphic symbiont described first by Costa *et al.* (1995) and more recently shown to be widespread in worldwide collections of *B. tabaci* using the 16S rRNA gene sequence for phylogenetic identification (Zchori-Fein and Brown 2002) is considered the primary symbionts of whiteflies. The primary symbiont in *B. tabaci* has been designated *Portiera aleyrodicarum* (GenBank accession no. AY268082) (Thao and Baumann 2004a). Based on analysis of the 16S-23S rDNA sequence, this pleomorphic bacterium was classified in the Gamma-subdivision of Proteobacteria (Baumann *et al.* 2004; Thao and Baumann 2004a). In contrast, the primary symbionts of aphids, mealybugs, and psyllids are placed in the genera *Buchnera*, *Carsonella*, and *Tremblaya*, respectively (Baumann *et al.* 2000; Thao *et al.* 2000, 2002) (Table 1.2). All of them belong to Proteobacteria in the *Gamma* subdivision except the mealybug symbiont, which is placed in the *Beta* subdivision (Baumann *et al.* 2004).

Whitefly primary symbionts also differ from the primary symbionts of the other hemipterans by the absence of an outer membrane of the Gram-negative cell wall (Costa *et al.* 1993b, 1995; Szklarzewicz and Moskal 2001), and are most closely related to
Table 1.2. Scientific name, taxonomic classification, and some properties of the primary endosymbionts of whiteflies, aphids, mealybugs, and psyllids.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Whiteflies</th>
<th>Aphids</th>
<th>Mealybugs</th>
<th>Psyllids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus</td>
<td>Portiera</td>
<td>Buchnera</td>
<td>Tremblaya</td>
<td>Carsonella</td>
</tr>
<tr>
<td>Species</td>
<td>aleyrodidarum</td>
<td>aphidicola</td>
<td>princeps</td>
<td>ruddii</td>
</tr>
<tr>
<td>Phylum</td>
<td>Proteobacteria</td>
<td>Proteobacteria</td>
<td>Proteobacteria</td>
<td>Proteobacteria</td>
</tr>
<tr>
<td>Class</td>
<td>γ</td>
<td>γ</td>
<td>β</td>
<td>γ</td>
</tr>
<tr>
<td>Outer membrane</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA size</td>
<td>30.2 Kb</td>
<td>641 Kb</td>
<td>64.4 Kb</td>
<td>37.1 Kb</td>
</tr>
</tbody>
</table>

Pseudomonads. The full genome of *Buchnera* is 641 Kbp, while the other groups have only been partially sequenced (Table 1.2).

*P. aleyrodidarum* is recognized as the primary symbiont for the entire *B. tabaci* complex. In two independent assessments, it was detected in 24 of 24 whitefly species examined (Thao and Baumann 2004a), whereas, Zchori-Fein and Brown (2002) found two examples among approximately 30 worldwide collections of *B. tabaci* from which they amplified by PCR 16S rRNA primers that were outliers to the main primary symbiont clade. Either the PCR primers employed facilitated the amplification of alternative symbionts, or certain *B. tabaci* harbor a different primary symbiont, or the whitefly samples analyzed were incorrectly identified as *B. tabaci*. It is suspected that the latter scenario will provide a satisfactory explanation.

C. Secondary Symbionts of *B. tabaci*. 
Secondary symbionts have also been found in *B. tabaci*; however, their diversity is greater than that found in primary symbionts. Zchori-Fein and Brown (2002) and Thao and Baumann (2004b) suggested that there were two types of secondary symbionts in 13 whitefly species they examined based on the 16S rRNA gene and/or the 16-23S rRNA sequence, respectively. The 16S-23S analysis indicated that *B. tabaci* AzA and AzB were infected by two T-type symbionts, whereas, four A-type symbionts (*Arsenophonus* spp., Enterobacteriaceae, Proteobacteria) were associated with the remaining 11 species. The two T-type symbionts are most closely related to T-type symbionts of aphids and to those found in the wasp, *Nasonia vitripennis* (Walker). Unlike primary symbionts, which are thought for aphids, mealybugs, psyllids, and now whiteflies, to have originated in their host through a single ancient infection, secondary symbionts may have infected whiteflies (and other hemipterans) multiple times, and also they may be horizontally transmitted (Thao and Baumann 2004b).

6. *Bacteroidetes* (Phylum) symbionts in arthropods

A symbiont related to *Bacteroides* (a common intestinal bacteria of humans) was first observed in bacteriocytes (internal cells housing microorganisms) of the leafhopper, *Heliochara communis* Fitch (Chang and Musgrave 1972). Endo (1979) observed similar organisms in cells of the soybean cyst nematode, *Heterodera glycines*. Costa *et al.* (1995) discovered what appeared to be the same bacteria in two biotypes of *B. tabaci*. Kurtii *et al.* (1996) found the same organisms in the cell culture of the blacklegged tick, *Ixodes scapularis* Say. To do so, PCR was used to amplify the 16S rDNA sequence and
construct a phylogeny with other well-studied bacteria. An identical bacterial morphology has been observed in the ovaries of several parasitoid wasps, including *Encarsia* spp. (Zchori-Fein *et al.* 2001, 2004), and also in eggs of the false spider mite, *Brevipalpus obovatus* Donnadieu (Weeks *et al.* 2001). Zchori-Fein *et al.* (2001) designated the symbiont in *Encarsia* spp. as EB (*Encarsia* bacterium) while more recently Weeks *et al.* (2003) and Hunter *et al.* (2003) referred to them as a *Cytophaga*-like organism (CLO). Subsequently, Weeks and Breeuwer (2003) also referred to the bacterium as *Cytophaga-Flavobacterium-Bacteroides* (CFB). The majority of these kinds of intracellular organisms are Gram-negative rods (occasionally appearing to be coccoid-like) with a dimension near 1.5 x 0.4 - 0.7 μm (Kurtii *et al.* 1996; Weeks and Breeuwer 2003). Cells live in direct contact with the host cytoplasm. The bacterial cytoplasm often contains parallel filaments (Kurtii *et al.* 1996) and are reminiscent of those described in the bacteriocytes of *B. tabaci* (Costa *et al.* 1995).

A. Characterization and classification of the *Bacteroidetes* symbionts

This group of undescribed intracellular symbionts was initially placed in the genus *Bacteroidetes* group (formerly, CFB) (Weeks *et al.* 2001; Weeks and Breeuwer 2003; Zchori-Fein *et al.* 2001). *Bacteroidetes* is a heterogeneous taxon from morphological, physiological and ecological standpoints. Most of the information on this taxon is from soil-water-inhabiting members (Reichenbach 1991). Hunter *et al.* (2003) specifically designated them as *Bacteroidetes*. Recently, based on the 16S rDNA and *gyrB* gene sequencing, the symbiont found in *Encarsia* spp. was identified as *Cardinium*
hertigii (GenBank accession number, AY331187 (16S rDNA gene) and AY332003 (gyrB gene) (Zchori-Fein et al. 2004). This symbiont is a Gram-negative, rod-shaped bacterium also placed in the Bacteroidetes (Garrity and Holt 2001) (Table 1.3).

Table 1.3. Taxonomic and hierarchical classification of the Bacteroidetes symbiont, Cardinium hertigii (Garrity and Holt 2001).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Bacteroidetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Sphingobacteria</td>
</tr>
<tr>
<td>Order</td>
<td>Sphingobacteriales</td>
</tr>
<tr>
<td>Family</td>
<td>Flexibacteraceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Cardinium</td>
</tr>
<tr>
<td>Species</td>
<td>hertigii</td>
</tr>
</tbody>
</table>

B. Distribution of the Phylum Bacteroidetes

Two recent surveys, together with available GenBank accessions, showed that Cardinium spp. symbionts appear to be largely restricted to three arthropod lineages, although they are also occasionally found in other arthropod taxa (Table 1.4) (Weeks et al. 2003; Zchori-Fein and Perlman 2004).

C. Phylogeny and systematics of the Bacteroidetes symbionts

Currently all characterized members of the Bacteroidetes group belong to the genus Cardinium. Recognized members to date exhibit a maximum divergence of 3-5% (Zchori-Fein et al. 2004). Ninety-five % similarity is considered the lower limit for a
Table 1.4. Host distribution of *Cardinium hertigii* and close relatives symbionts based in two surveys), and available sequences in GenBank.

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Scientific Name</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemiptera</td>
<td>Aleyrodidae</td>
<td><em>Bemisia tabaci</em></td>
<td>AY279409</td>
</tr>
<tr>
<td></td>
<td>Diaspididae</td>
<td><em>Aspidiotus nerii</em></td>
<td>AY279402</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aspidiotus paranerii</em></td>
<td>AY327469</td>
</tr>
<tr>
<td></td>
<td>Delphacidae</td>
<td><em>Dicranotropis hamata</em></td>
<td>AY279415</td>
</tr>
<tr>
<td>Hymenoptera</td>
<td>Aphelinidae</td>
<td><em>Aphytis lingnamensis</em></td>
<td>AY279404</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aphytis sp.</em></td>
<td>AY279403</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aphytis sp.</em></td>
<td>AY279405</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aphytis sp.</em></td>
<td>AY327473</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia berlesei</em></td>
<td>AY026336</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia citrina</em></td>
<td>AY026333</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia hispida</em></td>
<td>AY026334</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia lutea</em></td>
<td>AY279407</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia noyese</em></td>
<td>AY279408</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia pergandiella</em></td>
<td>AY026335</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia pergandiella</em></td>
<td>AF319783</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia sp.</em></td>
<td>AY279406</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Encarsia sp.</em></td>
<td>AY285777</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Marietta sp.</em></td>
<td>AY327470</td>
</tr>
<tr>
<td></td>
<td>Encyrtidae</td>
<td><em>Plagiomerus diaspidis</em></td>
<td>AY327472</td>
</tr>
<tr>
<td>Acari</td>
<td>Ixodidae</td>
<td><em>Ixodes scapularis</em></td>
<td>AB001518</td>
</tr>
</tbody>
</table>
bacterium to be included in the same genus (Ludwig et al. 1998). These include those found in parasitic wasps (Aphelinidae and Encyrtidae), a scale (Hemiptera/Homoptera: Diaspididae), certain mites (Phytoseiidae and Tetranychidae) and a tick (Ixodidae) (Zchori-Fein and Perlman 2004). Interestingly, based on the 16S rDNA sequence, two other hemipteran symbionts also have been identified as *Cardinium* and these were associated with the hosts *B. tabaci* (Riverside A biotype) and *Dicranotropis hamata* (Delphacidae), respectively (Zchori-Fein and Perlman 2004). Caballero (Chapter 2) has corroborated the presence of *Cardinium* in *B. tabaci* including the AzA, and two other A biotype colonies maintained in Riverside, CA [used by Perring et al. (1993) in mating studies to deduce that the A and B biotypes were different species], and Salinas, CA (J.E. Duffus) and have studied its putative effects on reproductive incompatibility for three
biotypes (Chapters 1, 2).

The genus *Cardinium* likely encompasses more than one species given the divergence among the characterized members at 1-5% (Zchori-Fein *et al.* 2004). According to the species definition for bacteria, organisms diverging from 2-3% are considered different species (Stackebrandt and Goebel 1994). The divergence between the parasitic wasps and the mites and tick symbionts is 3%, suggesting that mites and ticks should also be described as a different *Cardinium* species (Zchori-Fein *et al.* 2004).

Two other novel *Bacteroidetes* symbionts unrelated to *Cardinium* were recently discovered. These include those found in the scale, *Diaspis echinocactis* (Diaspididae) and the beetle, *Zigia versicolor* (Melyridae) (Zchori-Fein and Perlman 2004). Based on 16S rDNA sequences the two symbionts are 99% identical and belong to the *Flavobacteria* group of insect symbionts. The latter symbionts share an 83-85% identity with the primary symbiont of cockroaches and termites, the male-killing symbionts of certain lady bird beetles, and a symbiont of *Homalodisca coagulata* (Say), the glassy-winged sharpshooter (Zchori-Fain and Perlman 2004).

D. Host responses to the *Bacteroidetes* endosymbionts

The endosymbiont *Cardinium* spp. have recently been shown to cause sex-ratio distortion in certain arthropods (Zchori-Fein *et al.* 2001). These associations are known to result in feminization in *B. obovatus* (Weeks *et al.* 2001), parthenogenesis in *Encarsia* spp. (Hymenoptera: Aphelinidae) (Zchori-Fein *et al.* 2001, 2004), cytoplasmic incompatibility in the parasitic wasp, *E. pergandiella* Howard (Hunter *et al.* 2003), and
increased fecundity in the western predatory mite, *Galendromus* (=*Typhlodromus*=*Metaseiulus*) *occidentalis* Nesbitt (Acarina: Phytoseiidae) (Weeks and Breeuwer 2003). A search in the GenBank database identified two other symbionts that are closely related to those found in the parasitic wasps and the mites that also caused sex-ratio distortions. These symbionts are associated both with *B. tabaci* and the planthopper, *Dicranotropis hamata* Boheman (Hemiptera: Delphacidae) (Zchori-Fein and Perlman 2004). It is not known whether these symbionts have any effects on reproduction or other fitness characters.

7. *Wolbachia* (Proteobacteria) symbionts in *B. tabaci*

*Wolbachia* spp. are classified in the alpha subdivision of Proteobacteria phylum (Garrity and Holt 2001) (Table 1.5). *Wolbachia* spp. encompass a heterogeneous group of Gram negative bacteria of small rods and coccoid forms, resembling either *Rickettsia* or *Chlamydia*. Single *Wolbachia* species undergo both forms, rod-shape are 0.3-1.5 μm long; coccoid-shape are 0.4-1.0 μm in diameter (Weiss *et al.* 1984).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Proteobacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class</td>
<td>Alphaproteobacteria</td>
</tr>
<tr>
<td>Order</td>
<td>Rickettsiales</td>
</tr>
<tr>
<td>Family</td>
<td>Rickettsiaceae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Wolbachia</em></td>
</tr>
</tbody>
</table>

Table 1.5. Taxonomic and hierarchical classification of *Wolbachia* (Garrity and Holt 2001).
*Wolbachia* spp. are intracellular vertically-transmitted endosymbionts with mutual, neutral or parasitic effect. These bacteria are *sui generis* causing an extremely broad host phenotype such as cytoplasmic incompatibility, parthenogenesis induction, feminization of genetic males and male killing (Stouthamer *et al.* 1999). *Wolbachia* spp. are usually found on ovaries and testes; however, they may be also found in somatic tissues such as muscle, midgut, malphigian tubules, fat body, nervous tissue, wings, and hemolymph (Dobson *et al.* 1999). *Wolbachia* spp. are widely distributed with respect to host range, which includes nematodes and arthropods in the classes Arachnida, Crustacea and Insecta, in the orders Orthoptera, Homoptera, Hemiptera, Thysanoptera, Neuroptera, Lepidoptera, Coleoptera, Diptera and Hymenoptera (Werren 1997; Werren *et al.* 1995; Weeks *et al.* 2003).

*Wolbachia* sp. was first reported in the whitefly, *B. tabaci* Jatropha biotype (Bird 1957; Brown *et al.* 1998a,b; Caballero and Brown, in preparation). And, several *B. tabaci* field collections have been reported to harbor *Wolbachia* spp. (Jeyaprakash and Hoy 2000; Nirgianaki *et al.* 2003; Weeks *et al.* 2003; Zchori-Fein and Brown 2002); however, the whitefly host-*Wolbachia* relationship and potential for CI or other reproductive abberation-associated effects have not been studied.
CHAPTER 1

POSITIVE EVIDENCE FOR INTERBREEDING AND DIFFERENTIAL GENE FLOW BETWEEN THREE WELL CHARACTERIZED BIOTYPES OF THE *Bemisia tabaci* COMPLEX (GENNADIUS) (HEMIPTERA: ALEYRODIDAE) EXCLUDES GEOGRAPHIC AND HOST BARRIERS AS ISOLATING FACTORS

1. Abstract

Reciprocal and homologous crosses were carried out with single pairs or groups of 20 males and females for three well-characterized biological types (biotypes) of the whitefly *Bemisia tabaci*. Two polyphagous biotypes, the Arizona A biotype (AzA) of Western Hemisphere origen, the exotic B biotype (AzB) from the Eastern Hemisphere, and the monophagous, Jatropha (Jat) biotype from Puerto Rico (Western Hemiphere), were used in crossing experiments carried out in a growth chamber (27°C, 80% humidity, and 12:12 hr photoperiod). All crosses employed cotton as the oviposition host except for Jat female x AzA or AzB male crosses, for which *Jatropha gossypifolia* (Euphorbiaceae) was the host. Whitefly pairs and groups were caged together on the respective oviposition host for 7 and 10 days, respectively. Genetic identification of parents and selected F$_1$ and F$_2$ offspring was carried out using the maternally inherited mitochondria cytochrome oxidase I sequence. All homologous crosses yielded female offspring. Female offspring were produced in all but two reciprocal crosses. These exceptions were AzB♀ x AzA♂, or AzB♀ x Jat♂, for which female offspring were produced in a single
direction. Because this distinctive pattern could be attributable to endosymbiont-induced incompatibility, whitefly colonies were screened for bacterial presence using PCR and 16S rRNA primers. The AzA biotype was shown to harbor *Cardinium*, whereas, a *Wolbachia* spp. was associated with the Jat biotype. That *Wolbachia* and *Cardinium* are implicated in reproductive disorders that can be manifest as cytoplasmic incompatibility was intriguing, and so their discovery has been the subject of a follow-on study. The results reported herein provide the first evidence for gene flow between monophagous and polyphagous New World biotypes (AzA, Jat, respectively), and between an Old World (AzB) and two New World (AzA and Jat) biotypes, indicating that geographic and host barriers do not mandate reproductive isolation. These results challenge the proposed classification of the B biotype as a species unique from other *B. tabaci*. Based on the biological species concept, reproductively compatible organisms that produce viable offspring would be considered the same species. Several lines of evidence suggest that *B. tabaci* constitutes a single group of related genetic variants, of which some are reproductively compatible. Also, certain but not all variants are recognizable by a definitive set of (adaptive) behaviors (biotype), a designation that has contributed to taxonomic confusion. Even so, results of this study indicate that gene flow can occur between three reproductively compatible *B. tabaci* variants, in this instance ‘biotypes’, which have experienced prolonged physical separations imposed by geography and/or host plant. It is not known whether the AzA, AzB, Jat, or other biotypes and haplotypes have hybridized in nature and/or if so, whether they are naturalized. This result suggests that hybridization may serve as a more common means of diversification in this plastic
species than was previously thought.

2. Introduction

Whiteflies are classified in the family Aleyrodidae (Sternorrhyncha: Hemiptera/s.o. Homoptera) (Borror et al. 1989; Campbell 1993; Gill 1990; Martin 2003; Mound and Halsey 1978; Von Dohlen and Moran 1995). Whiteflies are unique among insects in that they employ a ‘modified’ paurometabolus metamorphosis, and they are haplo-diploid insects reproducing by arrhenotokyous parthenogenesis (Blackman and Cahill 1998; Byrne and Bellows 1991; Chapman 1998; White 1973). The closest relatives to whiteflies are aphids, mealybugs, psyllids, and scales, all which utilize piercing and sucking mouthparts that are specialized for feeding in plant phloem (Borror et al. 1989; Chapman 1998; Gill 1990). The suborder also is well known for harboring primary and secondary endosymbionts, some of which are known to synthesize amino acids that are in low supply in phloem sap (Buchner 1965; Douglas 1998). Among whiteflies, the Bemisia tabaci (Genn.) complex (Brown et al. 1995b) is the most widely distributed (Gill 1992b; Martin 2003) and the most economically important, worldwide. Both B. tabaci adults and nymphs cause feeding damage particularly when population levels are high (Byrne and Bellows 1991), and the adults transmit several genera of plant viral pathogens (Brown and Czosnek 2002) of cultivated eudicotyledonous food and fiber species. Members of the Bemisia tabaci complex (Brown et al. 1995b) are the exclusive insect vector of the genus, Begomovirus (Geminiviridae) (Brown 2001), a group of emergent single-stranded DNA plant viruses for which over 300 species are presently recognized
(Fauquet et al. 2005) and with which they have presumably co-adapted (Brown and Idris 2005).

The recognition of greater than expected genetic and biological variation for this species has give rise to the hypothesis that *B. tabaci* is a group of related genotypes, some of which have been characterized with respect to distinctive phenotype or ‘biological type’. They are particularly troublesome because among the phenotypic and genetic variants that have been recognized, none exhibit morphological characters by which they can be distinguished (Bedford et al. 1994; Berry et al. 2004; Bird 1957; Bird and Maramorosch 1978; Brown 2000, 2001; Brown and Idris 2005; Brown et al. 1995a,b, 2000; Burban et al. 1992; Costa and Brown 1991; Costa and Russell 1975; Costa et al. 1993c; De Barro et al. 2000, 2005; Frohlich et al. 1999; Gawel and Bartlett 1993; Gill 1990; Martin 2003; Mound 1963; Mound and Halsey 1978; Moya et al. 2001; Perring et al. 1993; Rosell et al. 1997; Sseruwagi et al. 2005, 2006; Viscarret et al. 2003; Wool et al. 1991). Consequently, the provisional term, *B. tabaci* complex, is used by many to refer to this species group that comprises definitive phenotypic (biotype) and/or genetic (haplotype) variation, in anticipation of eventual resolution of the taxonomy and systematics.

The B biotype is one of several polyphagous, highly fecund variants of *B. tabaci* that are well-adapted to native plant species in marginal habitats, while at the same time are capable of adapting to agricultural environments. Based on genetics studies, the B biotype is hypothesized to have originated in Africa or near the Middle East (Brown 2000; Brown et al. 1995b; in preparation, Frohlich et al. 1999). During 1986-1990 the B
biotype was introduced on infested ornamental plants multiple times into the Americas and Caribbean Basin, and presently is distributed nearly worldwide in tropical and protected temperate cropping systems (Cock 1986, 1993; Martin 2003). The subsequent displacement of indigenous B. tabaci by the B biotype has resulted in increased crop damage and coincided with the emergence of previously unrecognized begomoviral species (Brown 2000).

B. tabaci that were indigenous to the southwestern US, e.g. specifically, Arizona (Butler and Henneberry 1983) is referred to as the A biotype. Two phenotypically distinct biotypes, A and B, were differentiated by Costa and Brown (1991) based on unique general esterase pattern, host range, fecundity, and insecticide resistance (Costa et al. 1993c). One group proposed that the B biotype was sufficiently distinct to constitute a new species, and proposed the name Bemisia argentifolii Bellows & Perring. This conclusion was based on results of reciprocal crossing experiments that yielded no female offspring, genetic distance estimates (RAPDs, isozymes), and the presence or absence of the ASMS4 setae on the fourth instar (Bellows et al. 1994; Perring et al. 1993). Given the less than robust genetic distance estimate (Perring et al. 1993) the lack of hybridization provided the most convincing support of the ‘new species’ hypothesis, and this result also has been corroborated by another laboratory using colonies provided by our laboratory (Bedford et al. 1994). In contrast, a third study reported production of a few females from AzA x AzB crosses (Costa et al. 1993c), and finally, a single female offspring was obtained from crosses between AzB and an Old World, non-B biotype (Byrne et al. 1995). It is nonetheless clear that no taxonomic consensus has been
reached, nor has the common name, silverleaf whitefly been adopted by the
Nomenclature Committee of the Entomological Society of America. This prudent
conclusion is due to the paucity of rigorous population genetics studies and corroborative
reciprocal crosses employing representative phenotypes/genotypes, together with the
longstanding recognition that *B. tabaci* is phenotypically plastic. Immature instars for
this species have long been known to be capable of altering their external morphology in
response to leaf surface topology, whereas, adults are variable with respect to plant host
range and degree of polyphagy (Bird 1957; Brown *et al.* 1995b; Burban *et al.* 1992;
Costa and Brown 1991; Costa and Russell 1975; Gill 1990; Martin 2003; Mohanty and
Basu 1986; Mound and Halsey 1978; Russell 1957). Only recently have genetic studies
revealed that *B. tabaci* exhibits greater divergence than most species. Several molecular
markers have been examined, including the ITS1 (De Barro *et al.* 2000), the
mitochondria (mt) 16S, and the cytochrome oxidase I (mtCOI), the latter which has
proven invaluable for sorting the complex into ≈7 major phylogeographic groups (Brown

The objective of this study was to revisit the biological species status of *B. tabaci*,
employing three well-studied biological types that vary with respect to polyphagy,
fecundity, and geographical origin, and to carry out reciprocal crosses under controlled
conditions. Because whiteflies are haplo-diploid (arrhenotokous parthenogenesis) (White
1973) eggs that are unfertilized would be expected to yield haploid males (n=10)
(Blackman and Cahill 1998), whereas, fertilized eggs are expected to yield diploid
females. The biotypes included were the A biotype from North America, which is
moderately polyphagous and moderately fecund, the Jatropha biotype from Puerto Rico (Caribbean region), which is (nearly) monophagous with respect to several native euphorbiaceous species, and exhibits low fecundity, while the third was the highly polyphagous and fecund B biotype. All three were characterized with respect to their primary and secondary symbionts, which were found to be the same respectively, based on the 16S rRNA (Zchori-Fein and Brown 2002). We further tested the possibility that reproductive barriers would be imposed by host plant and geographical isolation. Finally, experiments were designed to determine whether single pairs or groups of *B. tabaci* were more or less likely to mate and/or produce viable offspring.

3. Materials and Methods

*Whitefly biotypes colonies.* Three biotypes of the *Bemisia tabaci* complex were employed in this study. The Arizona A (AzA) is a biotype indigenous to the southwestern US and Mexico. The colony was established from adults collected from cotton in Phoenix, Arizona in 1983. The AzA colony has been maintained since then by serial transfer to cotton, *Gossypium hirsutum* (L.) or pumpkin, *Cucurbita maxima* (Duschne) plants. The AzB biotype is an Old World isolate (Brown *et al.* 1995b), which was noted into Arizona on poinsettia plants in approximately 1987 (courtesy, W. Miller). It was first identified on poinsettia plants in the greenhouse on the campus of The University of Arizona in 1988 by Costa and Brown (1991), after genetics and behavioral studies were conducted and it was shown to differ from the local population in several key ways (Costa and Brown 1991). The AzB colony was maintained in a location
separate from the AzA biotype, by serial transfer to cotton or pumpkin (Costa and Brown 1991) approximately every 30 days. It has been maintained on cotton plants since 1995. The Jatropha biotype is native to Puerto Rico (Bird 1957) and was collected from *Jatropha gossypifolia* (L.) plants growing near Rio Piedras in 1993 and established in laboratory culture. Preliminary studies indicated that the Jat biotype could feed and breed on a limited number of euphorbiaceous species in the Caribbean region (Bird 1957). In our laboratory we noted that compared to the AzA and AzB biotypes it exhibited low fecundity (at 50 offspring/female), the AzA biotype exhibited moderate fecundity (100-150 offspring/female), and the B biotype exhibited the highest fecundity (200-300 offspring/female).

**Reciprocal crosses.** Cotton plants were grown in an insect-free growth chamber under artificial lights, and the *J. gossypifolia* plants were planted and maintained in insect-free cages housed in an insect-free greenhouse and/or a growth room. Crosses between the AzA and AzB types were carried out using cotton as the oviposition plant. Because of the monophagous behavior of the Jat biotype, which feeds and reproduces mainly on *J. gossypifolia*, crosses between the Jat and AzA were carried out using *J. gossypifolia* for oviposition (e.g. the plant host on which females were born and reared). For AzB x Jat crosses, a colony of AzB biotype was pre-adapted to *J. gossypifolia* plants for one-two generations, given it was found to colonize *J. gossypifolia* plants after its introduction into Puerto Rico in ≈1990.

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 adults appeared fully adjusted, at about 8-16 hrs post emergence, they were collected, sexed, and transferred to cages. Mating experiments were conducted in a growth chamber (1.75 m²) at 27°C constant temperature with a 12:12 hour photoperiod and 80% relative humidity.

*Mating studies.* Single crosses were most desirable because they enabled precise documentation of the mating behavior, making it relatively easy to identify specific parents and the associated offspring. Mating in groups was carried out to determine if mating behavior was negatively affected by mate-finding barriers that might possibly occur in single pair experiments, including behavioral cues or pheromone attractants about which little was known. For both kinds of experiments, a positive (homologous) control consisted of crosses between virgin female(s) and male(s) of each biotype.

Experiments were terminated after collecting the F₁ adult generation, approximately 3 weeks after parents were removed from the oviposition plant, and after the last egg batch had hatched and nymphs reached the adult stage. Live F₁ adult generations were frozen, sexed, and held frozen at -80°C until PCR analysis. In case of reciprocal crosses between AzA and Jat, experiments were carried out to determine if females were viable, and could thereby produce F₂ and F₃ offspring.

The sex ratio for each cross was determined as the proportion of males compared to the total number of offspring in each replicate. Parents and offspring for crosses were collected live and held at -80°C until analysis by PCR. The purity of whitefly colonies
used for reciprocal and homologous crosses was ascertained using the mtCOI sequence as a molecular marker for parents (all that were recoverable) and offspring from each cross.

Male:female pairs. Cylindrical, transparent cages with a radius of 7.5 cm and height of 35 cm were used to confine male-female pairs of newly emerged, unmated, adult whiteflies to a two-leaf cotton or *J. gossypifolia* plants, based on the maternal colony host. Courtship and mating behaviors were documented daily by leaf drawings to record distance between female and male of each replicate in each genetic cross. Pairs were allowed to pair and oviposit on the host plant for 7 days before adults were removed from the plant, collected into 95% ethanol and stored at -80C until PCR analysis.

Male:female groups. Each experimental cross was established in a cylindrical, transparent cage 30 x 60 cm. Groups of twenty virgin males and twenty virgin females were released onto cotton plants (5-leaf stage). Jat females were transferred to *J. gossypifolia* (5-leaf stage) seedlings previously established from cuttings. Observations were recorded at least once daily to document male and female number, presence, location, and quantity on leaves, and whether pairing was observed. Adults were allowed to mate and oviposit for 10 days before they were collected into 95% ethanol and stored at -80C until PCR analysis.

Lysis and mtCOI polymerase chain reaction. Total nucleic acids were extracted by grinding whole whiteflies on a petri-dish covered with aluminum foil and then parafilm, using the tip of a 0.4 ml micro-centrifuge tube. Lysis buffer was made fresh each time. Lysis buffer 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, and once ground, whitefly lysis was
kept in ice. Total extracts were briefly centrifuged, then incubated at 65°C for 15 min, and 95°C for 10 min, and held in ice. PCRs 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 1% agarose gel. Bands were stained with ethidium bromide and visualized under ultraviolet light. All methods were as described previously in Frohlich *et al.* (1999).

The mitochondria cytochrome oxidase I (mtCOI) fragment (780 bp) (Brown 2000; Frohlich *et al.* 1999) was amplified using the primers F-5’- TTGATTTTTTGGTCATCCAGAAGT (MTD 10) and (R) 5’- TCCAATGCACTAATCTGCCATATTA (MTD 12), which yielded 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 (Frohlich *et al.* 1999). Sequences were edited and trimmed to obtain a 780 base fragment that lacked the PCR primer sequence.

**DNA sequencing and sequence analysis.** The DNA sequence was determined for each PCR product in both directions using an ABI377 automated sequencer available in the Genomics Analysis Technology Core Facility at The University of Arizona, Tucson. The DNA sequences were aligned using Clustal W in the MEGALIGN software program (Lasergene, Madison, WI). Pairwise percentage nucleotide distances were calculated using MEGALIGN. The phylogenetic trees were reconstructed using maximum parsimony (MP) and maximum-likelihood (ML) methods available in PAUP (Phylogenetic Analysis Using Parsimony) software version 3.1.1 (Swofford 2002).
Under parsimony, heuristic searches employed stepwise addition and tree-bisection-reconnection (TBR) random branch-swapping options. For maximum likelihood trees, 1000 bootstrap replicates and the heuristic search option were performed.

**Statistical analysis of reciprocal crossing data.** An one-way ANOVA was performed to determined if there were differences between crosses based on sex ratio and fecundity. For crosses carried out in pairs, Linear contrasts were used to determine if there were differences in female offspring production for AzB♀ x AzA♂, AzB♀ x Jat♂, and for AzA♂ x Jat♀ and Jat♀ x AzA♂ crosses. For crosses established in groups, the Fisher’s exact test was used to detect differences among the crosses.

4. Results

All reciprocal and homologous control crosses employing the three biotypes yielded both female and male F₁ offspring. Unexpectedly however, when AzB females were mated with AzA or to Jat males, no female offspring were produced. This result was obtained for both types of crosses, irrespective of pairs or groups, with the proportion of males being consistently 1.00 (Table 2.1). Greater than two and seven times more male offspring were produced by pairs and groups of incompatible crosses, respectively, when males were the AzA biotype, compared to males of the Jat biotype (Table 2.1).

In compatible matings between AzB male x AzA/Jat female yielding female F₁ offspring, the following patterns were observed. The highest male/female sex ratio for female offspring was observed for AzB male x AzA female crosses in both pairs and
groups, with the proportion of males being 0.89 and 0.91, respectively (Table 2.1). In contrast, when females were the Jat biotype, the proportion of males was inconsistent across pairs and groups at 0.95 and 0.75, respectively (Table 2.1). For pairs, the number of F₁ females produced from crosses was not statistically significant (linear contrast, t = -1.021, \( P = 0.32 \)). However, there was a significant different in the number of females produced in certain crosses employing groups instead of pairs (Fisher’s exact test, \( P < 0.0001 \)).

Crosses between the AzA and Jat biotypes yielded female offspring in both directions, producing a similar sex ratio pattern irrespective of pairs or groups. When females of the AzA biotype were crossed with Jat males, the proportion of males was 0.77 and 0.58 from pairs and groups, respectively. In addition, reciprocal crosses revealed a similar sex ratio at 0.85 for pairs and 0.84 for groups (Table 2.1). For pairs there was not a significant difference in the proportion of females (linear contrast, t = -0.465, \( P = 0.65 \)), whereas, in groups, a significant difference was seen in the number of F₁ females produced from AzA x Jat crosses (Fisher’s exact test, \( P < 0.0001 \)). Experiments were terminated after the F₁ generation except for the female offspring resulting from the AzA x Jat reciprocal crosses, for which a second and third generation were carried out. These crosses also yielded female offspring indicating a viable, reproductive state (Table 2.2).

The mtCOI sequence analysis consistently demonstrated the genetic purity of each pair or group of parents, and yielded the expected mtCOI sequence (Brown 2000; Frohlich et al. 1999; Viscarret et al. 2003). Because the mtCOI is maternally inherited,
the \( F_1 \) females and males were expected to carry the maternal form of the gene and so parents and offspring could be differentiated, or in the cases of homologous crosses, both parents and all offspring had identical mtCOI sequences (Table 2.3, Figure 2.1). Whitefly mtCOI sequences characteristically grouped the AzB biotype with the Old World clade from northern Africa/Mediterranean region, while the AzA grouped with the North American group, and the Jat biotype is a member of a sister clade to the AzA and its closest New World relatives (Brown 2000).

5. Discussion

In this study, viable female offspring were produced in one direction or bi-directionally from crosses between three well-characterized biotypes of the \textit{B. tabaci} complex (Table 2.1). These patterns were for the most part irrespective of pairs or groups. In the several treatments for which significant differences were noted for pairs and groups, it has not been possible to attribute a particular biological explanation. Even though all attempts were made to standardize and optimize experiments, it is possible that the variability might be attributable to plant host used for oviposition, or possibly to the health of the colony at the time. Even so, the consistent production of a relatively high frequency of female \( F_1 \) offspring is herein considered a robust indication of successful fertilization for all possible combinations, indicating that reproductive isolation does not occur between these three candidate biological types of \textit{B. tabaci} that provided an opportunity to examine geographical and host-based isolations. For AzA x Jat crosses, the hybrid \( F_1 \) female offspring, and of the offspring by \( F_1 \) females (\( F_2 \)) were capable of
producing male and female offspring when crossed with males from their respective colony, demonstrating no indication of reproductive isolation (Table 2.2).

The results of this study for pairs or groups are not in line with those from previous studies that have reported no female offspring produced from AzA x AzB biotype crosses. In one study, reproductive isolation was attributed to incompatible courtship behavior (Perring et al. 1993). Our results are not in agreement with the results of Perring et al. (1993) or of Bedford et al. (1994), and the latter laboratory used precisely the same isolates of AzA and AzB as described here. However, we have carried out successful AzB x Riverside A unidirectional crosses using the same Riverside-A colony. It is known that the Riverside-A colony was established from adults collected in the Imperial Valley, CA (N. Prabhaker, personal communication) approximately 2 years after establishment of the AzA colony. Similarly, the results reported here are not in agreement with those of Costa et al. (1993c) who demonstrated a very low female offspring frequency for the AzA and AzB colonies as reported herein. Further, because the latter experiments were conducted in the AZ laboratory, it is known that the growth chambers used in the present experiment are far superior compared to those employed a number of years ago by Costa et al. (1993c), and that they have permitted the optimization of environmental parameters. Byrne et al. (1995) reported a similar result in demonstrating low frequency or lack of female offspring altogether from a B x non-B biotype from Sudan even though they are both Old World B. tabaci. And, De Barro and Hart (2000) reported incompatibility in B crosses with two local Australian isolates, the latter, which produced female offspring in reciprocal crosses, even though the frequency
of females was quite low.

Striking inconsistencies in the experimental result across studies, particularly when the same whitefly colony is used, albeit, but in different locations, may suggest that in some instances inconsistencies in environment and experimental approaches might influence whitefly behavior. If so, it is not feasible to compare results between experiments conducted under different conditions. The system adopted here utilized whole plants in relatively large, well-ventilated cages (and no clip cages), and prolonged mating potential at 7-10 days under 12 hr light/dark at 27°C and 80% RH. Such conditions appeared to elicit optimal whitefly behavior. Some studies have not reported particular practices instituted for controlling environmental conditions, including light intensity, day-length, plant host age and quality, among other parameters, and in some studies the duration of oviposition differed from our parameters. It would not be surprising to find that rather optimal conditions might be key to promoting successful courtship and mating. Indeed the moderately polyphagous Sida race, also indigenous to Puerto Rico (and also thought to have been displaced there by the B biotype), might well have been capable of hybridizing with the Jat biotype had Sida males approached Jat females on the Jat-compatible oviposition species. That neither the Jat or Sida biotype (Bird and Maramorosch 1978) are abundant on the island since the B has established widely, precludes exploration to collect putative hybrids, and adults of the two biotypes in order to establish colonies for experimentation (J. Bird and J.K. Brown, unpublished).

The three *B. tabaci* isolates examined exhibited some degree of isolation (Table 2.1). It can only be presumed that isolation can involve geographic barriers, and in the
case of the Jat biotype, host specialization (Bird 1957). However, our results confirm that geographical and/or host-induced isolation has not necessarily served as a biological species barrier for *B. tabaci*. Although the interspecific genetic variation appears greater than in most conventional species, the range and extent of variation together with the potential for hybridization, suggest that this taxon is still best described as a species group, comprising a number of closely related variants. From this standpoint it is possibly analogous to hemipterans of the Delphacidae and Membracidae (Wilson and Claridge 1991; Wood 1993).

Perring *et al.* 1993 reported a marginal genetic distance as a predictor that the A and B biotypes are distinct species. There is now ample evidence for extensive polymorphism at several loci for the *B. tabaci* complex, but this does not necessarily lead to a conclusion that divergence mandates speciation. Based on the most divergent sequence markers available, the mtCOI sequence demarcates *B. tabaci* populations (field collections, lab colonies) phylogeographically inferring an origin in either the Eastern or Western Hemisphere. Eastern hemisphere exemplars are moderately to highly divergent and further group by major geographical region. In contrast, exemplars from the North America and Caribbean region group somewhat geographically and exhibit minimal divergence within and when compared to the Eastern Hemisphere clades. In general samples are lacking for much of South America but preliminary data suggest that the South American *B. tabaci* diverge from the rest of the Americas by $\approx 4-6\%$ (Brown 2000; Frohlich *et al.* 1999; Viscarett *et al.* 2003). We hypothesize that the split is due to divergence that might be expected between populations isolated on different continents,
however, it is not yet known when the isolation and/or radiations occurred. Indeed, many Old World populations exhibit COI sequence divergence that are nearly as great as between the A and B biotypes (Brown 2000). Further, the ‘separate species’ proposal assumed that the B. tabaci group is composed of a small number of genetic variants, but the A and B biotypes clearly represent only two members of this complex (Perring et al. 1993). De Barro et al. (2005) likewise concur that the limited biological and molecular data for the species do not support a split into two species.

Screening (16S rRNA) of whitefly colonies to identify putative prokaryotes that are known to be associated with reproductive aberrations (data not shown), revealed that the AzA biotype was infected with a member of the recently described bacterial genus, Cardinium (Weeks et al. 2003; Weeks and Breeuwer 2003) (Bacteroidetes phylum), whereas, the Jat biotype was infected with a species of Wolbachia (Proteobacteria phylum) (O’Neill et al. 1992; Stouthammer et al. 1999; Werren, 1997, 1998) not previously associated with this genus. However, in neither case has either genus been associated with reproductive isolation in whiteflies. Also, the AzB biotype was shown to be infected with Rickettsia bellii (Gottlieb et al. 2006; data not shown); to date, this bacterium has not been implicated in reproductive incompatibility in B. tabaci. That Wolbachia and Cardinium have been shown to induce parthenogenesis, feminization, or cytoplasmic incompatibility (CI) in other insects (O’Neill et al. 1992; Stouthammer et al. 1999; Werren, 1997, 1998; Zchori-Fein et al. 2001) is intriguing and has become the subject of a follow-up study (Caballero et al., in preparation).

Herein we provide the first evidence of gene flow between a monophagous and
polyphagous biotype, respectively, from a well-known Old World and two New World biotypes of *B. tabaci*. Thus, geographical and/or plant host imposed isolation do not necessarily lead to reproductive isolation as surmised, and in the context of a biological species concept, these three biotypes are the same species. Consequently, the results challenge the proposed classification of the B biotype as a species unique from all other *B. tabaci* and support the contention that *B. tabaci* is best described as a group of related genetic variants, some of which but not all, are recognizable by sets of definitive adaptive (presumably) behaviors. It is not known whether the AzA, AzB, or Jat biotypes, or if other sympatric or allopatric variants have hybridized under natural conditions and/or if such hybrids have become or are capable of becoming naturalized.
Table 2.1. Mean number (± standard error), replicate (n), and sex ratio for offspring produced by single virgin male and female parents, or groups of 20 virgin females and 20 virgin males for three well-characterized biotypes of *B. tabaci*.

<table>
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<td></td>
<td>Sex ratio</td>
<td>Total</td>
<td>Sex Ratio</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>(X±SE)</td>
<td>(♂/♀+♂)</td>
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<td>AzB♀ x AzA♂</td>
<td>1.00 ± 0.00</td>
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<td>21.1 ± 6.54</td>
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<td>AzA♀ x AzB♂</td>
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<td>Jat♀ x Jat♂</td>
<td>0.68 ± 0.13</td>
<td>55.5 ± 13.50</td>
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Table 2.2. Proportion of male and female offspring produced in three sequential generations, from initial Jat and AzA biotype reciprocal crosses.

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<td></td>
<td></td>
<td>F₁</td>
<td>F₂</td>
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<tr>
<td>AzA♀ x Jat♂</td>
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<td>335</td>
<td>53</td>
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<td>♂</td>
<td>457</td>
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<td>♂</td>
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Table 2.3. Molecular analysis of parents and offspring involved in crosses established between single pairs, or groups of 20 males and females, based on the maternally inherited mitochondria cytochrome oxidase I (partial) gene.

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**Figure 2.1.** Phylogenetic analysis of the mitochondria COI gene sequences (780 bp) using PAUP v3.1.1. The maximum likelihood tree was reconstructed using the heuristic search option and 1000 bootstrap replicates. The bar indicates genetic distance in units of nucleotide substitution per site. The tree illustrates the relationships between AzA, AzB, and Jat biotypes of *B. tabaci*. The AzA and Jat biotypes from the Western Hemisphere group on distinct branches (∼3% divergent) in the New World clade, whereas, the AzB groups in the Eastern Hemisphere clade (at ∼8-9% divergence with the AzA and Jat biotypes). The tree was rooted using the greenhouse whitefly *Trialeurodes vaporarioum* as the outgroup genus.
A biotype and A-like relatives

Western Hemisphere clade

B biotype

Eastern Hemisphere clade
CHAPTER 2

CYTOPLASMIC INCOMPATIBILITY PHENOTYPES FROM RECIPROCAL CROSSES BETWEEN BIOTYPES OF THE *Bemisia tabaci* COMPLEX ARE ASSOCIATED WITH *Cardinium* (BACTEROIDETES) AND *Wolbachia* (PROTEOBACTERIA), AND APPARENT PARTIAL RESCUE OF CI YIELDING SEX BIAS

1. Abstract

We investigated the effects of two divergent, prospective cytoplasmic incompatibility (CI)-causing bacteria on gene flow among biotypes of the taxonomically confounded *B. tabaci* species group. We report the detection and identification by PCR, cloning, sequencing, and phylogenetic analysis of three bacteria in three biotypes of *B. tabaci*: the polyphagous New World (NW) Arizona A (AzA), the polyphagous Old World (OW) Arizona B (AzB), and the monophagous NW Jatropha (Jat). Based on PCR amplification of the 16S rRNA gene a *Cardinium* spp. (Bacteroidetes), was associated with the AzA and three other A biotype isolates (RivA, CulA, SalA colonies), whereas a *Wolbachia* spp. (Proteobacteria) was consistently associated with the Jat biotype. *Chlamydia* spp. and *Rickettsia bellii* also have been associated as secondary endosymbionts with certain *B. tabaci* but to date neither has not been implicated in CI-phenomenon. In a previous study, we reported partial and complete reproductive compatibility between three biotypes (AzA, AzB, Jat) examined in this follow up study to determine if the putative
CI-bacteria, *Wolbachia* and *Cardinium* are associated with these phenotypes. Results presented here permit the provisional attribution of bidirectional female offspring produced from Jat x AzA crosses to rescue of the incompatibilities imposed by the other. Further, previous results of crosses employing the *Wolbachia*-infected Jat♀ x *Cardinium*-infected AzA♂, indicated fewer female offspring, compared to AzA-*Cardinium*♀ x Jat-*Wolbachia*♂ crosses, which may suggest that *Cardinium* can better abate female offspring mortality induced by *Wolbachia* than the inverse. These results, taken together with the unequivocal demonstration of a *Cardinium*-mediated CI-phenotype for some but not all crosses, respectively, provide evidence that two phylogenetically divergent CI-bacteria contribute operatively to a range of *B. tabaci* reproductive incompatibilities, reflected by gene flow barriers and resulting in one case in sex bias. Herein we report a novel, rescue-like phenomenon between two phylogenetically divergent bacteria, which could possibly have evolved similar CI-inducing mechanisms, making possible the rescue of CI effects induced by the other. Due to the inability to rid *B. tabaci* of these suspect CI-bacteria using various treatments effective for other insects, an introgression experiment is in progress to explore CI-causality over CI-association.

2. Introduction

Many organisms have associations with intracellular, maternally transmitted endosymbionts with a neutral, mutual, or parasitic effect. Among sex-ratio distorting bacteria of arthropods, *Wolbachia* spp. (Proteobacteria) is among the best-studied, and it is known to cause multiple reproduction disorders in addition to cytoplasmic
incompatibility (CI) (Stouthamer et al. 1999). *Wolbachia* infect a broad range of hosts including arthropods in the classes Arachnida, Crustacea and Insecta (Orthoptera, Hemiptera, Thysanoptera, Neuroptera, Lepidoptera, Coleoptera, Diptera and Hymenoptera) and Nematoda (Weeks et al. 2003; Werren 1997; Werren et al. 1995). Recently, several populations of the sweetpotato whitefly *Bemisia tabaci* (Gennadius) have been associated with *Wolbachia* infection (Brown et al. 1998a,b; Jeyaprakash and Hoy 2000; Nirgianaki et al. 2003; Weeks et al. 2003; Zchori-Fein and Brown 2002). A second bacterium, *Cardinium* (Bacteroidetes), has been recently associated with sex-ratio distortions. This bacterium was first identified in a mite (Weeks et al. 2001) and parasitoid wasps (Zchori-Fein et al. 2001), and shortly thereafter in *B. tabaci* (Weeks et al. 2003). Herein we report the identification and biological effects of *Wolbachia* and *Cardinium* infecting two well-studied biotypes of *B. tabaci* from the Eastern Hemisphere.

Although there exists extensive *Wolbachia* literature, the cellular and molecular mechanisms involved in CI induction are not well understood. There is some evidence that *Wolbachia* modify paternal chromosomes during spermatogenesis, causing mitotic asynchrony during the first mitotic division (Tram and Sullivan 2002; Werren 1997). Molecular studies are in progress to identify the bacterial and insect proteins that function in CI (Braig et al. 1998; Harris and Braig 2001). Charlat et al. (2001, 2002) have summarized the properties of the mod/res model, in which closely related strains can overcome or rescue the respective CI-effects imposed by the other, including specific factors involved in these interactions. Very recently, since the genome sequence of *W. pipiens* has become available, a Type IV secretion system involving an ankyrin domain
protein has been implicated in *Wolbachia*-host communication and host reproduction aberrations (McGraw and O’Neill 2004).

The second unrelated endosymbiont known to cause CI-like sex-ratio distortion in arthropods, has been assigned the *Candidatus* genus name *Cardinium* spp. (Zchori-Fain *et al.* 2004). *Cardinium* is classified in the phylum Bacteroidetes (family Flexibacteraceae) (Garrity and Holt 2001). It has only been associated with three arthropod lineages, Acari, Hemiptera, and Hymenoptera (Weeks *et al.* 2003; Zchori-Fein and Perlman 2004), in which it causes variable host phenotypes, including feminization in the false spider mite, *Brevipalpus obovatus* (Weeks *et al.* 2001), parthenogenesis in parasitoid wasps, *Encarsia* spp. and in the oleander scale, *Aspidiotus nerii* (Provencher *et al.* 2005; Zchori-Fein *et al.* 2001, 2004), cytoplasmic incompatibility in the parasitoid wasp, *Encarsia pergandiella* (Hunter *et al.* 2003), increased fecundity in the predatory mite, *Metaceiulus occidentalis* (Weeks and Breeuwer 2003), and no negative effect in fitness of the mite, *Brevipalpus californicus* (Chigira and Miura 2005). Most recently, four Hemipteran/Homopteran insect species were shown to be hosts of *Cardinium*, including the A biotype of the whitefly, *Bemisia tabaci* (Aleyrodidae), the planthopper, *Dicranotropis hamata* (Delphacidae), the hardscales, *Aspidiotus nerii* and *A. paranerii* (Diaspididae), and the leafhopper, *Scaphoideus titanus* (Cicadellidae) (Marzaroti *et al.* 2006; Weeks *et al.* 2003; Zchori-Fein and Perlman 2004). Although multiple infections by *Cardinium* and *Wolbachia* were found in single wasps and mites (Weeks *et al.* 2003), the prospective contributions of these bacteria to sex-ratio distortion phenotypes are poorly understood. Thus, it is not known if these putative CI bacteria have discernable
effects on reproductive fitness, gene flow, or reproductive incompatibility, all of which are considered relevant to resolving the confounded systematics of the whitefly *B. tabaci* complex.

In this communication, we report for the first time evidence for a novel interaction between two distantly related CI-inducing bacteria that have been herein and/or elsewhere associated with distinct biotypes of the *B. tabaci* complex (Brown *et al.* 1995b). One, the ‘B’ biotype, has been considered by some to constitute a separate species (*B. argentifolii*) based on the results of mating experiments in which gene flow was shown to be impeded owing to behavioral barriers (Perring *et al.* 1993). Biotypes used in the reciprocal crossing experiments herein were found to be infected with one of the divergent bacteria, *Wolbachia* and *Cardinium*, and another biotype, the AzB, appears free of both types of bacteria. Our results demonstrate the unequivocal roles of *Wolbachia* and *Cardinium* in unidirectional reproductive incompatibility as important partial barriers to bidirectional gene flow, the former, which resulted from an unexpected ‘rescue of incompatibility’ for certain biotype combinations, and the latter, not observed at all. To our knowledge this is the first report of two unrelated bacteria involved in the reciprocal rescue of CI, one of which appears to more potently ‘protect’ the female offspring from the killing action of the other. These findings have important implications to the systematics of the *B. tabaci* complex. Moreover, they have potential for explaining differential fitness including sex ratio bias in *B. tabaci*, which may be relevant to upsurgence in some members of the *B. tabaci* complex.
3. Materials and Methods

**Whitefly biotypes colonies.** All biotypes and populations of *Bemisia tabaci* complex used in this study, including origin, collecting date, host range, and potential hosts of CI-inducing bacteria are shown in Table 3.1. For colonies no longer available, -80°C frozen material was used in the analysis. Caballero and Brown (in preparation) provided frozen material from their mating study to determine the effects of CI-bacteria in their results.

**Curing.** Three different approaches were tested to eliminate *Cardinium* and *Wolbachia* infections in AzA 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 8-10 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 regime 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 8-10 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.

**Polymerase chain of bacterial 16S rDNA.** Total nucleic acids were extracted by 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 by Frohlich *et al.* 1999. 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 1% agarose gel, bands stained with ethidium bromide, and visualized under ultraviolet light.

**Pedigree analysis.** The maternally-inherited mitochondria cytochrome oxidase I (mtCOI) gene fragment (780 bp) (Brown 2000; Frohlich *et al.* 1999) was used to verify purity of colonies and offspring from all crosses and backcrosses. The mtCOI sequence was amplified using primers F-5’-TTGATTTTTTGGTTCATCCAGAAGT (MTD 10) and (R) 5’-TCCAATGCACTAATCTGCCATATTA (MTD 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 (Frohlich *et al.* 1999). Sequences were edited and trimmed to achieve a 780 base fragment.

**CI bacteria.** PCR amplification of Cardinium or Wolbachia 16S rDNA in *B. tabaci* adults and eggs 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 for mtCOI.

Egg analysis was performed to verify horizontal transmission of potential CI bacteria. PCR analysis was carried out using 16S rDNA specific primers for detection of Cardinium, Wolbachia, Rickettsia, and Chlamydia. 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 for grinding. To obtain eggs from females no longer maintained in colonies, adults that had been archived in 95% ethanol at -80°C were dissected under a stereoscope and fully developed eggs were removed from the ovaries. Three replicates were examined for each biotype and six for Jat biotype. Five eggs per each replicate were ground in 20 µl of lysis buffer, and used for PCR, cloning, 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’-ACGGYTACCTTGTTACGACTT) (Weeks et al. 2003; Weisburg et al. 1991). From this reaction, 1µl of the PCR product was diluted with 99µl of H₂O and 5µl of the template was used for the second round of amplification. PCR conditions for the initial round of PCR were 4 min at 94°C (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.

**Cardinium 16S rDNA.** For detection and identification of *Cardinium*, the primers CLOf 5’-GCGGTGTAAAATGAGCGTG) and CLOr1 5’-ACCTMTTCTTTAACTCAAGCCT were used to amplify a short segment of ≈450 bp. To obtain a longer PCR product for phylogenetic analysis, the same forward primer was used with the reverse CLOr2 5’-TGTGTACAAGGTCCGAGACG to amplify a region of ≈1100 bp (Weeks *et al.* 2003). For PCR, the same conditions were employed, which involved 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).

**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 an expected size PCR product of ≈900 (O’Neill *et al.* 1992). PCR was performed using a hot start at 94°C 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.

**Rickettsia 16S rDNA.** For amplification of the 16S rDNA sequence for *Rickettsia*, the PCR primers Rb-F (5’-GCTCAGAACGAACGCTATC) and Rb-R (5’-GAAGGAAAGCATCTCTGC) were utilized, which yielded an expected size fragment of ≈900 (Gottlieb *et al.* 2006). PCR conditions were 95°C for 2 min, followed by 30 cycles of 30 seconds each at 92°C, 58°C, and 72°C, with a final extension for 5 min at
72°C (Gottlieb et al. 2006).

**Chlamydia 16S rDNA.** To amplify the 16S rDNA sequence for *Chlamydia*, primers were designed based on the accession number AF400484 available in GenBank. Primers were JCF (5’-CCTGGCTCAGATTGAATGCT) and JCR (5’-CTACGGCTACCTTGTTACGA) to obtain an expected size PCR product of ≈1500. PCR was performed using a hot start at 94°C for 4 min, followed by 35 cycles of 1 min each at 94°C, 55°C, and 72°C, with a final extension for 20 min at 72°C.

**DNA sequencing.** PCR products were sequenced in both directions either before or after cloning. When the DNA sequence was determined for PCR products directly, the primers used for PCR were also used as 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 for 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).

**Sequence analysis.** The DNA sequences (mtCOI, 16S rRNA genes) were aligned using Clustal W in the MEGALIGN software program (Lasergene, Madison, WI). Pairwise percentage nucleotide distances were calculated using MEGALIGN. Phylogenetic trees were reconstructed using maximum parsimony (MP) and maximum-likelihood (ML) methods available in PAUP (Phylogenetic Analysis Using Parsimony) software version 3.1.1 (Swofford 2002). Under parsimony, heuristic searches were used
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.

*Cardinium* 16S rDNA sequence. Sequences of four populations of the A biotype (AzA, RivA, SalA, CulA) were included in the analysis. Sequences of 1090 bp were aligned with 11 reference sequences representing the most diverse taxa, including the type sequence of *Cardinium hertigii* (AY331187). Trees were rooted using *Acanthamoeba* (AY549547) as outgroup. Reference sequences were obtained from GenBank and accession numbers and hosts and the respective systematics information are provided in Table 3.2.

*Wolbachia* 16S rDNA sequence. The analysis included a consensus sequence of Jat biotype (n=8) of 787 bp aligned with 32 sequences representing taxa of the six recently recognized groups (A, B, C, D, E, F). Trees were rooted with a *Rickettsia* (M21789) as outgroup. GenBank accession numbers and host systematics are provided in Table 3.3.

4. Results

**PCR amplification and DNA sequencing.** Extensive PCR amplification of individuals resulting from heterologous and homologous crosses detected an intracellular organism in the Jat and AzA biotype colonies, but never in the AzB biotype colony. For the Jat biotype, *Wolbachia*-specific 16S rDNA amplicon, consistently amplified *Wolbachia* yielding a ≈900 bp fragment of the 16S rDNA. Using these same primers,
Wolbachia was never detected in the AzA and the other A-biotype isolates, RivA, SalA, and CulA. Also, Wolbachia was not detected in the AzB biotype, or in two B-biotype isolates from Florida, referred to here as FlB1 and FlB2 (Table 3.1).

The CLO-specific 16S rDNA primers, however, amplified a ≈1100 bp fragment from the AzA biotype, which upon sequence comparisons, corresponded to a bacterium in the Bacteroidetes phylum. This bacterium was never found in Jat and AzB biotypes. This bacterium was also detected in the other three A-type isolates. Exhaustive attempts to amplify Wolbachia or Cardinium from the three B-biotypes yielded no PCR product for either CI-bacteria

**Vertical transmission of Wolbachia and Cardinium in whitefly eggs.** To demonstrate the maternal transmission of potential CI bacteria, eggs were analyzed by PCR. Eggs collected from the Jat, A-, and B-biotypes colonies were consistently infected with one of the two CI bacteria that were also detected by PCR in whitefly adults. Wolbachia was detected in 6/6 replicates of Jat biotype eggs (5 eggs pooled/replicate) (Table 3.4). Likewise, eggs dissected from female A-biotypes were infected with Cardinium, 3/3, 2/3, and 3/3 replicates (RivA, CulA, SalA) respectively (Table 3.4). Neither Wolbachia nor Cardinium were detected by PCR in eggs collected from the three B-biotype colonies. From other two potential CI bacteria targeted by PCR, none of the colonies were infected by Chlamydia but interestingly, Rickettsia bellii recently described by Gottlieb et al. (2006) and not known to cause CI was detected in the three B-biotype colonies (AzB, FlB1 and FlB2) (Table 3.4).

**Frequency of Wolbachia and Cardinium in whitefly colonies.** To determine the
percentage of adults infected by *Wolbachia* or *Cardinium*, extensive PCR screening for adults of the Jat, four A-biotypes and three B-biotypes was done (Table 3.5). *Wolbachia*, which was detected only in the Jat biotype was found to be present in 95.83% of adults screened from the Jat colony. *Cardinium*, on the other hand, was detected by PCR in all the four A-biotype colonies at a mean proportion of 90.64% (Table 3.5).

**Frequency of Wolbachia and Cardinium infection in whitefly adult offspring.**

Frequency of infection by *Wolbachia* and *Cardinium* was detected by PCR in a set of F$_1$ offspring from reciprocal crosses of the Jat and AzA biotypes (Table 3.6). When Jat females (*Wolbachia*-associated) were mated with AzA males (putatively harboring *Cardinium*), 100% of males (haploids) were infected with *Wolbachia*. None of the offspring were infected by *Cardinium*, as might have been predicted in an operational CI. As expected, from mating that yielded female offspring (demonstrating fertilization), 100% was infected by *Wolbachia* and none *Cardinium* (Table 3.6). In the reciprocal crosses, males that were produced by AzA females were infected with *Cardinium* 100% of the time, as would be predicted for operational CI. Thus *Cardinium* but not *Wolbachia* was detected in all F$_1$ females (Table 3.6). This was consistent with the detection of *Cardinium* in AzA and not in Jat colonies, and with the association of *Wolbachia* with only the Jat biotype colony.

**Curing Experiments.** After exhaustive attempts employing different treatments (antibiotics, heat, and both) to rid the Jat or A-biotype colonies of the putative CI-causing bacteria over a four year period of time, and for multiple (8-10) generations, we concluded that *B. tabaci* colonies were not curable of *Wolbachia* (Jat) or *Cardinium*
(AzA, RivA, CulA, SalA) in our hands. An observation made for all treatments, or combination of treatments, was that the respective whitefly colony characteristically declined gradually or rapidly, and when offspring survived for any period of time following treatment, the associated CI-bacterium was consistently detectable in offspring by PCR.

**Sequence analysis.** Pairwise percentage nucleotide distances (NJ) were calculated and phylogenetic trees reconstructed for *Cardinium* and *Wolbachia* 16 rDNA.

*Cardinium 16S rDNA.* The sequence analysis indicated that AzA, RivA, CulA, SalA 16S rDNA sequences shared 99.2-99.4% identity, and 99.4-99.6% identical to a previously published 16S rDNA for the Riverside A colony (AY279409) reported by Weeks *et al.* (2003) (Fig. 3.1). The four A-type isolates sequences have been deposited in the GenBank and have been assigned the accession numbers (submitted). Among hemipterans, the A-biotype shared 96.2-96.7% identity with the *Cardinium* associated with the scales (*Aspidiotus* spp.), the plant hopper (*Dicranotropis* sp.) at 96.2-96.5%, and the leafhopper (*Scaphoideus* sp.) at 96.4-96.6% identity. The outgroup *Acanthomoeba* (AY549547) shared 89.5-90.2% identity with the available *Cardinium* sequences (Fig. 3.1).

The A-biotype *Cardinium* 16S rDNA sequence ML and MP tree topologies did not differ significantly, and so only the ML tree is shown here. Both tree reconstructions revealed two major clades, one containing the four *B. tabaci*-associated *Cardinium* isolates reported herein, and a *Cardinium* sequence reported previously from *B. tabaci* (Riverside A) (Weeks *et al.* 2003) which grouped as a single monophyletic clade.
The sister group to the *B. tabaci Cardinium* clade contained the *Cardinium* spp. that has previously been identified from Hymenoptera and scales (Fig. 3.1). It contained two subclades, one with *Cardinium* spp. from scales (*Aspidiotus* spp.) and a wasp (*Aphytis*) (Aphelinidae) were 96.2-96.8% identical to *Cardinium* reported previously from *B. tabaci* (unknown biotypes/genotypes), and a second subclade with three wasps: *Encarsia pergandiella* (Aphelinidae), *E. hispida* (Aphelinidae) and *Plagiomerus* (Encyrtidae), at 96.7-97.3% shared identity with A biotype-*Cardinium* isolates (Fig. 3.1).

A second major clade contained the CI-bacterium from three families of mites (Tetranychidae, Tenuipalpidae, Phytoseiidae), a tick (Ixodidae), the planthopper (Delphacidae), and a leafhopper (Cicadellidae).

*Wolbachia 16 rDNA*. The Jat-*Wolbachia* sequence has been deposited in the GenBank and has been assigned the accession number (submitted). Pairwise analysis indicated that the Jat-*Wolbachia* consensus sequence (n=8) of 787 bp shared 99.1% identity with the only *B. tabaci* sequence available in GenBank (AY850932), indigenous from China. The Jat-*Wolbachia* sequence also shared 96.2 % identity with the whitefly *Aleurotrachelus duplicatus* (Fig. 3.2). Sequence has also been submitted to GenBank. A comparison of the Jat-*Wolbachia* with other hemipteran-associated *Wolbachia* indicated it shared 98.6, 99.0, and 99.0% identity with species from the Psyllidae, Delphacidae, and Cicadellidae, respectively. It also shared 98.7% identity with the *Wolbachia* infecting *Culex pipiens* (Culicidae) (X61768), which also was among its closest relatives. The Jat-*Wolbachia* and all in-group taxa shared 82.7-85.5% identity with the outgroup *Rickettsia* (M21789) (Fig. 3.2).
The ML and MP trees for *Wolbachia*-specific 16S rDNA consensus sequence for Jat 16S rDNA, 32 reference sequences from GenBank (representing taxa from the 6 groups) and outgroup placement revealed similar topologies, and so the ML tree was selected to represent the phylogeny. Here, the Jat sequence occupied a branch in the B group clade. Interestingly, the other available *Wolbachia* sequences from whitefly also clustered in the B group, together with three other hemipterans, a psyllid (Psyllidae), planthopper (Delphacidae), and leafhopper (Cicadellidae) (Fig. 3.2).

5. Discussion

Phylogenetic analysis of *B. tabaci* A-biotypes (AzA, RivA, CulA, SalA) associated *Cardinium* spp. exhibited greater than 96% identity to several other *Cardinium* spp. for which sequences are available in GenBank, and based on the working definition of >5% 16S rDNA divergence for distinct genera, would be classified in the genus *Cardinium* (Ludwig *et al.* 1998). This genus was erected as a result of the identification of a new bacterium associated with a tick, whitefly, and *Encarsia* wasp. The type species *Cardinium hertigii* (AY331187) is the first for which reproductive isolation has been demonstrated in *Encarsia hispida* wasps in which it caused parthenogenesis (Zchori-Fein *et al.* 2001, 2004). In this analysis, the *Cardinium* associated with AzA and Riverside A (AY279409) were identical, at 99.4-99.6% nt identity (Fig. 3.1), but were distinct from *C. hertigii* of *E. hispida* based on 96.9-97.2% shared nt identity. This conclusion is supported by a working cut-off for bacterial species at >97.5% identity (Stackebrandt and Goebel 1994). Similarly, the whitefly-associated *Cardinium* species represent a unique
species, with respect to other *Cardinium* identified in hemipterans at 96.2-96.7% identity (Fig. 3.1).

The high shared nt identity, at 99.6-99.99% between the *Cardinium* spp. associated with *Aspidiotus* spp. (Diaspididae) and its parasitoid *Aphytis* (Aphelinidae), is suggestive of horizontal transmission (Fig. 3.1). Correspondingly, the 99.1% shared identity between the *Cardinium* associated with the phytophagous mite *Oligonichus* (Tetranychidae) and its predator mite, *Metaseiulus* (Phytoseiidae), may likewise have resulted from horizontal transmission. Consequently, it would not be surprising to find a similar ecological interaction for the *B. tabaci-Cardinium* association described here. Thus, this bacterial genus may be found to have routinely co-evolved in predator-pray, or host-parasitod relationships (Hoy and Jeyaprakash 2005).

Studies have demonstrated that the 16S rDNA gene is not a robust marker for inferring bacterial phylogenies (Czarnetzki and Tebbe 2004; O’Neill *et al.* 1992; Vandekerckhove *et al.* 1999), however, the routinely employed 16S rDNA or *wsp* sequence permits provisional identification (Brown *et al.* 1998a,b; Hoy and Jeyaprakash 2005; Nirgianaki *et al.* 2003). The 16S rDNA analysis included a GenBank sequence corresponding to the mosquito, *Culex pipiens* (Culicidae), which is the type species for the genus and species *Wolbachia* (Hertig 1936). The nucleotide identity of *C. pipiens* of 98.7% with Jat associated *Wolbachia* sequence, suggests that the Jat-*Wolbachia* is similar species from *Wolbachia pipiensis* in mosquito. Contrariwise, *Wolbachia* detected in the whitefly, *Aleurothricelus duplicatus*, appears to be a distinct species at 96.7% and 96.2% identity with *C. pipiens* and Jat, respectively. The *Wolbachia* sequence of this
study, the other whiteflies, hemipterans, and mosquito sequences from GenBank clustered in the B group in the phylogenetic analysis (Fig 3.2). The diversity of taxa in the tree, including insects, mites, crustaceans and nematodes, strongly suggests horizontal transmission of *Wolbachia* (Czarnetzki and Tebbe 2004; Nyiro *et al.* 2002; O’Neill *et al.* 1992; Vandekerckhove *et al.* 1999).

The discovery of the Jat-*Wolbachia* isolate has been reported for the monophagous biotype from Puerto Rico (Brown *et al.* 1998a,b; Zchori-Fein and Brown 2002), and *Wolbachia* association was shown for several field collections of *B. tabaci* of unknown biotypes/haplotypes (Nirgianaki *et al.* 2003) and for a B biotype colony from Florida (Jeyaprakash and Hoy 2000), however, associated biological consequences have not been elucidated for those isolate-host combinations. And *Wolbachia* infection of the Jat-biotype, and *Cardinium*-like filaments associated with bacterial symbionts in the AzA biotype used in these studies was alluded in TEM micrographs published by Costa *et al.* (1995), thus the 16S rDNA sequence and biological data are corroborative. Extensive analysis using semi-nested, hot-start, and long PCR, respectively, to detect *Wolbachia* or *Cardinium* in the parents and offspring of the AzB biotype colony failed in all cases to reveal prospective CI-causing bacteria, including *Wolbachia*, which was reported previously for the B biotype (Jeyaprakash and Hoy 2000) referred to herein as FLB1. In contrast, the Jat biotype adults were found to harbor *Wolbachia* 96-100% of the time, and *Cardinium* also was detected in individuals of A-biotype isolates at high frequency (91-100%) (Table 3.5). Thus, these findings are consistent with bacterial-associated cytoplasmic incompatibility phenotypes in other insects. *Wolbachia* is widely recognized
for its induction of CI however the mechanism, in which zygote formation is arrested in the earliest developmental stages, is still unknown (Tram and Sullivan 2002; Werren 1997). In contrast, it is known that when identical or closely related *Wolbachia* CI-strains infect two interbreeding populations, a male-conferred CI factor passed via sperm modification to the egg can have a ‘rescue’ effect, thereby negating CI, and in the case of haplodiploidy, female offspring would be expected despite widespread CI bacterial infection in the paternal population (Werren 1997).

The association of *Cardinium* and *Wolbachia* with the AzA and Jat biotypes, respectively, and the production of only male offspring from AzB female x heterologous male crosses involving the Jat or AzA biotype, respectively, provides two additional, albeit, provisional (indirect) examples of CI-interrupted gene flow. In this haplodiploid insect, the CI phenotype is expressed either as unidirectional (AzB♀ x AzA♂ and AzB♀ x Jat♂) or bidirectional compatibility (Jat x AzA), which correlates positively with absence or presence of specific CI-bacteria. The essential requirement for demonstrating bacterial-induce CI is to ‘cure’ or rid the host of bacterial infection by administering antibiotics in the diet (Stouthamer *et al.* 1990). In this study, attempts to cure the Jat and AzA colonies of the associated CI bacteria were unsuccessful, presumably owing to the antibiotic-induced death of the host obligate primary endosymbiont. This corroborates other reports of the essential role of the primary endosymbiont in augmenting nutritional requirements of *B. tabaci* (Buchner 1965; Costa *et al.* 1997). Indeed, no aleyrodid has been reported curable of parasitic bacteria without also eliminating the primary symbiont. Thus, if CI can been demonstrated as causal using an alternative approach, the first
direct demonstration of bacterial-induced CI will be provided for whiteflies.

Here we observed the interruption of gene flow only when one of the two mated heterologous biotypes did not harbor a detectable CI-causing bacterium, and for those that did, gene flow was impeded in one direction only. In contrast, the two biotypes that harbored putative CI-causing bacteria showed evidence of positive gene flow in a bidirectional pattern, suggesting a ‘rescue’ affect. This effect was asymmetrical with respect to sex ratio, in which a bias was observed in the form of more abundant female offspring from Cardinium-associated AzA (maternal lineage), in contrast to a higher frequency of male offspring produced by Wolbachia harboring Jat-females (Caballero and Brown, in preparation). At present, an introgression experiment employing AzA females x AzB males is in progress to verify CI-causality over association. This involves the backcrossing of \approx 8 successive generations of AzAx AzB female hybrid with AzB males to produce the resultant 100% dilution of the AzA genome, while at the same time incorporating the Cardinium genome in backdrop of the AzB genome (Stouthamer et al. 1996) for performing reciprocal AzA and AzB crosses.

While extensive documentation has affirmed that CI is common among Wolbachia infected arthropods (Stouthamer et al. 1999), much less information is available for the new bacterial genus Cardinium (Weeks and Breeuwer 2003). Thus far, a single example (Encarsia wasp) for Cardinium-induced CI has been provided (Hunter et al. 2003), and so our data are suggestive of a second example. That B. tabaci utilizes haplodiploidy, whereas Encarsia can reproduce sexually or through CI-induced parthenogenesis indicates the potential for Cardinium to affect CI in at least two different insect orders,
each employing different reproductive modes.

Finally, we have shown for the first time that in the event of true CI, *Cardinium* may contribute a more potent offensive ‘female killing’ and/or defensive ‘female-killing prevention’ factors when confronted by another CI-inducer such as *Wolbachia*. That the two bacteria are classified in separate prokaryotic phyla is suggestive of a common mechanism among CI-causing bacteria. Although a novel proposition, it is possible that these divergent bacteria have inherited this capacity through horizontal transfer, known to occur commonly among bacteria, thereby enabling even distant relatives to out-compete one another, even if the outcome is subtle. In this example, *Cardinium* infection resulted in enhanced host fitness with respect to the production of female offspring, compared to the *Wolbachia* infected lineage. This would favor *Cardinium* over *Wolbachia* reproduction and increased spread. Likewise, reduced *Wolbachia* spread impeded by partial CI-rescue could reduce *Wolbachia* infected female numbers, thereby indirectly conferring greater fitness in *Cardinium*.

A number of prokaryotes have been associated with *B. tabaci*, including the primary symbiont, *Portiera*, and secondary or affiliated bacteria including *Hamiltonella, Rickettsia, Chlamydia, Cyanobacteria, and Wolbachia* among others (Baumann *et al*. 2004; Brown *et al*. 1998a,b; Gottlieb *et al*. 2006; Jeyaprakash and Hoy 2000; Nirgianaki *et al*. 2000; Thao and Baumman 2004a,b; Thao *et al*. 2003; Zchori-Fein and Brown 2002). Among these only *Wolbachia* has been implicated in CI for other arthropods (Weeks *et al*. 2002). Extensive analysis of the CI-like bacteria present in the adults (parents and offspring) and in eggs for the biotypes examined here, revealed detection of
Wolbachia only in the Jat colony (Table 3.4, 3.5). Likewise, only Cardinium was associated with the A-biotype (four isolates, 3 from the US and one from Mexico).

Neither bacterium was detectable in three B biotype isolates examined, including the AzB used for experiments described here (Table 3.4, 3.5). Likewise, Wolbachia was not detected in any of three B biotype isolates included here, including FIB1, which was previously implicated as a Wolbachia host (Jeyaprash and Hoy 2000). However, all three B biotype colonies were found to harbor Rickettsia bellii, a bacterium reported recently in B. tabaci from Israel by Gottlieb et al. (2006) (Table 3.4). The role of R. bellii in B. tabaci is not known, however, others and we have found no evidence for a CI association. Nevertheless, at least one rickettsial species has been implicated in male killing in beetles (Lawson et al. 2001; von der Schulenburg et al. 2001).

In summary, unexpectedly, female offspring were produced from the Jat x AzA reciprocal crosses, indicating that their respective CI-bacteria are potentially capable of rescuing incompatibility. Also Cardinium-AzA♀ x Wolbachia-Jat♂ crosses yielded more female offspring (58%♂), than Jat-Wolbachia♀ x A-Cardinium♂ (84%♂) cross, which is highly suggestive of assymetrical interference with female killing and/or its rescue (Caballero and Brown, in preparation). Clearly, Cardinium prevented Wolbachia-induced female mortality at a higher frequency than the inverse combination. This is the first report of a novel rescue-like phenomenon involving two distantly related bacteria. The outcome of introgression experiments will reveal whether the associated CI-bacteria known for their propensity to induce reproductive isolation among arthropods, will reveal unequivocally their role in this CI-like phenomenon in the B. tabaci complex.
Table 3.1. History of the *B. tabaci* complex isolates used in this study, and their geographical origin, host range, and associated CI-inducing bacteria.

<table>
<thead>
<tr>
<th>BIOTYPE</th>
<th>ABBREVIATION</th>
<th>GEOGRAPHIC ORIGIN</th>
<th>YEAR</th>
<th>HOST RANGE</th>
<th>ASSOCIATED CI BACTERIA</th>
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<tbody>
<tr>
<td>Jatropha²</td>
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<td>1990</td>
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<td>1998</td>
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<td><em>Cardinium</em></td>
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<tr>
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<td>1993</td>
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<td>1977</td>
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<td>Florida B¹</td>
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<td>Gainesville, FL</td>
<td>2001</td>
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</table>

¹ Lab colonies.
² Lab colonies no longer maintained/Frozen specimens used.
Table 3.2. *Cardinium* 16S rDNA GenBank accession numbers of representative arthropods used in the phylogenetic analysis.

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Table 3.3. *Wolbachia* 16S rDNA GenBank accession numbers for representative arthropods and nematodes used in the phylogenetic analysis.

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* Filarial worms (Nematoda).
Table 3.4. PCR detection of maternally inherited (via the egg) bacteria in *B. tabaci* isolates. Each replicate consisted of 5 eggs, pooled. Three replicates were carried out for each, except for Jatropha which employed six replicates.

<table>
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<tr>
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<tr>
<td>Florida B1</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Florida B2</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>
Table 3.5. Frequency of *Wolbachia* and *Cardinium* detected in adults of Jatropha, A-, and B-biotype isolates of *B. tabaci* using PCR.

<table>
<thead>
<tr>
<th>BIOTYPE</th>
<th>Cardinium</th>
<th>Wolbachia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jatropha</td>
<td>0/19 (0.00%)</td>
<td>23/24 (95.83%)</td>
</tr>
<tr>
<td>Arizona A</td>
<td>12/13 (92.31%)</td>
<td>0/13 (0.00%)</td>
</tr>
<tr>
<td>Riverside A</td>
<td>21/23 (91.30%)</td>
<td>0/21 (0.00%)</td>
</tr>
<tr>
<td>Culiacan A</td>
<td>12/13 (92.31%)</td>
<td>0/13 (0.00%)</td>
</tr>
<tr>
<td>Salinas</td>
<td>13/15 (86.66%)</td>
<td>0/13 (0.00%)</td>
</tr>
<tr>
<td>Arizona B</td>
<td>0/33 (0.00%)</td>
<td>0/15 (0.00%)</td>
</tr>
<tr>
<td>Florida B1</td>
<td>0/20 (0.00%)</td>
<td>0/12 (0.00%)</td>
</tr>
<tr>
<td>Florida B2</td>
<td>0/23 (0.00%)</td>
<td>0/18 (0.00%)</td>
</tr>
</tbody>
</table>
Table 3.6. Frequency of *Wolbachia* and *Cardinium* detected by PCR in F<sub>1</sub> offspring from reciprocal crosses of the Jat and AzA biotypes of *B. tabaci*.

<table>
<thead>
<tr>
<th>CROSS</th>
<th>F&lt;sub&gt;1&lt;/sub&gt;</th>
<th>CI-BACTERIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cardinium</td>
</tr>
<tr>
<td>Jat♂ xAzA♀</td>
<td>♀</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>AzA♀ xJat♂</td>
<td>♀</td>
<td>15/15 (100%)</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>15/15 (100%)</td>
</tr>
</tbody>
</table>
Figure 3.1. Phylogenetic analysis of *Cardinium* 16S rDNA sequences (1090 bp) using PAUP v3.1.1. The maximum likelihood tree was reconstructed using the heuristic search option and 1000 bootstrap replicates. The scale bar indicates genetic distance in units of nucleotide substitution per site. Names correspond to host species of *Cardinium* groups and the GenBank accession numbers are shown in Table 3.2. The tree was rooted using *Acanthamoeba* (AY549547) as the outgroup. The A-biotype whitefly sequences of *B. tabaci* are AzA, CulA, RivA, and SalA. *Wolbachia* hosts are designated as 1= Acari, 2= Hemiptera/Homoptera, and 3= Hymenoptera.
Figure 3.2. Phylogenetic analysis of *Wolbachia* 16S rDNA sequences (787 bp) using PAUP v3.1.1. The maximum likelihood tree was reconstructed using the heuristic search option and 1000 bootstrap replicates. The bar indicates the genetic distance in units of nucleotide substitution per site. The names correspond to representative host species of *Wolbachia* for six arthropod groups (A, B, C, D, E, F), and the respective GenBank accession numbers for each is shown in Table 3.3. The tree was rooted using the *Rickettsia* spp. (M21789) as the outgroup. The Jatropha-*Wolbachia* sequence of the Jatropha-*Wolbachia* sequence of the Jatropha biotype of *B. tabaci* is shown boxed. Two other whitefly sequences available in GenBank are pointed by arrows. Arthropod and nematode hosts are indicated by 1= Acari, 2= Crustacea, and 3= Nematoda, 4= Hemiptera/Homoptera, and 5= Aleyrodidae.
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