CURRENT MICROBIOLOGY Vol. 47 (2003), pp. 93–101
DOI: 10.1007/s00284-002-3969-1

Current Microbiology
An International Journal

Wolbachia Infections of the Whitefly *Bemisia tabaci*

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Received: 9 September 2002 / Accepted: 25 September 2002

Abstract. We report the first systematic survey for the presence of *Wolbachia* endosymbionts in aphids and whiteflies, particularly different populations and biotypes of *Bemisia tabaci*. Additional agriculturally important species included were predator species, leafhoppers, and lepidopterans. We used a polymerase chain reaction (PCR)-based detection assay with ribosomal 16S rDNA and *Wolbachia* cell surface protein (*wsp*) gene primers. *Wolbachia* were detected in a number of whitefly populations and species, whitefly predators, and one leafhopper species; however, none of the aphid species tested were found infected. Single, double, and triple infections were detected in some of the *B. tabaci* populations. PCR and phylogenetic analysis of *wsp* gene sequences indicated that all *Wolbachia* strains found belong to group B. Topologies of the optimal tree derived by maximum likelihood (ML) and a ML tree in which *Wolbachia* sequences from *B. tabaci* are constrained to be monophyletic are significantly different. Our results indicate that there have been at least four independent *Wolbachia* infection events in *B. tabaci*. The importance of the presence of *Wolbachia* infections in *B. tabaci* is discussed.

Wolbachia is an intracellular, maternally inherited bacterium that can invade numerous invertebrate host species and maintain itself by manipulating host reproduction. *Wolbachia*-associated reproductive alterations include the induction of parthenogenetic development in certain parasitic wasps, overriding chromosomal sex determination to convert infected genetic males into functional females in some isopod species, male-killing, and, most commonly in insects, the induction of cytoplasmic incompatibility (CI), a form of embryonic lethality in crosses between males and females with different *Wolbachia* infection status [41, 42, 45]. Although the actual distribution is not yet fully elucidated, *Wolbachia* has been shown to infect all major orders of insects, crustaceans, mites, and even nematodes [1]. PCR surveys have indicated that over 16% of the insect species tested carry *Wolbachia*, suggesting that more than a million insect species are infected [48]. This number may be a rather conservative estimate since recent data suggest that more than 70% of the arthropod species may be infected with *Wolbachia* [21].

Phylogenetic analyses based on the 16S rRNA gene have shown that the genus *Wolbachia* belongs to the alpha-Proteobacteria, forming a monophyletic group most closely related to the *Ehrichia* assemblage [30]. With the *ftsZ* gene, arthropod *Wolbachia* had been initially divided into two groups, A and B, which diverged from each other about 60 million years ago [49], while recent studies suggest that there may be up to six different groups present in infected invertebrate species [26]. Recently, a *Wolbachia* surface protein coding gene, *wsp*, was cloned, sequenced, and shown to be more variable in sequence than any other known *Wolbachia* gene [7, 51].

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Phylogenetic studies with *wsp* gene sequences have delineated over 20 subgroups of *Wolbachia* [11, 21, 31, 33, 36, 44, 51]. An additional use of the *ftsZ* and *wsp* genes has been as markers for the detection of *Wolbachia* superinfections [49, 51].

Aphids (Homoptera: Aphidoidea) and whiteflies (Homoptera: Aleyrodoidea) are serious agricultural pests. As plant sap-sucking insects, they cause major losses in crops by both direct feeding damage and via the transmission of many plant pathogens [2]. Both aphids and whiteflies are species with nutritionally restricted diets (plant sap) and carry symbiotic bacteria, which belong to the gamma subdivision of the Proteobacteria that provide them with limiting amino acids and vitamins [15]. Some aphid and whitefly species also carry secondary (S) endosymbiotic Gram-negative, rod-shaped bacteria (0.5–1.5 μm in diameter) that are also members of the gamma subdivision of the Proteobacteria [17]. Despite the importance *Wolbachia* may have in the evolution of biologically and genetically distinguishable populations and biotypes of their hosts, and its potential application as a tool for pest control [5, 6, 40, 46], there are very few published reports about the presence of this bacterium in whiteflies [21, 50].

In this study, we report the results of a systematic polymerase chain reaction (PCR) survey of important agricultural pests comprising whitefly species, aphids, some of their predators, leafhoppers, and lepidopteran species for the presence of *Wolbachia*. Our aim was to determine the prevalence of *Wolbachia* in these economically important insects, particularly in populations of the whitefly *Bemisia tabaci*, a major agricultural pest in many parts of the world. We also examine the phylogenetic relationships of the *wsp* gene sequences detected and discuss the possible role that *Wolbachia* infections may have in the biology of *B. tabaci*.

Materials and Methods

Insect samples. The various insect species and populations used in this study were either field collected or maintained as laboratory colonies for several generations. All insect material was kept in 100% ethanol at 4°C until DNA extraction. Whitefly field samples were identified by using taxonomic features of adults and pupae (visible with a hand lens), as described previously [10, 28]. The majority of aphid samples were provided as DNA by Paul Baumann (University of California, Davis, USA). Egg samples of *Pectinophora gossypiella* (pink bollworm) and *Anthonomus grandis thurberi* and adults of *Leptothrips mali*, *Leptothrips near mali* and *Delphastus pusillus* were used in the present study. It should be noted that *D. pusillus* was being fed on *Wolbachia*-free whiteflies, while caution was taken to insure that *L. mali* and *L. near mali* were reared in a whitefly-free environment.

DNA analysis. DNA was extracted according to standard protocols [12, 30]. Aphid DNA provided by Paul Baumann was extracted as described previously [29]. The presence of *Wolbachia* was determined by using the *Wolbachia*-specific 16S rDNA primers and *wsp* primers [12, 51]. At least nine individuals were assayed from each insect species or population tested. In the insect samples examined, the results of the 16S rDNA and *wsp* primers were in almost complete agreement; there were only a few cases where the *wsp* primers gave false positive results as confirmed later by sequencing PCR clones. We also used *wsp* primers that specifically amplify from A and B group of *Wolbachia* as described previously [51]. We carried out PCR control reactions to test the quality of the DNA template. For this, one of three sets of primers was used on the samples: eukaryotic 28S rDNA primers [49]; insect mitochondrial 12S rDNA primers [30]; and mitochondrial 16S rDNA primers [39]. One microliter (μL) of extract was used as template for PCR. All PCR analyses were carried out in 25-μL reactions and involved an initial denaturation step at 94°C for 5 min followed by 35 cycles of: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR reactions included 2.5 mM $MgCl_2$, dNTPs at 200 mM each, 1 μM of each primer, 1 unit of DNA Taq polymerase (Promega or Gibco BRL Life Technologies), and buffer supplied by the manufacturer. PCR products were visualized on 1.2% agarose gels stained with ethidium bromide (5μg/mL). PCR fragments were cloned into vector pGEM-T (Promega) following the manufacturer's instructions. Plasmid DNA was purified with the QIAprep Spin plasmid kit (Qiagen GmbH, Hilden, Germany). Sequencing reactions were performed with the d-Rhodamine dye-terminator cycle sequencing kit (Applied Biosystems) and run on an ABI377 sequencer (Applied Biosystems), all according to the manufacturer's protocols and instructions. We sequenced a fragment of the hypervariable gene of the *Wolbachia* surface protein (*wsp*) from all infected species and populations. Three to six clones were sequenced from each individual, and sequences from at least three different individuals from each infected population were obtained. The *wsp* sequences of this study have been deposited in the EMBL database under accession numbers AJ291370 to AJ291389.

Data analysis. Twenty new *wsp* sequences were obtained from the present study and were combined with 20 other known B group *wsp* sequences. *Wolbachia wsp* sequence from *Drosophila melanogaster* (*w*Mel) from the A group was used as outgroup. To obtain a manageable data set, we used only the representative *wsp* sequences from each of the subgroups of the *Wolbachia* B group revealed in previous studies (see Results and Discussion). Multiple sequence alignments were obtained by using the Clustal W1.8 algorithm. After alignment, nucleic acids at positions corresponding to the hypervariable regions of the peptide [7] were excluded from the analysis because those regions could not be accurately aligned (positions 1–30, 234–245, 530–571, 621–650). Distance, parsimony, and maximum-likelihood analyses were all performed with PAUP (Phylogenetic Analysis Using Parsimony) version 4.0 b8.0 for MacIntosh [43]. Distance matrices were constructed by using the Kimura-2 parameter model, and trees were constructed by neighbor-joining. For parsimony, 198 characters of 281 variable characters were informative (258 constant, 539 total), and bootstrapping was performed with the heuristic search option for 2000 replicates (5 random addition sequence replicates, tree-bisection-reconnection (TBR), and MULTREES in effect). ML trees were derived by using a general time-reversible model (GTR) [25] that incorporates gamma-distributed rate heterogeneity across sites (+G) and a proportion of invariable positions (+I). Choice of the model was based on work by Schulenburg et al. [36], who showed that simpler substitution models that did not consider rate heterogeneity or a proportion of invariant sites (e.g., HKY85) [18] led to systematic error for a similar set of sequences from *Wolbachia*. Model parameters were estimated with the program MODELTEST [34]. Bootstrapping was performed with the heuristic search option for 100 replicates [five random addition sequence replicates, branch swapping by nearest-neighbor interchange

(NNI)]. Estimated base frequencies were A=0.312216, C=0.161375, G=0.202788, T=0.323621. The estimated value of the proportion of invariable sites was (+I) was 0.135327, and the estimated value of the gamma shape parameter (+G) was 0.683655. To test for monophyly of *Wolbachia* sequences, a tree was constructed with MACCLADE 4.03 [27] from the optimal ML tree wherein all *B. tabaci wsp* sequences were forced to be monophyletic. The topology of the optimal tree was then compared with the topology of the constrained tree by the Shimodaira-Hasegawa test [37] as executed in PAUP, under both RELL approximation and full optimization, with non-parametric bootstrapping (1000 replicates).

***Wolbachia* nomenclature.** The *Wolbachia* strain names were based on the nomenclature system proposed by Zhou et al. [51] with minor modifications. Each strain's name is defined by *w* (in italics) denoting *Wolbachia*. This is followed by a capital letter derived from the first letter of the genus name and by three lower case letters coming from the first three letters of the species name. Multiple strains present in a given species are distinguished by numbers added at the end. For example, *Wolbachia* strains present in *B. tabaci* were named as *w*Btab1, *w*Btab2. If a sample has been classified only as far as genus, we used the first three letters of the genus name followed by "sp". For example, *Wolbachia* strains present in *Aleurotrachelus* sp. were named as *w*Alesp.

Results and Discussion

Prevalence of *Wolbachia*. We performed an extensive targeted survey for *Wolbachia* infection in insect species of economic interest and screened 78 populations from 36 different insect species for *Wolbachia* by PCR amplification with 16S rDNA (99F/994R) and *wsp* (81F/691R) primers. Six out of the 36 insect species assayed were infected (16.7%), very similar to previous reports but significantly lower than others [21, 48]. At least nine adult insects from each population were tested individually by PCR (unless stated otherwise). All individuals tested from the infected populations were carrying *Wolbachia*. On the basis of a PCR assay using specific *wsp* primers, all infected samples of the present study carried B group *Wolbachia* (data not shown). As is evident from Table 1, *Wolbachia* infections were detected in whiteflies (see below), Thysanoptera (*Leptothrips mali*), Coleoptera (*Delphastus pusillus*), and a leafhopper species (*Cicadulina mbila*), but was absent from the two Lepidoptera species tested. It is also noteworthy that no infection was detected in 24 populations from 22 aphid species tested. To exclude the possibility that presence of *Buchnera aphidicola* DNA at high frequency could interfere with detection of *Wolbachia*, we mixed aphid DNA with infected insect DNA and checked it by PCR with *Wolbachia*-specific primers. In no case was detection of *Wolbachia* affected.

***Wolbachia* infections in whiteflies.** As shown in Table 1, three out of seven whitefly species were infected with *Wolbachia* (43%): *Aleurotrachelus* sp., *Bemisia afer*, and a wide range of populations of *Bemisia tabaci* collected worldwide. Since *B. tabaci* is of major economic importance and might well comprise a species complex [14, 16], we performed an extensive survey of populations for *Wolbachia* infections. Interestingly, not all populations of *B. tabaci* were infected with *Wolbachia* (Table 1). It must be noted here that the systematics of *B. tabaci* is still a subject of intense scientific debate, and the renaming of the silverleafing whitefly, or B biotype as *B. argentifolii*, a new species, is controversial. Herein we use the term silverleafing (SL) whitefly instead of *B. argentifolii* or the B biotype. Only one out of 13 SL populations of *B. tabaci*, the one from Yemen, was positive for *Wolbachia*. On the other hand, 10 out of 26 surveyed non-SL, indigenous populations of *B. tabaci* were infected with *Wolbachia*. In total, 11 out of 39 populations tested were infected (28%). A recent study reported a similar rate of detection of *Wolbachia* (about 30%) of *B. tabaci* populations, but the infections were not characterized molecularly [50].

***Wolbachia* relationships.** The *wsp* gene PCR products were cloned from at least three different adult insects from each of the infected populations. Three clones from each infected adult insect were sequenced. An additional three clones were sequenced if sequence variation was detected in the first three clones. Majority-rule consensus sequences were produced for each specimen, and these consensus sequences were used for subsequent phylogenetic analyses. The sequencing of the *wsp* gene fragments and the subsequent phylogenetic analysis confirmed that all the newly detected *Wolbachia* strains in this study belonged to group B. Most of the infected insect populations were carrying a single *Wolbachia* strain, so that no variation was detected between individuals from the same species or population. However, there were some exceptions; some individuals from *B. tabaci* populations from Benin and Kenya (Nairobi, cabbage) carried double infections; and in a *B. tabaci* sample from a Kenyan Ossimium plant, some individuals appeared to be triple infected.

We combined our data set (20 *wsp* sequences) with 20 other B group *wsp* sequences, which included representatives from all currently published B subgroups plus two more *wsp* sequences of *Wolbachia* strains (*w*Cuc and *w*AscA) present in tephritid species [20]. The sequences showed a maximum divergence of 27.4% within group B. All methods used to reconstruct phylogenies yielded virtually identical relationships, and only differences in the arrangement of terminal taxa in the myriad polytomies derived were observed. The three methods—distance, parsimony, and maximum-likelihood—make different evolutionary assumptions, but their congruence provides strong support for the deduced phylogeny. For

Table 1. Insect samples used in the present study and their *Wolbachia* infection status

Insect samples[a]	Origin	Source	Sample type[b]	*Wolbachia*
Whiteflies:				
Aleurodicus dispersus	Spain (Canary Islands)	*Musa sapientum* (banana)	F	− (0/15)
Aleurotrachelus sp.	Benin	*Asystasia* sp.	F	+ (13/13)
Bemisia afer	Malawi	*Manihot esculenta* (cassava)	F	+ (15/15)
Bemisia hancocki	Pakistan	sheesham	F	− (0/13)
Lecanoides floccissimus	Spain (Canary Islands)	*Musa sapientum* (banana)	F	− (0/15)
Trialeurodes vaporariorum	Greece	*Lycopersicum esculentum* (tomato)	F	− (0/9)
Trialeurodes vaporariorum	Spain	*Lycopersicum esculentum* (tomato)	F	− (0/13)
B. tabaci (SL)	USA	*Gossypium hirsutum* (cotton)	L	− (0/13)
B. tabaci (SL)	Antigua	*Lycopersicum esculentum* (tomato)	F	− (0/9)
B. tabaci (SL)	Australia	*Gossypium hirsutum* (cotton)	F	− (0/13)
B. tabaci (SL)	Brazil	*Lycopersicum esculentum* (tomato)	F	− (0/13)
B. tabaci (SL)	Cyprus	*Gossypium hirsutum* (cotton)	F	− (0/13)
B. tabaci (SL)	Egypt	*Gossypium hirsutum* (cotton)	F	− (0/12)
B. tabaci (SL)	Florida	*Mirabilis* sp. (night shade)	F	− (0/13)
B. tabaci (SL)	Israel	*Gossypium hirsutum* (cotton)	F	− (0/13)
B. tabaci (SL)	Japan	*Citrullus vulgaris* (watermelon)	F	− (0/9)
B. tabaci (SL)	New Zealand	*Euphorbia pulcherrima* (poinsettia)	F	− (0/13)
B. tabaci (SL)	Taiwan	*Euphorbia pulcherrima* (poinsettia)	F	− (0/3)
B. tabaci (SL)	Yemen	*Citrullus vulgaris* (watermelon/squash)	F	+ (9/9)
B. tabaci (non-SL)	Australia	*Gossypium hirsutum* (cotton)	F	+ (13/13)
B. tabaci (non-SL)	Benin	*Asystasia* sp.	F	+ (13/13)
B. tabaci (non-SL)	Colombia	*Phaseolus vulgaris* (beans)	F	+ (13/13)
B. tabaci (non-SL)	Greece	*Gossypium hirsutum* (cotton)	F	+ (13/13)
B. tabaci (non-SL)	Hainan	unidentified weed	F	+ (13/13)
B. tabaci (non-SL)	India	*Gossypium hirsutum* (cotton)	F	− (0/9)
B. tabaci (non-SL)	Kenya	*Ossimium* sp.	F	+ (12/12)
B. tabaci (non-SL)	Kenya (Nairobi)	*Brassica oleracea*(cabbage)	F	+ (13/13)
B. tabaci (non-SL)	Kenya (North)	*Manihot esculenta* (cassava)	F	− (0/8)
B. tabaci (non-SL)	Kenya (West)	*Manihot esculenta* (cassava)	F	− (0/8)
B. tabaci (non-SL)	Kenya (West)	*Lycopersicum esculentum* (tomato)	F	− (0/8)
B. tabaci (non-SL)	New Zealand	*Euphorbia pulcherrima* (poinsettia)	F	− (0/13)
B. tabaci (non-SL)	Nigeria	*Manihot esculenta*(cassava)	F	− (0/9)
B. tabaci (non-SL)	Nigeria	*Vigna unguiculata*(cowpea)	F	− (0/4)
B. tabaci (non-SL)	Pakistan	dalbergia	F	+ (13/13)
B. tabaci (non-SL)	Portugal	*Gossypium hirsutum* (cotton)	F	− (0/12)
B. tabaci (non-SL)	Singapore	*Euphorbia pulcherrima* (poinsettia)	F	− (0/13)
B. tabaci (non-SL)	Spain	*Ipomea indica*	F	+ (13/13)
B. tabaci (non-SL)	Spain (Almeria)	*Lycopersicum esculentum* (tomato)	F	− (0/9)
B. tabaci (non-SL)	Spain 1992	*Gossypium hirsutum* (cotton)	F	− (0/9)
B. tabaci (non-SL)	Spain 1993	*Gossypium hirsutum* (cotton)	F	− (0/13)
B. tabaci (non-SL)	Spain 1994 (Rodriguez)	*Capsicum annuum* (pepper)	F	− (0/13)
B. tabaci (non-SL)	Spain 1997	*Lycopersicum esculentum* (tomato)	F	− (0/4)
B. tabaci (non-SL)	Sri Lanka	*Solanum melongena*(aubergine)	F	− (0/13)
B. tabaci (non-SL)	Sudan	*Gossypium hirsutum* (cotton)	F	− (0/13)
B. tabaci (non-SL)	Turkey	*Gossypium hirsutum* (cotton)	F	+ (13/13)
Aphids				
Acyrthosiphon pisum	USA	*Vicia faba* (broad bean)	L	–
Aphis craccivora	Tanzania	ground nut	F	–
Aphis craccivora	Malawi	ground nut	F	–
Aphis craccivora	Uganda	ground nut	F	–
Diuraphis noxia	unknown	unknown	F	–
Melaphis rhois	unknown	unknown	F	–
Myzus persicae	unknown	unknown	F	–
Rhopalosiphum padi	unknown	unknown	F	–
Rhopalosiphum maidis	unknown	unknown	F	–
Schizaphis graminum	USA	wheat	L	–
Uroleucon aeneum	Sweden	*Cirsium vulgare*	F	–

Table 1. (*Continued*)

Insect samples[a]	Origin	Source	Sample type[b]	*Wolbachia*
Uroleucon ambrosiae	USA	*Ambrosia trifida*	F	–
Uroleucon astronomus	USA	*Aster macrophyllus*	F	–
Uroleucon caligatum	USA	*Solidago altissima*	F	–
Uroleucon erigeronense	USA	*Conyza canadese*	F	–
Uroleucon helianthicola	USA	*Helianthus* sp.	F	–
Uroleucon jaceae	Sweden	*Centaurea jacea*	F	–
Uroleucon jaceicola	Sweden	*Centaurea jacea*	F	–
Uroleucon obscurum	Sweden	*Hieracium* sp.	F	–
Uroleucon rapunculoidis	Sweden	*Campanula rapunculoides*	F	–
Uroleucon rudbeckiae	USA	*Rudbeckia hirta*	F	–
Uroleucon rurale	USA	*Actinomeris alternifolia*	F	–
Uroleucon solidaginis	Sweden	*Solidago virgaurea*	F	–
Uroleucon sonchi	USA	*Sonchus oleraceum*	F	–
Leafhoppers				
Cicadulina mbila	Kenya	millet	F	+ (15/15)
Nephotettix virescens	Thailand	*Oryza sativa* (rice)	F	− (0/15)
Nephotettix virescens	Bali	*Oryza sativa* (rice)	F	−(0/13)
Lepidoptera				
Pectinophora gosspiella	USA		L	–
Anthonomous grandis thurberi	USA		L	–
Thysanoptera				
Leptothrips mali	USA		L	+(9/9)
Leptothrips near mali	USA		L	−(0/9)
Coleoptera				
Delphastus pusillus	USA		L	+(9/9)

[a] SL = silverleafing; non-SL = non-silverleafing.
[b] F = field sample; L = laboratory colony.

brevity, we show only the tree derived by maximum-likelihood estimation. Under parsimony, an open-ended heuristic search identified an island of over 10,000 equally parsimonious reconstructions of length 742. Bootstrapping produced a 50% majority-rule consensus tree identical to the tree derived by maximum likelihood (Fig. 1), with strong support ($p > 0.95$) for the same groups. The maximum likelihood tree shown in Fig. 1 was derived under a general time-reversible (GTR) substitution model [25] that incorporated gamma-distributed rate heterogeneity across sites (+G) and a proportion of invariable positions (+I) [36] (-ln L score = 4733.02019) and shows the bootstrap values for the internal nodes. When the topology of this optimal tree is compared with the topology of a tree (not shown) wherein *Wolbachia* sequences from *B. tabaci* are constrained to be monophyletic (-ln L score = 5251.02547), by the Shimodaira-Hasegawa test [37], the difference is significant ($p < 0.05$), suggesting that *Wolbachia* strains of *B. tabaci* do not form a monophyletic group (see also below).

By using, with one exception, the grouping criterion of 2.5% sequence difference as proposed by Zhou et al. [51], 20 subgroups can be detected in the B group. Most of them have been described in earlier studies [11, 21, 31, 33, 36, 44, 51], while three new subgroups were revealed by the present study. The *Wolbachia* strain from *C. mbila* forms a new subgroup Cmbi because the shared sequence identity, based on uncorrected distances between *w*Cmbi and *w*Prn in the closest sister subgroup, is only 93.7%. Similarly, *Wolbachia* strains *w*Btab7 and *w*Btab8 from *B. tabaci* collected off a Kenyan cabbage tree form two new subgroups Btab1 and Btab2 respectively, because their shared sequence identity between each one of them and representative strains from all the other subgroups is less than 93%. The exception to the grouping criterion of 2.5% sequence difference concerns the bacterial strains that grouped with the *Wolbachia* strains present in *Tribolium confusum* and *Sphaeroma rugicauda* (Con and Rug subgroups). Sequences within this cluster showed maximum divergence up to 3.4%. The resolution within this cluster is rather poor as shown by the percentage confidence values for the internal nodes. We suggest that these sequences should be provisionally kept in one subgroup (referred to as the Con/Rug subgroup in the following text) until more strains are sequenced and/or a better molecular marker is identified to resolve their phylogenetic relationships. This

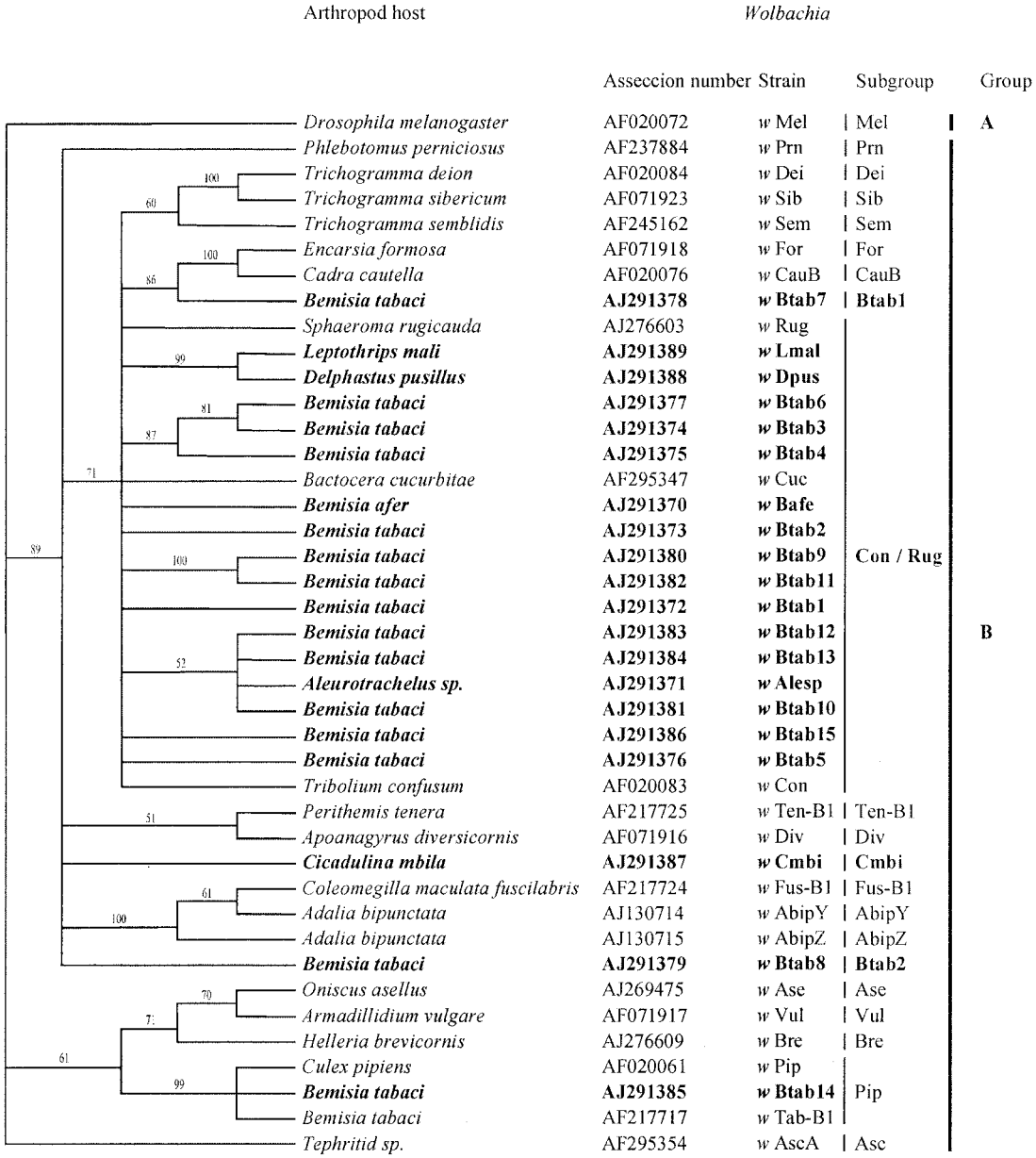

Fig. 1. Phylogenetic tree of *Wolbachia* based on *wsp* sequences. The tree has been constructed by maximum-likelihood analysis under a GTR+G+I substitution model (see experimental details), and the *w*Mel *wsp* sequence is the outgroup. Numbers on the nodes indicate bootstrap values. New *Wolbachia* strains and subgroups are shown in bold.

subgroup contains in total 14 whitefly *Wolbachia* strains as well as the strains present in the two *Wolbachia*-infected predator species detected in the present study, *L. mali* and *D. pusillus*. It should be mentioned that these groupings are based on *wsp* gene sequences alone, and it is yet to be determined whether or not they reflect the true phylogeny of *Wolbachia* strains. The *wsp*-based group-classification system, as introduced by Zhou et al. [51], may have limited biological meaning. According to this classification system, taxa are placed into different groups if they show more than 2.5% nucleotide divergence. However, this criterion may be misleading, mainly owing to the fact that the substitution rate of the *wsp* gene shows extreme differences between lineages as shown by Schulenburg et al. [36]. In this paper, the authors also specifically point out: "The presence of significant substitution rate heterogeneity between lineages in both *ftsZ* and *wsp* gene sequences forbids reliable estimation of divergence dates and also limits the applicability of a simple sequence-based classification

system such as that proposed for the *wsp* gene." Moreover, the recent reports about recombination in *Wolbachia* and within the *wsp* gene complicate phylogenetic analyses of these bacteria and, in particular, the phylogenetic relationships of the different strains [22, 47].

The *B. tabaci Wolbachia* strains belong to four different subgroups; interestingly, these groupings are similar to those formed for *B. tabaci* populations based on the mitochondrial 16S rRNA gene (Banks et al. unpublished data). The majority of the strains, 12, are of the Con/Rug subgroup. One is present in the Pip subgroup together with a recently reported *B. tabaci Wolbachia* strain [21], while two others are those that define two new groups (Btab1 and Btab2). The newly detected *Wolbachia* strains also exhibit size variation in their *wsp* gene fragments, which appears to be related to their respective subgroup: the lengths of the fragments were 558 bp for *B. tabaci* from Colombia (Pip subgroup), 555 bp for *B. afer*, *Aleurotrachelus* sp., *D. pusillus*, *L. mali*, and for some of the *B. tabaci* strains (Con/Rug subgroup), 552 bp for *C. mbila* (Cmbi subgroup), and 549 bp and 546 bp for *B. tabaci* collected off a Kenyan cabbage tree (Btab1 and Btab2 subgroups respectively). It is also noteworthy that the partial *wsp* gene sequences of the 15 *B. tabaci Wolbachia* strains reported in the present study differ from each other and from the one already reported [21] by one or more substitutions. Although there are other host species with *Wolbachia* strains exhibiting high levels of sequence differences, e.g., *Wolbachia* strains from *D. simulans* [51], the two *Wolbachia* strains from *Adalia bipunctata* [19, 36], or the *Wolbachia* strains from *Acraea encedon* [22–23], such extensive sequence variation has not been reported previously. Recent studies provided very strong evidence for the presence of positive selection on the outer membrane protein of *Wolbachia* and other species in the Rickettsiaceae family, suggesting that this protein plays an important role in symbiont host interactions [24, 36]. Finally, the *wsp* sequences of whitefly and predator *Wolbachia* strains, although closely related, are not identical, and thus there is not strong evidence of horizontal transmission events.

The significance of *Wolbachia* infections in whiteflies. *B. tabaci* is a major agricultural pest whose taxonomy and classification is very complex and controversial. *B. tabaci* comprises many populations, some of which can be distinguished by biological, biochemical, and molecular assays and on this basis have been referred to as races or biotypes [8, 14, 16, 32]. However, none of the populations or biotypes can reliably be distinguished on the basis of morphological characters alone [35]. The *Wolbachia* infection prevalence and *wsp* sequence data now offer yet another means to differentiate populations and biotypes of *B. tabaci*. In addition, *Wolbachia* may be implicated in cytoplasmic incompatibility phenomena in *B. tabaci* since there have been a number of reports in the literature regarding mating incompatibilities between different populations or biotypes of this species [3, 8, 9, 13, 32], although controlled crossing experiments between infected and uninfected strains having the same nuclear background have not yet been performed. Since the biotypes of *B. tabaci* may form a species complex [14, 16], the presence of *Wolbachia* could have played a role in the evolution of some of these biologically and genetically distinguishable populations and biotypes thus supporting the notion that *Wolbachia* should be considered as a driving force in speciation [4, 38, 46]. Finally, the presence of *Wolbachia* in *B. tabaci* may open novel ways for the control of this important insect pest [5, 6, 40].

ACKNOWLEDGMENTS

The authors thank Dr. Paul Baumann for providing aphid DNA; Drs. Ruth Osterlind, Leo P. Schouest Jr., and Frances Kimmins for providing insect samples; Drs. E. Hernandez-Suarez and Aurelio Carnero for identification of insects; Jacques Lagnel for technical assistance; Drs. Pattamaporn Kittayapong and Einat Zchori-Fein for communicating unpublished data; Drs. John H. Werren, Richard Stouthamer, Hinrich Schulenburg, and Stefan Oehler for comments on earlier versions of the manuscript; and Dr. Carol Von Dohlen for phylogenetic advice. K. Bourtzis thanks Dr. Scott O'Neill for his encouragement to initiate this study. Funding was provided by intramural funds of the University of Ioannina to K. Bourtzis, by BBSRC to the John Innes group, and by the California Cotton Pest Control Board to T. Miller.

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