Bulletin of Entomological Research (2012) **102**, 353–365
doi:10.1017/S0007485311000757

Population genetic structure and secondary endosymbionts of Q *Bemisia tabaci* (Hemiptera: Aleyrodidae) from Greece

A. Tsagkarakou[1]*, L. Mouton[2], J.B. Kristoffersen[1], E. Dokianakis[1,3], M. Grispou[1] and K. Bourtzis[3,4†]

[1]National Agricultural Research Foundation, Plant Protection Institute, Laboratory of Entomology and Agricultural Zoology, PO Box 2228, 71003 Heraklion, Greece: [2]Université Lyon1, CNRS, UMR 5558, Laboratoire de Biométrie et Biologie Évolutive, F-69622, Villeurbanne, France: [3]Department of Environmental and Natural Resources Management, University of Ioannina, 2 Seferi St., 30100 Agrinio, Greece: [4]Biomedical Sciences Research Centre, Al. Fleming, 16672 Vari, Greece

Abstract

We investigated the molecular diversity of the major agricultural pest *Bemisia tabaci* and of its associated secondary endosymbionts in Greece. Analyzing mitochondrial DNA, we found that the Q1 (=Q west) is predominant. We used eight microsatellite polymorphic markers to study the genetic structure of 37 populations from mainland and insular Greece, collected on different host species from outdoor and protected crops as well as from non-cultivated plants. In some cases, gene flow was found to be low even between populations separated by just a few kilometres. Bayesian analysis identified two main genetic groups, the first encompassing populations from south Crete and the second composed of populations from north Crete, two other Aegean islands and mainland Greece. Genetic differentiation was not correlated with different host plant species or habitat, or greenhouse versus open environment populations. Gene flow significantly decreased with geographic distance, but no isolation by distance existed when only the samples from mainland Greece or only the samples from Crete were considered. The secondary symbionts *Wolbachia* and *Hamiltonella* were present at high frequencies while *Arsenophonus, Cardinium* and *Rickettsia* were absent from Greek populations. Multilocus sequence typing of *Wolbachia* identified two *Wolbachia* strains. These two strains were found together in most of the populations studied but never in the same host individual. Their role on the observed population structure is discussed.

Keywords: Q *Bemisia tabaci*, population structure, microsatellites, secondary endosymbionts, *Wolbachia*

(Accepted 7 November 2011; First published online 27 January 2012)

*Author for correspondence
Fax: 0030 2810245858
E-mail: tsagkarakou@nagref.gr
†Current address: Department of Environmental and Natural Resources Management, University of Western Greece, 30100 Agrinio, Greece

Introduction

The species complex *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) has a global distribution, and its members are major pests of agricultural crops, causing damage directly by feeding and indirectly by transmitting many plant viruses (Jones, 2003). Their propensity to develop

insecticide resistance, in combination with the high genetic variability, makes their control problematic. Although morphologically indistinguishable, members of this species complex display a large number of biological, physiological and genetic variations, which had led to the characterization of >30 biotypes (references in Xu *et al.*, 2010). Recently, it has been proposed, based on a global analysis of mitochondrial cytochrome oxidase I gene (mtCOI) as well as the results of crossing experiments, that *B. tabaci* is a complex of at least 28 cryptic species (Dinsdale *et al.*, 2010; De Barro *et al.*, 2011; Hu *et al.*, 2011 and references therein). These papers point out the erroneous and misleading use of the term biotype. Following their considerations, we use hereafter '*Bemisia tabaci* complex' to refer to the species complex. In addition, we refer to the species corresponding to the clusters which have been determined by the Bayesian analysis of the mtCOI haplotypes, using the cluster's names proposed by Dinsdale *et al.* (2010) and adopted by Hu *et al.* (2011). For example, we refer to the Middle East–Asia Minor 1 cluster (known commonly as biotypes B and B2) by using MEAM1. However, we refer to the species of the Mediterranean cluster (known commonly as biotypes Q, J and L) and which includes sequences from Greek whiteflies using *Bemisia tabaci* given that the lectotype of *B. tabaci* is from Greece. Greece, both mainland and insular regions, is the geographic origin of the whitefly populations of the present study, including the area of Agrinio in west Greece from which Gennadius (1889) first described the species. Moreover and in order to establish a connection with previous works, we continue to use the associated 'biotype' designation (B, Q) when appropriate.

Bemisia tabaci complex is known to harbour seven different vertically-transmitted bacteria (Zchori-Fein & Brown, 2002). Besides *Portiera aleyrodidarum*, the obligatory primary endosymbiont of whiteflies, which has a long co-evolutionary history with all members of the Aleyrodinae (Thao & Baumann, 2004), six additional facultative secondary endosymbionts were detected (Zchori-Fein & Brown, 2002). The interaction of *Fritschea bemisiae*, so far only reported in the New World species of the *B. tabaci* complex (Thao *et al.*, 2003), with its host is as yet uncharacterized. *Hamiltonella defensa*, which induces parasitoid resistance in the pea aphid (Oliver *et al.*, 2010) seems to play a major role in virus transmission of MEAM1 (Gottlieb *et al.*, 2010). Four others (*Wolbachia, Cardinium, Rickettsia, Arsenophonus*) are known to manipulate host reproduction in many different insect species (Hunter *et al.*, 2003; Duron *et al.*, 2008; Saridaki & Bourtzis, 2010), but their effects on populations of the *B. tabaci* complex have yet to be determined. *Rickettsia* seems to play a role in the response to parasitization of the MEAM1 by the wasp *Eretmocerus mundus* (Mahadav *et al.*, 2008). Moreover, it has been shown that this bacterium increased the survival and fecundity of MEAM1 populations in the southwestern United States, leading to its spread in host populations in this area (Himler *et al.*, 2011). These different species of symbionts often co-infect individuals of *B. tabaci* complex, and their community composition is closely related to the host species (Chiel *et al.*, 2007; Gueguen *et al.*, 2010; Skaljac *et al.*, 2010) although differences occur even within host species, as is the case for MEAM1 and *B. tabaci* (Gueguen *et al.*, 2010).

MEAM1 (B biotype) and Q *B. tabaci*, both considered native to the Mediterranean Basin area, are highly invasive and currently occur worldwide (McKenzie *et al.*, 2009, Hu *et al.*, 2011). The examination of the mtDNA polymorphism of Greek *B. tabaci*, both from insular and mainland Greece, had shown a high homogeneity of the COI sequences (Tsagkarakou *et al.*, 2007). In addition, phylogenetic and diagnostic analysis disclosed that the Q and, more specifically, the western Mediterranean Q1 is predominant in Greece (Tsagkarakou *et al.*, 2007; Roditakis *et al.*, 2009). The eastern Mediterranean Q2 has not been found in Greece, whereas the B seems to be restricted to some areas (Papayannis *et al.*, 2008).

Studies on the population genetics, using microsatellites, showed that both Q and B populations of the Mediterranean Basin are highly differentiated (Simón *et al.*, 2007). Microsatellite markers have been used to study the genetic structure of B and Ms populations of the southwest part of the Indian Ocean (Delatte *et al.*, 2006, 2011) and the characterization of at least six Asia-Pacific *B. tabaci* genetic populations with little or no gene flow between them (De Barro, 2005).

An exploratory study using only six *B. tabaci* samples from the island of Crete, Greece, showed that differentiation might be significant even at a small geographic scale, and the Bayesian approach disclosed that the individuals clustered into at least two groups based on their genotypes (Tsagkarakou *et al.*, 2007). However, only six sampling populations, all from Crete, were included in that study, and any attempt to explain the factors affecting the observed structure would have led to speculations. To explore this issue, in the present study, we examined the polymorphism of eight microsatellite loci in a large set of sampling populations. By using populations coming from insular and continental Greece and collected on different plant species grown in both greenhouse and open environments, we addressed the role of the geographic distance, type of the habitat and host plant in shaping the genetic structure of the Q *B. tabaci*. In addition, with focus on *Wolbachia*, we characterized the bacterial symbiotic community known to induce reproductive alterations of host species, and thus possibly affecting (or affected by) the genetic structure of *B. tabaci*.

Material and methods

Whitefly samples

Samples were taken in mainland Greece (ten samples) and on the islands of Crete (26 samples), Santorini (one sample) and Schoinoussa (one sample) between 2002 and 2007 (fig. 1). A detailed record for each population is given in table 1. Of the total of 38 samples, seven were collected on non-cultivated plants (mainly *Ipomoea* sp. and *Solanum nigrum*) and 31 were from cultivated vegetables (mainly melon, cucumber, eggplant, pepper) or non-food crops (cotton and tobacco). Twelve samples, all from Crete, were from greenhouse plants.

A unique identification code was assigned to each collected population and is used for reference along with a three-letter designation: MGr for samples from mainland Greece, SCr for samples from south Crete, NCr for north Crete, ISc for the island of Schoinoussa and ISa for the island of Santorini. Most of the Cretan samples were collected in the south, from the three main greenhouse crop regions, Ierapetra, Arvi and Tympaki, where pest management relies on intensive spraying of neonicotinoids, organophosphates, carbamates and pyrethroids (Roditakis *et al.*, 2009). At each location, adult whiteflies were collected from several plants within the same field (open environments or greenhouses) and stored until use at either −80°C or in 70% ethanol.

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

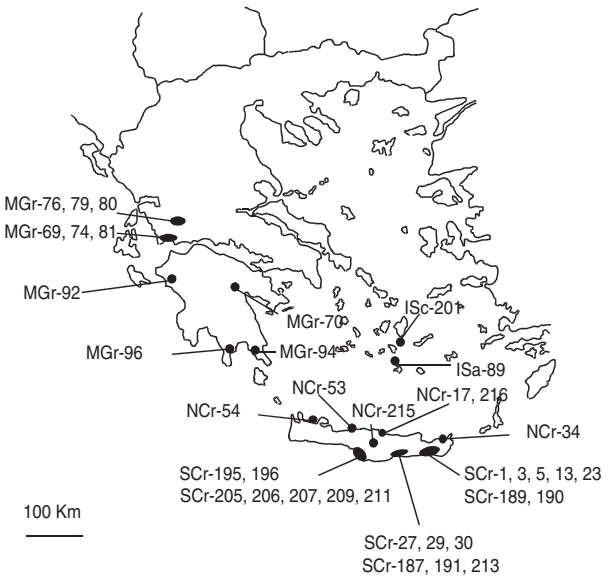

Fig. 1. Geographical sites of *B. tabaci* collections. A unique identification code was assigned to each collected population and is used for reference along with a three-letter designation: MGr for samples from mainland Greece, SCr for samples from south Crete, NCr for north Crete, ISc for the island of Schoinoussa and ISa for the island of Santorini.

Genomic DNA was extracted from individual females as described previously (Tsagkarakou *et al.*, 2007) and used for polymerase chain reactions (PCR).

Microsatellite genotyping

B. tabaci is haplo-diploid: males hatch from unfertilized eggs and are haploid. All genotyping was done using adult, diploid females. Each insect was genotyped at eight variable microsatellite loci. Details on the microsatellite markers used are given in the table 2. Two multiplex PCRs were performed for each individual. The first for loci BT-4, BT-b159 and BT-d26 and the second for loci BT-b34, BT-b155, 11, 145 and 177 with annealing for 45 s at 63°C and 59°C, respectively. One or two µl of DNA extract were used in each 10 µl PCR reaction, containing 0.2 mM dNTPs, 1.6 mM $MgCl_2$, 1 × enzyme buffer (Minotech) and 0.5 units *Taq* polymerase (Minotech). The final primer concentrations and fluorescent labels are shown in table 2. The PCR product was diluted 1/8, one µl from the dilution was mixed with 11 µl of Hi-Di™ formamide and 0.2 µl Genescan Liz 400 size standard (Applied Biosystems), denatured for 5 min at 96°C, and electrophoresed with an ABI 3700 sequencer. Micro-Checker v. 2.2.3 (Oosterhout *et al.*, 2004) was used to evaluate the genotyping results for null-alleles and scoring errors.

From most sampling sites, 20 to 33 individuals were genotyped, resulting in about 1000 individuals for each locus (table 1).

Species identification

Species identity was examined first through the allele size of microsatellite locus BT159, which has been shown to be diagnostic for the B and Q populations and through a PCR-RFLP test to further discriminate between the Q1 (west Mediterranean) and the Q2 (east) populations (Tsagkarakou *et al.*, 2007). The latter assay was performed for all the individuals of the 19 populations included in the endosymbiont study.

Microsatellite data analysis

Linkage disequilibrium, departure from Hardy-Weinberg Equilibrium (HWE), inbreeding coefficient (F_{IS}), expected (H_E) and observed (H_O) heterozygosities, as well as allele frequencies and mean number of alleles per locus were tested/calculated using software GENEPOP, version 4 (Rousset, 2007). When multiple tests were conducted, sequential Bonferroni correction of the *P*-values was performed (Holm, 1979). The distributions of two groups of *H* or F_{IS} estimates were compared by performing a Mann-Whitney *U*-test.

F_{ST} estimates were computed according to Weir & Cockerham (1984). Genotypic differentiation between and among populations were analyzed by computing an unbiased estimate of the *P*-value of a F_{ST} based exact test, as implemented in GENEPOP, version 4. The overall significance of multiple tests for each locus was estimated using the Fisher combined probability test. In addition to *F*-statistics which rely on a predefined population organisation, a model-based method (Pritchard *et al.*, 2000) was used to identify clusters of individuals. This Bayesian approach, implemented in the software STRUCTURE, identifies the number of different subpopulations (*K*) and estimates the ancestry of the sampled individuals on the basis of their genotypes. We used a burn-in of 50,000 Markov Chain Monte Carlo (MCMC), a run length of 1,000,000 MCMC and a model allowing for admixture and correlated allele frequencies. Log-likelihood estimates were calculated for $K = 1$ to 10 with six replicates each. The modal value of ΔK, a quantity based on the second order rate of change of the likelihood function with respect to *K*, was used to detect the number of clusters according to Evanno *et al.* (2005). Finally, the program DISTRUCT was used for graphical display of structure results (Rosenberg, 2002).

Isolation by distance was examined using the regression between $F_{ST}/(1 - F_{ST})$ and the natural logarithm of distance (in kilometres) between sample populations. The null hypothesis of no geographical correlation to genetic divergence was examined by testing the significance of the rank correlation, using a nonparametric one-sided Mantel test based on 10,000 permutations. This was performed with the ISOLDE programme implemented in GENEPOP v. 4 (Rousset, 2007).

The effect of host plant and habitat (greenhouse versus open environment) was examined by performing an analysis of molecular variance (AMOVA) using Arlequin 3.0 (Excoffier *et al.*, 2005) to estimate the different genetic variance components (among groups, among populations within groups, within populations).

Endosymbiont monitoring

To detect the presence of secondary symbionts, 16S rDNA for *Rickettsia*, *Hamiltonella* and *Cardinium* and 23S rDNA for *Arsenophonus* were amplified using genus-specific primers (Chiel *et al.*, 2007 and references therein). The presence of *Wolbachia* was determined using *wsp* primers (Zhou *et al.*, 1998). PCR conditions were as described in Chiel *et al.* (2007) and in Zhou *et al.* (1998). A total of 357 individuals from 19 populations (16–25 females per population with exception of

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

Table 1. Collection information and genetic characteristics of the populations.

Acronym		Locality	Host plant	Collection date					
South Crete					*N*	N_A (±SD)	H_O	H_E	F_{IS}
G	SCr-1	Ierapetra	Eggplant	Feb-2002	27	3 (±1.73)	0.311	0.325	0.042
G	SCr-3	Ierapetra	Zucchini	Feb-2002	24	2.72 (±0.49)	0.318	0.316	−0.007
G	SCr-5	Ierapetra	Eggplant	Feb-2002	30	3.29 (±0.76)	0.326	0.377	0.134**
F	SCr-13	Ierapetra	*Amaranthus retroflexus*	Jun-2002	29	2.86 (±0.90)	0.401	0.373	−0.077
F	SCr-23	Ierapetra	Zucchini	Aug-2002	27	3.29 (±1.11)	0.314	0.366	0.142*
F	SCr-189	Ierapetra	*Solanum nigrum*	May-2007	31	3.14 (±1.21)	0.376	0.393	0.042
G	SCr-190	Ierapetra	Pepper	May-2007	27	3.43 (±1.27)	0.380	0.414	0.083
F	SCr-27	Arvi	Eggplant	Aug-2002	29	2.86 (±0.90)	0.283	0.321	0.119**
F	SCr-29	Arvi	*Ipomoea* sp.	Aug-2002	28	2.86 (±1.57)	0.287	0.285	−0.007
F	SCr-30	Arvi	*Solanum nigrum*	Aug-2002	30	2.57 (±0.79)	0.254	0.314	0.192*
G	SCr-187	Arvi	Cucumber	May-2007	30	3.43 (±1.39)	0.382	0.415	0.079
G	SCr-191	Arvi	Bean	Jun-2007	29	3.28 (±1.11)	0.457	0.448	−0.020
G	SCr-213	Arvi	Cucumber	Sep-2007	29	3.43 (±1.27)	0.396	0.390	−0.014
G	SCr-195	Tympaki	Melon	Jun-2007	26	2.86 (±0.90)	0.240	0.346	0.307**
G	SCr-196	Tympaki	Melon	Jun-2007	31	3.29 (±1.25)	0.326	0.341	0.043
G	SCr-205	Tympaki	Cucumber	Sep-2007	27	3.43 (±1.72)	0.384	0.431	0.111**
F	SCr-206	Tympaki	Tomato	Sep-2007	30	3 (±1.15)	0.345	0.384	0.101
F	SCr-207	Tympaki	Eggplant	Sep-2007	25	3.29 (±0.76)	0.429	0.422	−0.015
G	SCr-209	Tympaki	Pepper	Sep-2007	26	3.86 (±1.07)	0.431	0.427	−0.009
F	SCr-211	Tympaki	Tomato	Sep-2007	29	3.29 (±0.95)	0.371	0.420	0.116***
North Crete									
G	NCr-17	Heraklion	*Ibiscus mutabilis*	Jul-2002	28	3 (±1.41)	0.361	0.387	0.067
F	NCr-34	Siteia	*Ipomoea* sp.	Sep-2002	26	3.71 (±2.06)	0.383	0.412	0.069
F	NCr-53	Rethymno	*Ipomoea* sp.	Jun-2003	26	3.57 (±1.72)	0.299	0.407	0.265***
F	NGr-54	Chania	Melon	Jun-2003	31	4.71 (±1.79)	0.423	0.449	0.058
F	NCr-215	Assimi	Cucumber	Oct-2007	26	3.86 (±1.77)	0.321	0.423	0.242***
F	NCr-216	Heraklion	Lantana	Oct-2007					
Island of Santorini									
F	ISa-89	Santorini	Melon	Jul-2004	29	4.14 (±1.77)	0.415	0.432	0.041
Island of Schoinoussa									
F	ISc-201	Schoinoussa	*Ibiscus mutabilis*	Sep-2007	17	3.14 (±1.46)	0.337	0.444	0.243**
Mainland Greece									
F	MGr-76	Agrinio	Tobacco	Jun-2004	33	3.71 (±1.25)	0.385	0.436	0.116***
F	MGr-79	Agrinio	Eggplant	Jun-2004	32	4.43 (±1.62)	0.382	0.454	0.158***
F	MGr-80	Agrinio	Tobacco	Jun-2004	32	3.86 (±1.22)	0.399	0.427	0.065*
F	MGr-69	Mesolongi	Cotton	Jun-2004	31	4 (±1.53)	0.449	0.506	0.112**
F	MGr-74	Mesolongi	Cotton	Jun-2004	28	3.43 (±0.98)	0.340	0.410	0.17
F	MGr-81	Mesolongi	Zucchini	Jun-2004	31	4.43 (±1.90)	0.407	0.462	0.119***
F	MGr-70	Argos	Tobacco	Jun-2004	29	4.28 (±2.14)	0.414	0.462	0.105*
F	MGr-92	Amaliada	Zucchini	Jun-2004	33	4.71 (±1.89)	0.406	0.487	0.167***
F	MGr-94	Assopos	Eggplant	Aug-2004	33	4.43 (±1.62)	0.420	0.488	0.139***
F	MGr-96	Gythio	Cucumber	Aug-2004	33	4.71 (±1.60)	0.403	0.449	0.103

N, number of individuals genotyped; N_A, mean number of alleles per population; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; G, samples collected in greenhouses; F, samples collected from field cultivations. Significant Hardy-Weinberg departures after Bonferroni's correction are given in bold: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

NCr-23, 11 individuals) were examined by PCR for the presence of the above symbionts.

Wolbachia *characterisation*

Ten individuals randomly chosen from nine *B. tabaci* populations separated genetically and geographically were used for Multi Locus Sequence Typing (MLST) and *wsp* analysis (Baldo *et al*., 2006). Amplification of the genes *gat*B, *cox*A, *hcp*A, *fbp*A, *fts*Z and *wsp* and sequencing were performed using the universal primers described at http://www.pubmlst.org/Wolbachia/.

PCR amplifications were performed in 50 µl reactions containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, each primer at a concentration of 1µM, *Taq* buffer (Minotech), 5 U *Taq* DNA polymerase (Minotech) and 2 µl DNA. Cycling conditions were: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 1.5 min and a final elongation step at 72°C for 10 min. PCR products were purified using the Nucleospin Extract kit (Macherey-Nagel) and both strands directly sequenced (Macrogen, Korea). Editing of the sequences was performed using BioEdit v.7.0 (Hall, 1999). Multiple sequence alignment was carried out using the software package Clustal W v.1.7 (Higgins *et al*., 1996). The sequences are deposited in the GenBank under the accession numbers JQ013512 and JQ013511, for *Wolbachia fts*Z and *cox*A, respectively, JQ013509 and JQ013510 for *Wolbachia gat*B, JQ013507 and JQ013508 for *Wolbachia fbp*A, JQ013513 and JQ013514 for *Wolbachia hcp*A, and JQ013515 and JQ013516 for *Wolbachia wsp*.

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

Table 2. Characteristics of the microsatellite loci used in the population genetic study of *Bemisia tabaci*.

Locus	Genbank accession number	Repeat unit	Primers (5′ to 3′)	Primer concentration (μM)	A	Allele range	*Ho*	H_E
BT-4	AY183673	$(GT)_3(CA)_8$	GAGATCATATCCCCATTGTTTC-Fam ATCACGGGTCATAATCACG	0.60	10	282–316	0.4745	0.5297
BT-b159	AY183681	$(AC)_{12}$	ACTCCATTTGGCTTATGTGC-Ned TTATCGTCTGAAAACTGGTGG	0.20	12	260–286	0.2141	0.2218
BT-b34	AY183675	$(AC)_{10}CCTC(AC)_9$	AAATTAACTGCCGCTCAACG-Vic ATATCGATACAATCTTACCCG	0.80	18	268–314	0.5171	0.6277
BT-b155	AY183680	$(CA)_8(CACCCT)_2(CA)_8$	ACATGTTTCCTTGTCGCACT-Ned ATTAGTGCTCGGTTCGATTC	0.20	11	162–198	0.3343	0.3548
11	AJ866708	(GT)9	CCAGAAAAGTGGACTTAAGA-Fam GATCTGGGTGTTTTCTTCTA	0.30	7	167–199	0.2542	0.2891
145	AM779550	(AC)9	CCTACCCATGAGAGCGGTAA-Vic TCAACAAACGCGTTCTTCAC	0.10	4	171–217	0.3884	0.4356
177	AM779551	(AC)12	AGACCGGCAGAGACCCTAAT-Ned TGATGCTCCTGAATGGAGTG	0.16	16	237–275	0.4169	0.4267
BT-d26	AY183682	$(GA)_{10}$	ACGACACAAATTGGCATTACAT-Vic ACAAGTCAACATCCTCTAGGTA	0.20	12	201–237	0.3178	0.6027

Locus BT-d26 was removed from further studies due to evidence for the presence of null alleles, see results for details.
A, number of alleles; H_O, observed and H_E, expected heterozygosity per locus. Loci BT-4, BT-b159, BT-d26, BT-b34, BT-b155, are from Tsagkarakou & Roditakis (2003), locus 11 from Delatte *et al.* (2006) and loci 145 and 177 are from Dalmon *et al.* (2008). Fluorochroms used for PCR product detection are indicated as Fam, Ned and Vic.

Wolbachia *typing*

Polymorphisms were detected in genes *gat*B, *hcp*A, *fbp*A and *wsp*. Each gene had two allelic forms shared by five individuals, indicating the existence of two distinct *Wolbachia* strains. Sequences from our study, as well as sequences of *Wolbachia* from *Bemisia* in GenBank were aligned and subsequently examined for restriction recognition site with an in-house developed Perl script. For *hcp*A and *gat*B, no restriction sites suitable for routine discrimination between the two strains were found. Enzymes *Alu*I and *Hha*I, respectively, allowed easy distinction between the two allelic forms of *wsp* and *fbp*A.

A 610 bp fragment of the *wsp* gene was amplified using the universal primers 81F and 691R (Zhou *et al.*, 1998) and subsequent digested with *Alu*I. Digestion yields a restriction pattern with nine (27 bp, 23 bp, 24 bp, 48 bp, 50 bp, 54 bp, 84 bp, 93 bp and 207 bp) or ten (27 bp, 23 bp, 24 bp, 48 bp, 50 bp, 54 bp, 55 bp, 84 bp, 93 bp and 152 bp) fragments for the first and second allelic form, respectively. A 512 fragment of *fbp*A gene amplified using primers fbpA_F1 and fbpA_R1 (Baldo *et al.*, 2006) and subsequently digested with *Hha*I yields a restriction pattern with four fragments of 22 bp, 145 bp, 147 bp and 198 bp for the first allelic form and four fragments of 22 bp, 80 bp, 147 bp and 263 bp for the second allelic form.

PCR reactions were performed in 10 μl containing 0.2 mM dNTP, 0.2 μM primers, 0.5 U *Taq* DNA polymerase (DreamTaq, Fermentas) and 1 μl of DNA template. Cycling conditions were the same as for endosymbiont screening (see above). The PCR products were digested at 37°C for 3 h with 5 U of the respective enzyme (Fermentas Life Sciences), then incubated at 65°C for 20 min, and analyzed by electrophoresis on a 2% agarose gel.

The two PCR-RFLP based diagnostic assays were used to determine the prevalence of these *Wolbachia* strains in 19 Greek populations (approximately 12 individuals per population) representative of the geographic distribution of our sampling as well as of the different genetic populations.

The distributions of *Wolbachia* infection frequencies and the two *Wolbachia* strains between populations or groups of populations were compared with Fisher's exact test using the R statistical software (http://www.R-project.org). Mann-Whitney *U*-tests were also employed for some two by two comparisons.

Results

The sampling procedure does not allow discriminating males and females, so mixed sex individuals were collected from the field. However, only females were used in the genotyping and in all the molecular experiments including the monitoring of the symbionts. The selection of the female individuals was done under a binocular stereoscope based on the size and the shape of the posterior part of their bodies. This procedure revealed that in all of our samples both sexes were present.

Species identification

Among 38 Greek *B. tabaci* populations, the MEAM1 discriminative allele 268 of locus BT159 (called 273 in Tsagkarakou *et al.*, 2007) was found in 28 of 30 individuals from population NCr-216 collected on *Lantana camara* in a garden in north Crete. NCr-216 had also unique alleles for loci

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

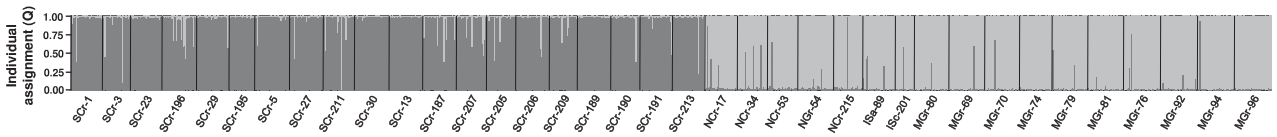

Fig. 2. Results of STRUCTURE clustering analysis ($K=2$) of 1072 *B. tabaci* belonging to 37 sampling populations. Individuals are presented by vertical lines, divided into segments that represent their inferred membership into each of the two clusters.

b34 and 145, whereas PCR primers for loci d26, 155 and 11 failed to amplify. The PCR-RFLP assay further demonstrated that population NCr-216 was a B population of MEAM1, and thus it was eliminated from further analysis. All other populations belonged to the Q *B. tabaci*. The PCR-RFLP assay, which discriminates between populations Q1 and Q2 (Tsagkarakou *et al.*, 2007), was performed for 357 individuals of 19 populations, in which the bacterial community was also studied. All were of the Q1 *B. tabaci*. This confirms previous findings (Tsagkarakou *et al.*, 2007; Roditakis *et al.*, 2009) that Q1 are the predominant *B. tabaci* in Greece.

Genetic polymorphism of the microsatellite loci

In all, 1134 females were genotyped at eight microsatellite loci. Heterozygote deficiency was especially high for locus BT-d26 in almost all populations (33/37). For this locus, analysis with Micro-Checker revealed the presence of null alleles. Thus, this marker was excluded from further analysis. Sixty-two individuals amplified for fewer than four loci and were removed from the analysis. The polymorphism of seven microsatellite markers for 1072 females belonging to 37 sampling populations were further analyzed. Mean number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities as well as F_{IS} values are given in table 1.

The number of alleles over all individuals ranged from four for locus 145 to 18 for locus BT-b34. The mean number of alleles per sample ranged from 2.57 ± 0.79 to 4.71 ± 1.81. The mean number of alleles was significantly lower in Crete ($n=25$, range from 2.57 to 4.71) than in mainland Greece, including the ISa and ISc samples ($n=12$, range from 3.42 to 4.71) as assessed by the Mann-Whitney test $U=343$, $P<0.001$.

H_O values ranged from 0.24 to 0.45 and H_E ranged from 0.285 to 0.506. In seven out of 37 populations, $H_O \geq H_E$, resulting in negative F_{IS}. Significant heterozygote deficits were found in 18 of the 37 populations. Most of the samples from mainland Greece (8/10) had significant heterozygote deficits. F_{IS} values were also significantly lower in Crete ($n=25$) compared to mainland Greece ($n=10$) as assessed by the Mann- Whitney test $U=235$, $P=0.042$.

In order to test whether high F_{IS} are associated with the sampling period, the relationship between significant heterozygote deficits and the colonization phase of the crop was investigated: samples collected during September–October for greenhouse crops and May–June for field crops were considered to be part of the early colonization phase. It was found that there were no significant differences (Fisher's exact test: $P=0.191$) between the samples collected early and after establishment on the crops.

Among the 720 tests performed for linkage disequilibrium, 25 were significant (in 17 populations) but none remained significant after the Bonferroni correction. Thus, the seven microsatellite loci carried independent genetic information.

Population structure

Bayesian clustering analysis of multilocus genotypes with STRUCTURE identified at least two clusters ($K=2$): lnP(D) increased from $K=1$ to $K=4$ and reached a plateau for $K>4$. The smallest value of K that captured the major structure in the data is 2. Most individuals (1005/1072) are clearly assigned to one of the two clusters ($Q>90$) (fig. 2). The first cluster contains only the individuals from mainland Greece, the islands of Santorini and Schoinoussa and north Crete. The second genetic cluster contains only samples from south Crete. In terms of allelic frequencies, the most striking difference between the two clusters concerns locus Bt-155. Allele 198 of this locus has in the first cluster a frequency of 0.001 and in the second cluster a frequency of 0.708, while allele 196 is found with a frequency of 0.001 in the second and with a frequency of 0.644 in the first cluster. Only a few individuals (67/1072) had admixed ancestry.

Gene flow in relation to geographic distribution

Genetic differentiation was analyzed by relating genotypic distribution with F_{ST} estimates. Differentiation was highly significant ($P<10^{-5}$) over all samples ($F_{ST}=0.15$) (table 3), as well as among samples collected in Crete ($F_{ST}=0.13$). The same was true for samples from south Crete $F_{ST}=0.1$, $P<10^{-5}$. Significant differentiation ($P=0.04$) was also found for samples collected in mainland Greece; however, there was more gene flow than among Cretan samples with lower F_{ST} ($F_{ST}=0.0046$). Among the ten samples from mainland Greece, only MGr-69 was significantly different from samples MGr-76, 92 and 94 ($F_{ST}=0.014$, 0.018, 0.017; $P=0.012$, 0.044, 0.012, respectively). All other samples from mainland Greece were not differentiated from each other, with pairwise F_{ST} ranging from -0.0001 to 0.018 and $P>0.05$, even where geographic distances exceed 500 km. For the Cretan samples, however, significant differentiation ($P<0.05$) was identified in most of the pairwise comparisons (260/300). The highest level of differentiation among the Crete populations, and at the same time among all pairwise comparisons in this study, was found between samples SCr-190 and SCr-29 ($F_{ST}=0.331$, $P<0.001$).

Isolation by distance

Mantel tests were performed to assess isolation by distance. The relationship between gene flow and geographic distance was analyzed by examining the slope of the regression function between multilocus pairwise estimates of $F_{ST}/(1-F_{ST})$ and log (geographic distance), computed for each pair of samples. We analyzed (i) all sampled populations of *B. tabaci*, (ii) all populations from Crete, (iii) all populations from mainland Greece and (iv) all populations from south Crete. For all populations, the slope of the regression was

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

Table 3. F_{ST} estimates for population subdivisions of *B. tabaci* at different geographic scales.

	N^2	F_{ST}[1]							
		BT-4	BT-b159	BT-b34	BT-b155	11	145	177	All[3]
All samples	37	0.0094	0.0514***	0.0777***	0.5248***	0.0073**	0.0096	0.0732***	0.154***
Continental Greece	9	−0.0075	0.0195**	−0.0046	0.0142*	0.0023	0.0029	0.0141	0.0046*
All Crete	25	0.0057*	0.0739***	0.0137***	0.5253***	0.0110**	0.0075	0.0093***	0.1268***
South Crete	20	0.0073*	0.0737***	0.0104***	0.5427***	−0.0016	0.0059	0.0075***	0.1001***

[1] Estimated by the estimator of Weir & Cockerham (1984).
[2] Number of samples considered.
[3] "All" refers to the multi-locus estimate.
Significant genotype differentiation: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Table 4. Analysis of molecular Variance (AMOVA) for *B. tabaci* using seven microsatellite loci (1) among all populations, (2) between the two groups identified by STRUCTURE, (3) among three groups of plants (see text for details) and (4) between greenhouses versus field samples (only south Cretan samples are considered).

	Source of variation	df	Sum of squares	Variance components	Percentage of variation	Statistics	*P*-value
1	Among populations	36	482.110	0.21	15.28	$F_{ST}=0.15278$	0
	Within populations	2107	2465.219	1.17	84.72		
2	Among groups	1	272.97	0.25	16.62	$F_{CT}=0.16621$	0
	Among populations within groups	35	209.13	0.083	5.52	$Fsc=0.06623$	0
	Within populations	2107	2465.219	1.17	77.86	$F_{ST}=0.22144$	0
3	Among groups	2	33.75	0.0067	0.48	$F_{CT}=0.0047$	0.258
	Among populations within groups	29	368.866	0.209	14.90	$Fsc=0.14975$	0
	Within populations	1828	2169.99	1.187	84.62	$F_{ST}=0.15379$	0
4	Among groups	1	8.39	−0.00221	–0.18	$F_{CT}=-0.00181$	0.44
	Among populations within groups	23	228.014	0.15650	12.83	$Fsc=0.12803$	0
	Within populations	1389	1480.527	1.06589	87.36	$F_{ST}=0.12645$	0

positive (b = 0.024) with a significant positive rank correlation between $F_{ST}/(1-F_{ST})$ and (ln) geographic distance (Mantel test, $P<0.001$). The significant positive correlation remains (b = 0.01281, $P<0.003$) for the samples from mainland Greece and north Crete, which clustered together in the same genetic group (see above). However, when considering only samples from mainland Greece ($n=10$) or only samples from Crete ($n=23$) there is no significant correlation: b = −0.0001, $P=0.34$ and b = 0.0094, $P=0.13$ for mainland Greece and Crete, respectively. These results indicate that gene flow between populations decreases with geographic distance but that, at a smaller geographic scale, factors other than geographic isolation dominate in shaping the genetic structure.

The role of host plant species and habitat in B. tabaci *differentiation*

When considering all populations, AMOVA analysis showed that the variance within populations explained 84.72% of the total variance, while the rest was due to variance among populations (table 4). Considering the two genetic groups, AMOVA showed that 16.62% of the variance was attributed to differences between these two clusters and 5.52% to differences among populations in the two clusters.

Host plant adaptation should restrict gene flow between populations colonizing different plant species. Similarly, adaptation to specific conditions in greenhouses should restrict gene flow between populations inhabiting greenhouses and populations in the open environment. To determine the impact of the host plant on the genetic affinities of the *B. tabaci* samples, an analysis based on clusters of the three representative families of host plants sampled in this study was performed. When considering the three groups with respect to the three host plant families from which *B. tabaci* was mainly sampled (*Solanaceae*: 16 samples, *Curcubitacea*: 12 samples and *Malvacea*: four samples) or to the habitat type (only for the Cretan populations, 12 samples from greenhouses versus 11 samples from open environment), the genetic variability among groups accounted only for the 0.48% (host plant) and −0.18% (greenhouse/open environment) of the global genetic diversity and the fixation index among groups (*Fct*) was not significant ($Fct=0.0047$, $P=0.258$; and $Fct=-0.00181$, $P=0.44$), suggesting that populations were still structured within at least one group. This indicates that neither host plant species nor the habitat type play a role in the genetic differentiation in our samples.

The results of the AMOVA confirmed the presence of structure in our samples, which is not associated with the host plants or the habitat type.

Presence of secondary symbionts

The presence of secondary symbionts in 309 individuals from 19 populations was examined. All individuals were

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

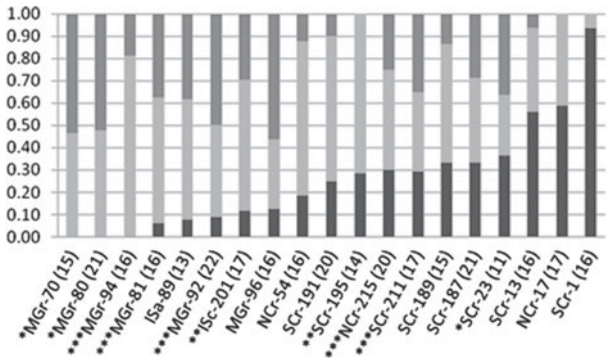

Fig. 3. Frequency of *Wolbachia* negative and of *Wolbachia* strain W1 and *Wolbachia* strain W2 in 19 populations of *B. tabaci* from Greece. Populations are ranked with increasing frequency of W1. The number of individuals tested for the presence of *Wolbachia* is given in brackets after the name of the population. Significant heterozygote deficits are given before the name of each population: $*P<0.05$, $**P<0.01$, $***P<0.001$; ■, not infected; ■, W2; ■, W1.

associated with *Hamiltonella*, while there was no evidence for the presence of *Arsenophonus*, *Rickettsia* or *Cardinium*. The three reactions failed to produce amplification products with the test insects but produced successful amplification with known positive controls (Q2 from Israel for *Arsenophonus*, and *Rickettsia* and Q1 from Spain for *Cardinium*). We partially sequenced the PCR products of the 16S ribosomal RNA gene of *Hamiltonella* from six *B. tabaci* individuals from different localities, finding no polymorphism among individuals. The sequences were identical to other *Hamiltonella* sequences already published.

The prevalence of *Wolbachia* varied among populations from 45% to 100% (average: 75%; fig. 3). The variation in *Wolbachia* prevalence was significant when all the 19 populations were considered separately (Fisher's Exact test, $P<0.0005$) but also when populations from the same region (SCr, NCr, MGr) were grouped together ($P<0.0005$). *Wolbachia* prevalence is lower in mainland Greece (average 59%) than in south (84%) or north (91%) Crete ($P<0.0001$). The difference between south and north Crete is not significant ($P=0.47$). Within regions, the highest variability of *Wolbachia* prevalence among populations is found in south Crete ($P=0.008$), while differences are not significant among populations in north Crete or in mainland Greece ($P>0.08$).

Wolbachia *characterisation*

Ten individuals randomly chosen from nine *B. tabaci* populations separated genetically and geographically were used for MLST and *wsp* analysis (Baldo *et al.*, 2006).

Among the six genes tested, no polymorphism was detected for *cox*A and *fts*Z, while the others (*gat*B, *hcp*A, *fbp*A, *wsp*) showed two allelic forms, each shared by five individuals, indicating the presence of two distinct *Wolbachia* strains (table 5). However, we can't exclude that other strains are present in these populations. Indeed, as PCR products were directly sequenced, in case of multiple infections, a strain in very low proportion may not be detected.

Comparison against the multilocus sequence typing (MLST) database (http://pubmlst.org/Wolbachia/) and the *wsp* database showed that strain W1 carries alleles 9, 88, 13, 7, 9 and 160 of loci *gat*B, *cox*A, *hcp*A, *fts*Z, *fbp*A and *wsp*, respectively. The second stain (W2) carries alleles 105, 88, 106, 7, 165 and 535 of loci *gat*B, *cox*A, *hcp*A, *fts*Z, *fbp*A and *wsp*, respectively. Phylogenetic analysis based on the *fts*Z sequences indicated that the strains belong to the *Wolbachia* group B, as previously described *Wolbachia* strains found in *B. tabaci* (Nirgianaki *et al.*, 2003).

Typing of Wolbachia

Digestion with *Alu*I of a 610 bp fragment of the *wsp* gene yielded two distinct restriction patterns discriminative for W1 and W2, respectively (fig. 4). Similarly, digestion with *Hha*I of a 512 bp fragment of the *fbp*A gene yielded two distinct restriction patterns discriminative for W1 and W2 (see fig. 4).

This PCR-RFLP diagnostic test was applied to 233 infected individuals from 19 populations to determine *Wolbachia* infections. For half of the individuals (randomly chosen), PCR-RFLP were done with the two marker-enzyme pairs. Since we always found the same *wsp* and *fbp*A alleles to be coupled, the other half of samples was only tested with *fbp*A/*Hha*I.

We did not detect double infections in any of the individuals. However, we cannot exclude double infections in which one of the co-infecting strains is strongly underrepresented.

The two strains did not occur with equal frequency: W2 was present in 68% of the *Wolbachia*-infected *B. tabaci* individuals. This varied from 6% to 100% in different populations (Fisher's Exact test, $P<0.0005$) and was significant different between MGr and SCr or NCr ($P<0.0005$). The highest frequency of W2 was found in mainland Greece (90.5%). In south and north Crete the frequencies were 50.3% and 59.9%, respectively, but not significantly different ($P=0.38$). Within each of the three regions, the differences are significant only in SCr ($P=0.002$), $P>0.09$ for MGr and NCr. In Crete, the strain W1 was found in higher frequency in samples collected in 2002 (table 1 and fig. 3), but its presence decreased in samples from subsequent years.

Infection patterns in relation to the population structure

There was no significant correlation between either F_{IS} and the frequency of *Wolbachia*-infected whiteflies ($R=0.148$, $P=0.5456$) or F_{IS} and the prevalence of the one or the other *Wolbachia* strains ($R=-0.2945$ and $R=0.2412$ for strains W1 and W2, respectively; $P>0.05$). When the distributions of *Wolbachia* infection and *Wolbachia* strains are compared between populations with significant HW departures and populations that are under HW equilibrium, no differences in the frequency of infected individuals were found (mean infection frequency in populations with significant heterozygote deficits $=66.19\pm16.59$, $n=10$, and in populations under HW equilibrium $=81.63\pm19.02$, $n=9$; $U=99$, $P=0.22$); however, a significantly lower prevalence of *Wolbachia* strain W1 is found in the populations with heterozygote deficits (mean frequency of W1 in populations with significant heterozygote deficits $=15.15\pm13.08$, $n=10$, and in populations under HW equilibrium $=37.71\pm27.45$, $n=9$; Fisher's Exact test, $P<0.0005$).

The two different genetic groups (populations SCr versus populations MGr/NCr) have distinct infection patterns: (i) in the first group there is a higher frequency of *Wolbachia*-infected

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

Table 5. Results of the MLST analysis as disclosed by the partial sequences of five genes obtained for ten *B. tabaci* individuals.

Individual	*gat*B	*cox*A	*hcp*A	*fts*Z	*fbp*A	*wsp*	HRV1	HRV2	HRV3	HVR4
ISc-89.21	9	88	13	7	9	160	2	17	3	23
SCr-187.16	9	88	13	7	9	160	2	17	3	23
SCr-1	9	88	13	7	9	160	2	17	3	23
SCr-13	9	88	13	7	9	160	2	17	3	23
NCr-34.2	9	88	13	7	9	160	2	17	3	23
NCr-54	105	88	106	7	165	535	159	17	89	23
MGr-70	105	88	106	7	165	535	159	17	89	23
ISc-89	105	88	106	7	165	535	159	17	89	23
SCr-189	105	88	106	7	165	535	159	17	89	23
MGr-96.36	105	88	106	7	165	535	159	17	89	23

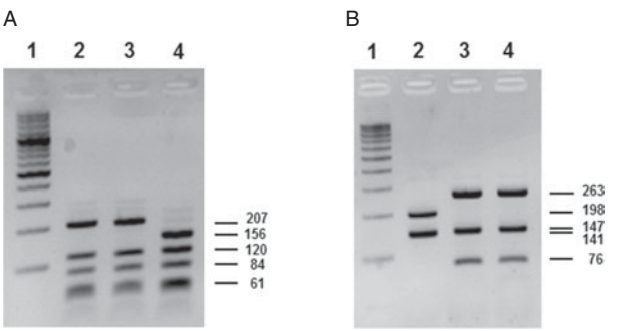

Fig. 4. PCR-RFLP assays to discriminate between the two *Wolbachia* strains. (A) Couple marker-enzyme *wsp*/*Alu*I. 1, 100 bp Ladder; 2, 3, strain W1; 4, strain W2. The strains W1 and W2 can be distinguished by a band at 207 and 156 bp, respectively. (B) Couple marker-enzyme *fbp*A/*Hha*I. 1, 100 bp ladder; 2, 3, strain W1; 4, strain W2. The strains W1 and W2 can be distinguished by a band at 263 and 198 bp, respectively. Note that all other fragments <60 bp expected after digestion (22 bp, 23 bp, 24 bp, 27 bp, 48 bp, 50 bp, 54 bp and 55 bp) were not detected as they were too small to be visualized by electrophoresis in a routine agarose gel.

individuals compared to the second genetic group (Fisher's Exact test, $P = 0.003$), with mean infection in the MGr/NCr group of 66.04 ± 18.57 and mean infection in the SCr group of 83.77 ± 15.09; and (ii) the two genetic groups differ in relation to the *Wolbachia* strain prevalence (Fisher's Exact test, $P < 0.0005$). The latter is due to the lower frequency of *Wolbachia* strain W1 in the MGr/NCr samples (mean frequency of infected individuals with strain W1 per population is 14.08 ± 17.34, $n = 11$) compared to the south Cretan samples (mean frequency of infected individuals with strain W1 per population = 42.00 ± 22.96, $n = 8$).

However, when looking at pairwise comparisons of populations, there is no clear influence of *Wolbachia* on gene flow. For example, population SCr-195, infected mostly with the W2 strain (fig. 3), is genetically closer to SCr-1, which is mostly infected with W1 ($F_{ST} = 0.036$, $P = 0.0043$) than to population SCr-191 with a similar infection pattern ($F_{ST} = 0.2212$, $P < 0.001$).

Discussion

The genetic structure of *B. tabaci* from mainland and insular Greece was studied by investigating polymorphic microsatellite loci. Thirty-seven out of 38 populations were assigned to the Q *B. tabaci*, which is predominant in this geographic area (Tsagkarakou *et al.*, 2007; Roditakis *et al.*, 2009). All Q individuals examined belonged to the west Mediterranean Q1 populations (Tsagkarakou *et al.*, 2007). Strong genetic differentiation was found among the sampled populations of the whole data set. A Bayesian approach disclosed that the individuals clustered into at least two groups (G1 and G2) based on their genotypes. Two genetic populations in Greek samples were also found in a previous exploratory work (Tsagkarakou *et al.*, 2007); however, due to the limited number of samples, no correlation to the geographic origin of the samples was possible. In the present study, we found that *B. tabaci* were highly differentiated and displayed a strong geographic structure: those from south Crete all formed one genetic group (G1), while those from north Crete were in the same cluster (G2) together with samples from mainland Greece and from the islands Schoinoussa and Santorini. Within G1, the *B. tabaci* populations were highly differentiated. Populations within G2 were also differentiated; however, the differentiation within the populations from mainland Greece was the lowest observed, even when separated by more than 100 km. These results show that *B. tabaci* populations from Greece are genetically structured.

Host plant

The formation of sympatric differentiated populations as a consequence of host plant adaptation to plant species or even at the finer level of individual plant genotypes has been suggested for phytophagous insects (Mopper, 1996). *B. tabaci* is one of the few whitefly species associated with herbaceous hosts with an ability to attack multiple crop, weed and ornamental hosts. The different performance on diverse host species has been attributed to different biotypes (Maruthi *et al.*, 2004) and might be in the origin of the genetic differentiation found among *B. tabaci* populations (De Barro, 2005). In our study, considering the family level, the analysis of molecular variance indicates that the differentiation does not originate in the different host plants colonized. Gene flow was not higher between populations collected on species belonging to the same plant family compared to gene flow between populations from different plant families.

Habitat

Populations from south Crete were sampled from greenhouses and plants in the open environment. There was significant differentiation among them, even between

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

populations separated by only a few meters (e.g. pop SCr189 and SCr190 (F_{ST} = 0.035). However, the genetic differences were not explained by the different habitats. The analysis of molecular variance showed no correlation of the type of habitat with the molecular differences between samples from southern Crete. Populations from greenhouses were not genetically closer among them than to *B. tabaci* from outside greenhouses. Due to the mild weather (minimum temperatures are often above 10°C in winter), populations of *B. tabaci* can be found in southern Crete all year, infesting vegetable crops and weeds. During the six colder months (November–April), when cultivation occurs mostly in greenhouses, *B. tabaci* invades protected crops but can also be found outside greenhouses on weed hosts. During the six summer months, it infests the outdoor cultivations. This continuous cycle does not permit a homogeneous 'greenhouse' or 'outdoor' population to be formed. On the other hand, the repetitive colonisations promote genetic differences due to genetic drift. This phenomenon is accentuated in south Crete due to the intensive use of insecticide treatments, which induces large fluctuations of population sizes.

Intense selection through extensive *Bacillus thuringiensis* applications used in some greenhouses significantly impacted the genetic structure of *Trichoplusia ni* populations in Canada and resulted in a strong positive correlation between *Bt* resistance, and genetic differentiation (Franklin *et al*., 2010). A similar relationship was found in the French codling moth, *Cydia pomonella*, where the mean number of insecticide sprays was positively correlated with levels of genetic differentiation (Franck *et al*., 2007). In southern Crete, where *B. tabaci* control relies on intensive insecticide use and high resistance levels are found (Roditakis *et al*., 2009), the frequency of the different resistant alleles varied, underlying the presence of differentiated populations (Tsagkarakou *et al*., 2009).

Geographic distance

Significant isolation by distance is found when the whole data set is considered; increase of geographic distance decreases gene flow between populations. However, populations from northern Crete are genetically closer to those from mainland Greece than those from southern Crete. Also, the absence of isolation by distance when considering only samples from mainland or only samples from Crete indicate that factors other than the geographic distance play a major role in the genetic structure of *B. tabaci* in Greece. The landscape fragmentation due to the mountain range in the middle of Crete may contribute to the differences between samples from northern Crete and southern Crete. Landscape fragmentation does not play a significant role in mainland Greece since samples collected there formed almost a panmictic population (except one sample). The absence of high differentiation in mainland Greece is in accordance with the findings of Dalmon *et al*. (2008) for greenhouse *B. tabaci* in France where recent colonization of the species, together with human activities, have been evoked to explain the high F_{ST} among samples. However, conditions are different for *B. tabaci* in mainland Greece where the species has been established at least since 1889, when it was first described by Gennadius (1889). The homogenization observed in mainland Greece may be due to the fact that *B. tabaci* inhabits mainly outdoor cultivations and not greenhouses, where *Trialeurodes vaporariorum* remains the main whitefly pest. Also, members of the *B. tabaci* complex can disperse over long distances despite its small size. Dispersal is not entirely passive and may not be considered weak since it is capable of sustaining flight in the field for distances of up 7.0 km in a 12-h period (Byrne, 1999).

Before 2000, *B. tabaci* was not considered an important pest in greenhouses in Crete (Roditakis N, personal communication). Since then, it has replaced *T. vaporariorum* (at least in south Crete) and is currently considered the major pest in combination with virus transmission outbreaks. One hypothesis explaining this situation is that a particular genetic population of *B. tabaci* has been installed in southern Crete, which does not move much since suitable host plants and climate conditions are present year round. Under these conditions, southern Cretan populations of *B. tabaci* would rarely move more than a few meters although their dispersal capacities are higher. This, together with the intensive bottlenecks imposed by the repetitive heavy pesticide applications in the confined environment of greenhouses could explain the absence of isolation by distance in Crete, as well as the high differentiation among samples due to genetic drift.

Secondary symbionts

Insects have established symbiotic associations with diverse groups of bacterial species. These associations affect various aspects of insect host biology, including development, nutrition, reproduction, speciation, immunity, vector competence, as well as host preference (Moran *et al*., 2008). We tested for the presence of *Hamiltonella, Rickettsia, Wolbachia, Cardinium* and *Arsenophonus* in Greek populations of Q1 *B. tabaci*. Only *Hamiltonella* and *Wolbachia* were present, while *Rickettsia, Cardinium* and *Arsenophonus* were absent from all individuals tested. *Hamiltonella* infection is fixed in all populations, while only a patchy distribution of *Wolbachia* was observed.

The role of *Hamiltonella* in the Greek populations of *B. tabaci* is currently unknown. In aphids, the presence of *Hamiltonella* (and its APSE phage) provides protection against natural enemies (Oliver *et al*., 2010); however, in the present study, we did not examine whether the *Hamiltonella* of the Greek *B. tabaci* is infected by the APSE phage. Gottlieb *et al*. (2008) reported that *Hamiltonella* was located within the bacteriocytes of *B. tabaci* and MEAM1 together with the primary symbiont *Portiera*. Whether *Hamiltonella* is acting as a co-primary symbiont or is competing with *Portiera* is still under question (Gottlieb *et al*., 2008). Alternatively, these two endosymbionts may have a complementary metabolic role, like *Buchnera aphidicola* and *Serratia symbiotica* in tryptophan provision in the aphid *Cinara cedri* (Gómez-Valero *et al*., 2004).

It is also worth noting that the presence of *Hamiltonella* and the expression of its GroEL protein were recently correlated with the transmission efficiency of *Tomato Yellow Leaf Curl Virus* (TYLCV) by MEAM1 (Gottlieb *et al*., 2010). Furthermore, a mutualistic relationship between plant virus and the insect host has been shown in MEAM1 from China, which increased their fecundity, longevity and population density when feeding on Begomovirus-infected plants (Jiu *et al*., 2007). If such an impact on host phenotype applies in the case of TYLCV and members of the *B. tabaci* complex, this fitness benefit could indirectly explain the high prevalence of *Hamiltonella* associated with TYLCV transmission efficacy. This hypothesis cannot explain on its own the 100% fixation of *Hamiltonella* in the present study. Jiu *et al*. (2007) showed that the mutually beneficial relationships between the invasive whitefly and the plant viruses are indirect via the plants;

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

however, our study included also *B. tabaci* from plants which are not infected by TYLCV.

It is known that the presence of *Wolbachia* in insects is usually involved in the manipulation of the host's reproductive system, including feminization, parthenogenesis, male killing and cytoplasmic incompatibility (Saridaki & Bourtzis, 2010). *Wolbachia* was detected in Greek populations of *B. tabaci*. Although the exact sex ratio was not determined in each population studied here, it is worth mentioning that both sexes were present in the tested *B. tabaci*. The prevalence of *Wolbachia* varied between populations and localities, with the lowest abundance in mainland Greece. Previous studies have reported the presence of *Wolbachia*, as a single infection, in natural populations of the *B. tabaci* complex (Nirgianaki *et al*., 2003; Li *et al*., 2007). Gueguen *et al*. (2010) recently reported the presence of the so-called W1 *Wolbachia* strain in Q1 populations, while the W2 *Wolbachia* strain is associated with the Q2 from Israel. However, no individual of *B. tabaci* has ever been reported to be double- or multiple-infected. We report for the first time the presence of two *Wolbachia* strains (W1 and W2) in the same Q1, at the population but not at the individual level. The apparent lack of double infections could be due to: (i) the presence of a *Wolbachia* strain at a low, PCR non-detectable infection level. Such low-titer *Wolbachia* strains have been recently described in different insect host species (Arthofer *et al*., 2009) and (ii) the recent invasion of a novel *Wolbachia* strain into previously infected natural populations. Indeed, our data suggest that hypothesis (ii) may be valid. Although, in general, W2 was the most frequent strain in infested individuals from mainland Greece and W1 the most frequent strain in infested individuals from south Crete, there is no clear association of the two *Wolbachia* strains (W1 and W2) with the two genetic groups (SCr and MGr/NCr). However, two findings suggest a possible role of the W2 *Wolbachia* strain in the genetic structure of the Greek populations of *B. tabaci* Q1: (i) *Wolbachia* strain W1 is present at a low frequency in populations with significant heterozygote deficiency (MGr/NCr genetic group) compared to the Hardy-Weinberg equilibrium populations (SCr genetic group) and (ii) *Wolbachia* strain W2 is more prevalent than strain W1 in all populations studied, except in four populations (SCr-23, 13, 1 and NCr-17) from southern and northern Crete. It is important to note here that these four samples are the oldest included in our survey and were originally collected in 2002. If the *Wolbachia* strain W2 is incompatible with strain W1 (the 'ancient' strain), then our data are consistent with the scenario of a replacement of W1 by W2 in Greek populations of Q1 *B. tabaci*. Compatibility assays, as well as monitoring of the *Wolbachia* strains W1 and W2 in a *B. tabaci* collection over time, are both required in order to evaluate the CI properties of these strains.

It was recently shown that *Wolbachia* is present both inside and outside bacteriocytes of Israeli populations of Q *B. tabaci*, presumably Q2, which is known to harbor the *Wolbachia* strain W2 (Gottlieb *et al*., 2008; Gueguen *et al*., 2010). Another recent study showed that *Wolbachia* is confined inside bacteriocytes in Croatian populations of Q *B. tabaci* (Skaljac *et al*., 2010). It is known that both Q1 and Q2 *B. tabaci* exist in Croatia (Dinsdale *et al*., 2010); however, neither the whitefly populations nor the *Wolbachia* strain(s) present in them were genotyped (Skaljac *et al*., 2010) to know if the outside- and/or inside-bacteriocyte localization pattern is a specific genetic property of a particular *Wolbachia* strain or symbiotic association. This question, as well as the mode and efficiency of transmission of W1 and W2 *Wolbachia* strains, are currently under investigation in our laboratories. Given the fact that recent reports suggest dynamic interactions of *Wolbachia* with different viruses (Bian *et al*., 2010) the role of both W1 and W2 *Wolbachia* strains in the transmission of plant viruses by Q *B. tabaci* also requires a thorough analysis.

In conclusion, we show that among Q1 *B. tabaci* gene flow is often very restricted. Bayesian analysis suggests that whiteflies from Greece group in two genetic populations; geographic and anthropogenic parameters seem to play a major role in this grouping. The high prevalence of *Hamiltonella* and *Wolbachia* may not be related with the occurrence of these two genetic groups; however, the role of the *Wolbachia* strains W1 and W2 in the shaping of the population genetic structure has to be examined in a broader *B. tabaci* sampling and by performing reproductive compatibility assays.

Acknowledgements

We thank Hélène Henri for her valuable contribution in performing the PCR-RFLP diagnostic assay and Dr Antonis Magoulas for giving us access to sequencing facilities in the Marine Biology and Genetics Institute, HCMR, Heraklion. This work was partially supported by a General Secretariat of Research and Technology bilateral Greece-France (229-E) to AT and by EU CSA-REGPROT 203590 – MicrobeGR and intramural funds of the University of Ioannina to KB. KB also benefited by travel grants from EU Cost Action FA0701: 'Arthropod Symbioses: From Fundamental Studies to Pest and Disease Management'.

References

Arthofer, W., Riegler, M., Schneider, D., Krammer, M., Miller, W.J. & Stauffer, C. (2009) Hidden *Wolbachia* diversity in field populations of the European cherry fruit fly, *Rhagoletis cerasi* (Diptera, Tephritidae). *Molecular Ecology* **18**, 3816–3830.

Baldo, L., Dunning Hotopp, J.C., Jolley, K.A., Bordenstein, S.R., Biber, S.A., Choudhury, R.R., Hayashi, C., Maiden, M.C., Tattelin, H. & Werren, J.H. (2006) Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. *Applied and Environmental Microbiology* **72**, 7098–7110.

Bian, G., Xu, Y., Lu, P., Xie, Y. & Xi, Z. (2010) *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLos Pathogens* **6**, e1000833.

Byrne, D..N. (1999) Migration and dispersal by the sweet potato whitefly, *Bemisia tabaci*. *Agricultural and Forest Meteorology* **97**, 309–316.

Chiel, E., Gottlieb, Y., Zchori-Fein, E., Mozes-Daube, N., Katzir, N., Inbar, M. & Ghanim, M. (2007) Biotype dependent secondary symbiont communities in sympatric populations of *Bemisia tabaci*. *Bulletin of Entomological Research* **97**, 1–7.

Dalmon, A., Halkett, F., Granier, M., Delatte, H. & Peterschmitt, M. (2008) Genetic structure of the invasive pest *Bemisia tabaci*: evidence of limited but persistent genetic differentiation in glasshouse populations. *Heredity* **100**, 316–325.

De Barro, P.J. (2005) Genetic structure of the whitefly *Bemisia tabaci* in the Asia-Pacific region revealed using microsatellite markers. *Molecular Ecology* **14**, 3695–3718.

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press

De Barro, P.J., Liu, S.-S., Boykin, L.M. & Dinsdale, A.B. (2011) *Bemisia tabaci*: A Statement of Species Status. *Annual Review of Entomology* **56**, 1–19.

Delatte, H., David, P., Granier, M., Lett, J.M., Goldbach, R., Peterscmitt, M. & Reynaud, B. (2006) Microsatellites reveal extensive geographical, ecological and genetic contacts between invasive and indigenous whitefly biotypes in an insular environment. *Genetical Research* **87**, 109–124.

Delatte, H., Holota, H., Warren, B.H., Becker, N., Thierry, M. & Reynaud, B. (2011) Genetic diversity, geographical range and origin of *Bemisia tabaci* (Hemiptera: Aleyrodidae) Indian Ocean Ms. *Bulletin of Entomological Research* **101**, 487–497.

Dinsdale, A., Cook, L., Riginos, C., Buckley, Y.M. & De Barro, P. (2010) Refined global analysis of *Bemisia tabaci* (Hemiptera, Sternorrhyncha: Aleyrodoidea, Aleyrodidae) Mitochondrial cytochrome oxidase 1 to identify species level genetic boundaries. *Annals of the Entomological Society of America* **103**, 196–208.

Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J. & Hurst, G.D. (2008) The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. *BMC Biology* **6**, 27–39.

Evanno, G., Regnaut, S. & Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE, a simulation study. *Molecular Ecology* **14**, 2611–2620.

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47–50.

Gennadius, P. (1889) Disease of tobacco plantations in the Trikonia. The aleyrodid of tobacco. *Ellenike Georgia* **5**, 1–3 (in Greek).

Franck, P., Reyes, M., Olivares, J. & Sauphanor, B. (2007) Genetic architecture in codling moth populations, comparison between microsatellite and insecticide resistance markers. *Molecular Ecology* **16**, 3554–3564.

Franklin, M.T., Ritland, C.E. & Myers, J.H. (2010) Spatial and temporal changes in genetic structure of greenhouse and field populations of cabbage looper, *Trichoplusia ni*. *Molecular Ecology* **19**, 1122–1133.

Gómez-Valero, L., Soriano-Navarro, M., Pérez-Brocal, V., Heddi, A., Moya, A., García-Verdugo, J.M. & Latorre, A. (2004) Coexistence of *Wolbachia* with *Buchnera aphidicola* and a secondary symbiont in the aphid *Cinara cedri*. *Journal of Bacteriology* **186**, 6626–6633.

Gottlieb, Y., Ghanim, M., Gueguen, G., Kontsedalov, S., Vavre, F., Fleury, F. & Zchori-Fein, E. (2008) Inherited intracellular ecosystem: symbiotic bacteria share bacteriocytes in whiteflies. *Faseb Journal* **22**, 2591–2599.

Gottlieb, Y., Zchori-Fein, E., Mozes-Daube, N., Kontsedalov, S., Skaljac, M., Brumin, M., Sobol, I., Czosnek, H., Vavre, F., Fleury, F. & Ghanim, M. (2010) The transmission efficiency of tomato yellow leaf curl virus by the whitefly *Bemisia tabaci* is correlated with the presence of a specific symbiotic bacterium species. *Journal of Virology* **84**, 9310–9317.

Gueguen, G., Vavre, F., Gnankine, O., Peterschmitt, M., Charif, D., Chiel, E., Gottlieb, Y., Ghanim, M., Zchori-Fein, E. & Fleury, F. (2010) Endosymbiont metacommunities, mtDNA diversity and the evolution of the *Bemisia tabaci* (Hemiptera: Aleyrodidae) species complex. *Molecular Ecology* **19**, 4365–4376.

Hall, T.A. (1999) BioEdit, a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Higgins, D.G., Thompson, J.D. & Gibson, T.J. (1996) Using Clustal for multiple sequence alignments. *Methods Enzymology* **266**, 383–402.

Himler, A.G., Adachi-Hagimori, T., Bergen, J.E., Kozuch, A., Kelly, S.E., Tabashnik, B.E., Chiel, E., Duckworth, V.E., Dennehy, T.J., Zchori-Fein, E. & Hunter, M.S. (2011) Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science* **332**, 254–256.

Holm, S. (1979) A simple sequentially rejective multiple test procedure. *Scandinavian Journal of Statistics* **6**, 65–70.

Hu, J., De Barro, P., Zhao, H., Wang, J., Nardi, F. & Liu, S.-S. (2011) An extensive field survey combined with a phylogenetic analysis reveals rapid and widespread invasion of two alien whiteflies in China. *PLoS ONE* **6**, e16061 (doi:10.1371/journal.pone.0016061).

Hunter, M.S., Perlman, S.J. & Kelly, S.E. (2003) A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. *Proceedings of the Royal Society of London, Series B* **270**, 2185–2190.

Jiu, M., Zhou, X.-P., Tong, L., Xu, J., Yang, X., Wan, F.H. & Liu, S.-S. (2007) Vector-Virus Mutualism Accelerates Population Increase of an Invasive Whitefly. *PLoS ONE* **2** (1), e182 (doi:10.1371/journal.pone.0000182).

Jones, D.R. (2003) Plant viruses transmitted by whiteflies. *European Journal of Plant Pathology* **109**, 195–219.

Li, Z.X., Lin, H.Z. & Guo, X.P. (2007) Prevalence of *Wolbachia* infection in *Bemisia tabaci*. *Current Microbiology* **54**, 467–471.

Mahadav, A., Gerling, D., Gottlieb, Y., Czosnek, H. & Ghanim, M. (2008) Parasitization by the wasp *Eretmocerus mundus* induces transcription of genes related to immune response and symbiotic bacteria proliferation in the whitefly *Bemisia tabaci*. *BMC Genomics* **9**, 342.

Maruthi, M.N., Colvin, J., Thwaites, R.M., Banks, G.K., Gibson, G. & Seal, S.E. (2004) Reproductive incompatibility and cytochrome oxidase I gene sequence variability among host-adapted and geographically separate *Bemisia tabaci* populations. *Systematic Entomology* **29**, 560–568.

McKenzie, C.L., Hodges, G., Osborne, L.S., Byrne, F.J. & Shatters, R.G. Jr (2009) Distribution of *Bemisia tabaci* (Hemiptera, Aleyrodidae) biotypes in Florida investigating the Q invasion. *Journal of Economic Entomology* **102**, 670–676.

Moran, N.A., McCutcheon, J.P. & Nakabachi, A. (2008) Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics* **42**, 165–190.

Mopper, S. (1996) Adaptive genetic structure in phytophagous insect populations. *Trends in Ecology and Evolution* **11**, 235–238.

Nirgianaki, A., Banks, G.K., Frohlich, D.R., Veneti, Z., Braig, H. R., Miller, T.A., Bedford, I.D., Markham, P.G., Savakis, C. & Bourtzis, K. (2003) *Wolbachia* infections of the whitefly *Bemisia tabaci*. *Current Microbiology* **47**, 93–101.

Oliver, K.M., Degnan, P.H., Burke, G.R. & Moran, N.A. (2010) Facultative symbionts in Aphids and the horizontal transfer of ecologically important traits. *Annual Review of Entomology* **55**, 247–266.

Oosterhout, C., Hutchinson, W.F., Wills, D.P.M. & Shipley, P. (2004) Micro-Checker software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* **4**, 535–538.

Papayiannis, L.C., Brownm, J.K., Hadjistylli, M. & Katis, N.I. (2008) *Bemisia tabaci* biotype B associated with tomato yellow

leaf curl disease epidemics in Rhodes Island, Greece. *Phytoparasitica* **36**, 20–22.

Roditakis, E., Grispou, M., Morou, E., Kristoffersen, J.B., Roditakis, N., Nauen, R., Vontas, J. & Tsagkarakou, A. (2009) Current status of insecticide resistance in Q biotype *Bemisia tabaci* populations from Crete. *Pest Management Science* **65**, 313–322.

Rousset, F. (2007) GENEPOP'007, a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Notes* **8**, 103–106.

Pritchard, J.K., Stephens, M. & Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.

Rosenberg, N.A. (2002) Distruct, a program for the graphical display of structure results. Available online at http://www.cmb.usc.edu/~noahr/distruct.html.

Saridaki, A. & Bourtzis, K. (2010) *Wolbachia*, more than just a bug in insects genitals. *Current Opinion in Microbiology* **13**, 67–72.

Simón, B., Cenis, J.L. & De La Rúa, P. (2007) Distribution patterns of the Q and B biotypes of *Bemisia tabaci* in the Mediterranean Basin based on microsatellite variation. *Entomologia Experimentalis et Applicata* **124**, 327–336.

Skaljac, M., Zanic, K., Goreta Ban, S., Kontsedalov, S. & Ghanim, M. (2010) Co-infection and localization of secondary symbionts in two whitefly species. *BMC Microbiology* **10**, 142.

Thao, M.L., Baumann, L., Hess, J.M., Falk, B.W., Ng, J.C.K., Gullan, P.J. & Baumann, P. (2003) Phylogenetic evidence for two new insect associated *Chlamydia* of the family Simkaniaceae. *Current Microbiology* **47**, 46–50.

Thao, M. & Baumann, P. (2004) Evolutionnary relationships of primary prokaryotic endosymbionts of whiteflies and their hosts. *Applied and Environnemental Microbiology* **70**, 3401–3406.

Tsagkarakou, A. & Roditakis, N. (2003) Isolation & characterization of microsatellite loci in *Bemisia tabaci* (Hemiptera: Aleyrodidae). *Molecular Ecology Notes* **3**, 196–198.

Tsagkarakou, A., Tsigenopoulos, C., Gorman, K., Lagnel, J. & Bedford, I. (2007) Biotype status and genetic polymorphism of the whitefly *Bemisia tabaci* (Hemiptera, Aleyrodidae) in Greece, mitochondrial DNA and microsatellites. *Bulletin of Entomological Research* **97**, 29–40.

Tsagkarakou, A., Nikou, D., Roditakis, E., Sharvit, M., Morin, S. & Vontas, J. (2009) Molecular diagnostics for detecting pyrethroid and organophosphate resistance-associated point mutations in the white fly *Bemisia tabaci*. *Pesticide Biochemistry and Physiology* **94**, 49–54.

Weir, B.S. & Cockerham, C.C. (1984) Estimating *F*-statistics for the analysis of population structure. *Evolution* **38**, 1358–1370.

Xu, J., De Barro, P.J. & Liu, S.S. (2010) Reproductive incompatibility among genetic groups of *Bemisia tabac*i supports the proposition that the whitefly is a cryptic species complex. *Bulletin of Entomological Research* **100**, 359–366.

Zchori-Fein, E. & Brown, J.K. (2002) Diversity of prokaryotes associated with *Bemisia tabaci* (Gennadius) (Hemiptera, Aleyrodidae). *Annals of the Entomological Society of America* **95**, 711–718.

Zhou, W., Rousset, F. & O'Neil, S. (1998) Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London, Series B* **265**, 509–515.

https://doi.org/10.1017/S0007485311000757 Published online by Cambridge University Press