Research Article

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Nuclear and cytoplasmic differentiation among Mediterranean populations of *Bemisia tabaci*: testing the biological relevance of cytotypes

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Abstract

BACKGROUND: The taxonomy of the species complex *Bemisia tabaci* is still an unresolved issue. Recently, phylogenetic analysis based on *mtCOI* identified 31 cryptic species. However, mitochondrial diversity is observed within these species, associated with distinct symbiotic bacterial communities forming associations, which here are called cytotypes. The authors investigated the biological significance of two cytotypes (Q1 and Q2) belonging to the Mediterranean species, which have only been found in allopatry in the Western Mediterranean to date. Sampling was done over a few years in Western Europe, and sympatric situations were found that allowed their reproductive compatibility to be tested in the field with the use of microsatellites.

RESULTS: The field survey indicated that, in spite of its recent introduction, Q2 is well established in France and Spain, where it coexists with Q1. Microsatellite data showed that, in allopatry, Q1 and Q2 are highly differentiated, while there is little or no genetic differentiation when they coexist in sympatry, suggesting a high rate of hybridisation. Crossing experiments in the lab confirmed their interfertility.

CONCLUSION: Q1 and Q2 hybridise, which confirms that they belong to the same species, in spite of the high degree of genetic differentiation at both the cytoplasmic and nuclear levels, and also suggests that their symbiotic bacteria do not prevent hybridisation.

Keywords: *Bemisia tabaci*; MED species; hybridisation; microsatellites; insecticide resistance gene; bacterial endosymbionts

1 INTRODUCTION

Bemisia tabaci is one of the most devastating pests in the world, both directly through plant sap feeding and indirectly by virus transmission.[1] Historically, based on significant variations between populations, biotypes have been identified using ecological traits such as host-plant range, virus transmission abilities, insecticide resistance, enzymatic variability and genetic markers such as the mitochondrial cytochrome oxidase gene (*mtCOI*).[2–4] Because some of these characters are shared between genetic groups or show within-group variability that is higher than between-group variations, the term biotype often appears to be inappropriate.[5] Based on a 3.5% *mt*DNA sequence divergence criterion, it has been proposed that *B. tabaci* is actually made up of at least 31 morphologically indistinguishable species.[5–10] Belonging to 11 major groups, these putative species regroup the previously described biotypes. This view is consistent with some genetic data accumulated on reproductive incompatibilities within the *B. tabaci* species complex.[7,8] Besides the number of species in the *B. tabaci* complex, several questions remain, such as the relevance of the *mt*DNA sequence divergence threshold used for species boundary identification (3.5%). It has been suggested that this value is probably underestimated. For example, Lee *et al.*[9] proposed that this threshold be raised to 4% because there is much variability within species, particularly within the Mediterranean (MED) species. The MED species appears to be genetically diverse, not only because it includes the J, ASL, L and highly damaging Q biotypes[5] but also because high genetic variability has been observed within the Q biotype, leading to the recognition of 4 *mtCOI* haplotypes (Q1 to Q4).[10,11] This variability may regroup separate entities with high genetic differentiation and possible different species, challenging MED species status. Therefore, more information is clearly needed to determine what the true interbreeding limits of the putative species are within the *B. tabaci* complex, and whether reproductive incompatibilities may occur within the putative species that challenge the 3.5% *mt*DNA sequence divergence as a criterion for species delimitation.

In addition to the uncertainty of the *mt*DNA divergence threshold proposed for species recognition, another challenging point concerns the mitochondrial DNA marker, which is probably not the best marker to identify species limits. As in a number of other

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arthropods, *mt*DNA undergoes indirect selection owing to vertically transmitted microorganisms invading host populations.[12] Linkage disequilibrium between *mt*DNA haplotypes and bacterial endosymbionts may blur the clade history and rather reveal the history of infection. Moreover, horizontal transfers of bacterial endosymbionts followed by their invasion in the host population can result in the rapid spread of *mt*DNA lineages without any reproductive barrier.[13,14] This is achieved by their ability to manipulate host reproduction in different ways, especially by inducing cytoplasmic incompatibility (for a review, see Engelstädter and Hurst[15]). In addition to their ability to manipulate host reproduction, symbiotic bacteria may also play an important role in host ecology, host evolution and speciation (see the review in Ferrari and Vavre[16]). Symbiotic bacteria are involved in resistance to natural enemies,[17] resistance to insecticides and host-plant specialisation.[18,19] They can also increase their host fitness under certain environmental conditions.[20] Moreover, they can be involved in resource exploitation and specificity, which confer ecological isolation, leading to speciation.[21]

B. tabaci, like most phloem-feeding insects, harbours an obligatory maternally transmitted endosymbiont, *Portiera aleyrodidarum*, which provides necessary essential nutrients.[22,23] *B. tabaci* also hosts a wide diversity of facultative bacteria, all of which are maternally transmitted. To date, seven secondary endosymbionts have been reported, which form specific association in multi-infected individuals.[24,25] These symbiotic communities appear to be species-specific but also show variations within putative species.[10,26–28] For example, the four haplotypes identified within the MED species harbour distinct bacterial communities.[10,26,27] Q1 harbours *Hamiltonella* and sometimes *Cardinium*, whereas Q2 is infected by *Rickettsia* and *Arsenophonus*. Both Q1 and Q2 are infected by *Wolbachia*[10] but belong to different lineages. Q3 is free of *Wolbachia* but hosts *Arsenophonus* and *Rickettsia*. Q4 is a rare haplotype that has only been detected in Croatia, for which bacterial infection remains unknown. In the present paper, the term 'cytotype' is used to designate an identical *mtCOI* haplotype associated with a specific symbiotic bacterial community. As there is variability in infection frequencies both within and among populations, none of the individuals belonging to one cytotype harbours all of the members of this symbiotic community but rather a combination of symbionts. These cytotypes show different geographic localisations, as Q3 has been recorded only in Africa, whereas Q1 is localised in a large western part of the Mediterranean basin: Morocco, Tunisia, Portugal, France, Greece and Spain.[10,29–31] All data available on the *B. tabaci* MED species in France and Spain indicate the mere presence of the Q1 cytotype.[29,32–35] This cytotype has been found in several parts of the world, in China and America for example, while Q2 is restricted to the Middle East countries of the Mediterranean basin, Israel and Cyprus, where Q1 is absent.[27,36,37] Q1 and Q2 cytotypes overlap in neighbouring countries such as Turkey, Egypt and Syria (GenBank records).[6,33] As these two cytotypes harbour very different bacterial communities,[10] the MED species provides a good framework to test and to understand the role of these communities in the complex ecology and evolution of the *B. tabaci* species. This particular situation also provides a good opportunity to test whether the 3.5% *mtCOI* threshold reflects the true limit of species within the *B. tabaci* species complex in which bacterial endosymbiont infection is pervasive. This is particularly important from pest management perspectives.

In the present study, the authors report the presence of the Q2 cytotype in Western Europe in sympatry with Q1, which was the only cytotype previously known in this area. The dynamics of colonisation of the Q2 cytotype in France and Spain, which coexists locally with the Q1 cytotype, was followed. These sympatric situations provided the opportunity to test for possible hybridisation or the presence of a reproductive barrier among Q1 and Q2 cytotypes via the use of microsatellite markers (expected to be neutral). Because of the extensive use of insecticides to control *B. tabaci* populations,[38] two genes that are known to be involved in insecticide resistance against pyrethroids and organophosphates were also studied. Resistant alleles and their possible different frequencies in both cytotypes may explain patterns of cytotype distribution. They also could drive the outcome of competition in the case of reproductive isolation.

2 MATERIALS AND METHODS

2.1 Insect lines and rearing

Two lines belonging to Q1 and Q2 were used for crossing experiments. The Q1 line, originating from Sigean (Languedoc Roussillon, France), was infected by *Hamiltonella* and *Cardinium*. The Q2 line, originating from Lyon (Rhône-Alpes, France), was infected by *Rickettsia* and *Arsenophonus*. Both lines were fixed for bacterial infection. These lines were maintained in the lab for at least 1 year (about 17 generations) before the experiments were initiated. They were reared on *Hibiscus moscheutos* var. *Galaxy* plants at 25 °C with an L:D cycle of 16:8 and 70% relative humidity. Their cytotypes (*mtCOI* and associated symbionts) were regularly verified by PCR.

2.2 Crossing experiments

Pupae from the Q1 and Q2 lines were isolated, and adults were sexed from 1 to 4 h after emergence to ensure female virginity. All four possible crosses were performed (crosses between Q1 males and females, crosses between Q2 males and females, crosses between Q1 males and Q2 females and reciprocal crosses), with ten replicates each. Five females and males were placed on 30 mm diameter excised *Hibiscus moscheutos* var. *Galaxy* leaf discs on 1% agar medium in a 50 mm petri dish for 6 days. Laid eggs were counted 3 days after removing the females, as were emerging adults. In order to test for the fertility of F1 individuals, offspring were kept together, which allowed them to mate and produce progeny. Thus, the presence of both males and females in the progeny was checked.

2.3 *B. tabaci* field sampling

A total of 531 females from ten localities were collected in France and Spain from 2006 to 2010 for a temporal survey. Adults were collected in greenhouses on different hosts, including ornamental plants and vegetables (Table 1). Sampled adults were placed alive in ethanol (96%) and stored at −20 °C until DNA extraction. In order to have Q1 and Q2 'reference' cytotypes, use was made of samples from Greece and Israel, where only one Q cytotype had been reported to date: Q1 in Greece and Q2 in Israel. This was confirmed by the recent study of Gauthier and collegues.[39]

2.4 Molecular assays

2.4.1 DNA extraction

For each individual, total DNA was extracted in 26 µL of a buffer containing 50 mM of KCl, 10 mM of Tris-base pH 8, 0.45% Nonidet P-40, 0.45% Tween 20 and 50 mg mL^{-1} of Proteinase K. After 3 h at 65 °C, samples were incubated at 100 °C for 15 min. A volume of 35

Table 1. Characteristics of the sample site locations

Country	Region	Locality	Acronym[a, b]	Year	Host plant	*N*[c]
France	Midi-Pyrénées	Lavaur	LavO	2007	Ornamental	24
		Castelmaurou	CastM	2007	Manoelila	15
		Launaguet	LaunH	2007	Hibiscus	23
	Languedoc	Sigean	SigT	2006	Tomato	20
	Provence Alpes Cote d'Azur	St Martin de Crau	CrauP	2007	Sweet pepper	97
		Saint Laurent du Var	SlvE*	2010	Eggplant	45
		Fréjus	FroH*	2010	Hibiscus	48
	Rhône-Alpes	Lyon	LyoV	2007	Various plants	19
Spain	Catalonia	Cabrils	CabV	2007	Various plants	91
			CabM*	2010	Melon	31
			CabT*	2010	Tomato	40
		Viladecans	VilT*	2010	Tomato	31
			VilC*	2010	Cucumber	47
Greece		Thessaloniki	TskC*	2010	Cucumber	10
		Kasteli-Chania	KscE*	2010	Eggplant	10
		Ierapetra	IerC*	2010	Cucumber	10
Israel		Hof Carmel	HfcV*	2010	Various	29

[a] The first three or four letters of the acronyms correspond to the locality; the last capital letter indicates the host plant.
[b] *: Populations used for microsatellite analysis (all the individuals of these populations have been genotyped) and insecticide-resistant allele detection (the number of individuals studied per population is specified in Table 3).
[c] *N*: the number of sampled individuals genotyped for mitochondrial haplotype and screened for the presence of endosymbionts.

µL of pure water was added to this extract, which was then stored at −20 °C until use.

2.4.2 mtCOI *haplotype determination*

For all of the 531 individuals collected, mitochondrial haplotypes were determined using a PCR-RFLP diagnostics tool as described in Henri *et al.*,[40] which allowed the discrimination of all of the mitochondrial haplotypes described within the MED species. Some of the PCR products (2–10 individuals per population) were also sequenced by Biofidal (Lyon, France) for confirmation (GenBank accession numbers: KJ411776–KJ411814).

2.4.3 *Detection of endosymbiotic bacteria*

The six most frequent secondary symbiotic bacteria found in *B. tabaci* (*Wolbachia*, *Cardinium*, *Hamiltonella*, *Rickettsia*, *Arsenophonus* and *Fritschea*) were screened using specific PCR primers for each of the 531 individuals (Table 2). One simplex PCR was realised for each symbiont. All samples were tested for the presence of the obligatory symbiont *Portiera aleyrodidarum* to test for the quality of the DNA extraction. PCR reactions were performed in 25 µL volumes containing 200 µM of dNTP, 200 nM of primers, 0.5 IU of Taq DNA polymerase (Eurobio) and 2 µL of DNA template. PCR products were visualised in 1% agarose gels stained with Gelred™ (5 µL 100 mL^{-1}) under UV illumination.

2.4.4 *Insecticide-resistant allele detection*

Resistant (r) and susceptible (S) alleles were identified in two genes associated with insecticide resistance in *B. tabaci*: the *para*-type voltage-gated sodium channel and the *ace1* genes. Two mutations at two different sites in the *para*-type voltage-gated sodium channel gene, L925I and T929V (r1 and r2 alleles), were screened for,

Table 2. Characteristics of primers used for endosymbiont screening

Endosymbiont	Targeted gene	Primers	Primer sequence	T_m (°C)	Reference
Portiera aleyrodidarum	*rDNA 16S*	28F 1098R	5′-TGCAAGTCGAGCGGCATCAT-3′ 5′-AAAGTTCCCGCCTTATGCGT-3′	58	24
Rickettsia	*rDNA16S*	Rb-F Rb-R	5′-GCTCAGAACGAACGCTATC-3′ 5′-GAAGGAAAGCATCTCTGC-3′	58	73
Hamiltonella	*rDNA 16S*	Hb-F Hb-R	5′-TGAGTAAAGTCTGGGAATCTGG-3′ 5′-AGTTCAAGACCGCAACCTC-3′	58	24
Cardinium	*rDNA 16S*	CFB-F CFB-R	5′-GCGGTGTAAAATGAGCGTG-3′ 5′-ACCTMTTCTTAACTCAAGCCT-3′	56	74
Wolbachia	*wsp*	81F 691R	5′-TGGTCCAATAAGTGATGAAGAAAC-3′ 5′-AAAAATTAAACGCTACTCCA-3′	56	75
Arsenophonus	*rDNA 23S*	Ars-23S1 Ars-23S2	5′-CGTTTGATGAATTCATAGTCAAA-3′ 5′-GGTCCTCCAGTTAGTGTTACCCAAC-3′	60	76
Fritschea	*rDNA 23S*	Frit-F Frit-R	5′-GAGTTTGATCATGGCTCAGATTG-3′ 5′-GCTCGCGTACCACTTTAAATGGCG-3′	62	77

Table 3. Frequencies of sodium channel and *ace-1* resistant mutations in *B. tabaci* per host plant, cytotype and locality

			Sodium channel									*ace*				
			Locus 1 (L925I) genotypes				r1 allele resistance	Locus 2 (T929V) genotypes			r2 allele resistance	Genotypes				R allele resistance
Country	Locality	Cytotype	*n*	r1r1	r1s1	s1s1	frequencies	*n*	r2r2	r2s2	s2s2	frequencies	*n*	RR	RS	SS	frequencies
Greece	Various	Q1	29	16	11	2	0.74	29	0	1	28	0.02	29	29	0	0	1
Israel	Hof-Carmel	Q2	23	12	8	3	0.70	27	3	11	13	0.29	27	27	0	0	1
Spain	Cabrils	Q1	7	3	4	0	0.71	7	0	3	4	0.21	7	7	0	0	1
		Q2	25	6	16	3	0.56	25	0	15	10	0.30	25	25	0	0	1
	Viladecans	Q1	28	12	14	2	0.68	28	0	6	22	0.11	28	28	0	0	1
		Q2	9	6	2	1	0.78	9	0	3	6	0.17	9	9	0	0	1
France	Saint-Laurent du Var	Q1	12	11	1	0	0.96	12	0	0	12	0	12	12	0	0	1
		Q2	7	6	1	0	0.93	7	0	0	7	0	7	7	0	0	1
	Frejus	Q1	8	8	0	0	1	8	0	0	8	0	8	8	0	0	1
		Q2	15	12	3	0	0.90	15	0	2	13	0.07	15	15	0	0	1

along with one mutation in the acetylcholinesterase enzyme *ace1* (F331W, R allele). These mutations confer resistance to pyrethroids (Pyrs) and organophosphates (OPs) respectively. L925I and F331W resistance mutations were identified using the PCR-RFLP tool developed by Tsagkarakou *et al.*[41] The T929V resistance mutation was detected by PCR amplification of specific alleles.[41] Screening was performed on 7–29 individuals per population, to a total of 111 (Table 3). Twelve ambiguous PCR products were sequenced for verification.

2.4.5 *Microsatellite genotyping*

Seven microsatellite loci were used as neutral nuclear markers to study genetic diversity and differentiation of *B. tabaci* populations and cytotypes (Table 4). *B. tabaci* is a haplodiploid species, i.e. haploid males hatch from unfertilised eggs; therefore, genotyping was performed on females only. As only one Q cytotype has been described to date in Greece and Israel (Q1 and Q2 respectively), they were expected never to have been hybridised. In the present study, populations located in these countries were considered as references for Q1 and Q2 cytotypes. Around 30 individuals were genotyped from each population (Table 1). Among the 531 individuals collected in France and Spain between 2006 and 2010, 242 individuals were genotyped, originating from localities where Q1 and Q2 cytotypes were found in sympatry (two sites per country, indicated with an asterisk in Table 1).

PCR reactions were performed separately for each primer pair in 10 µL volumes containing 200 µM of dNTP, 200 nM of each primer (fluorescently labelled 5′ primer) and 0.5 IU of Blue Taq DNA polymerase (Eurobio). Cycling conditions were initial denaturing at 94 °C for 2 min, followed by 35 cycles of 30 s at 94 °C for denaturation, 30 s at 52 or 57 °C, depending on the primer set (Table 4), for annealing and 1 min at 72 °C for elongation, with a final elongation at 72 °C for 10 min. Fluorescent amplicons were loaded on an ABIPrism 3100-Avant genetic analyser (Applied Biosystems, Foster City, CA) automated sequencer, and allele sizes were calculated using GeneMapper software (Applied Biosystems).

2.5 Genetic data analyses

The mean number of alleles per locus (A^-), the observed heterozygosity (H_o) and the unbiased expected heterozygosity (H_e) were calculated using Genetix.[42] Weir and Cockerham's estimators of F_{IS} within populations and genetic groups were calculated using Genepop, and exact tests were used for Hardy–Weinberg equilibrium.[43,44] Linkage disequilibrium between all pairs of loci

Table 4. Characteristics of the primers used for microsatellite genotyping

Locus	Primer name	Primer sequence	Reference	T_m (°C)
BtIS2.3	BtIs2.3F	5′-CAGAACGACAGGTCGAG-3′	50	50
	BtIs2.3R	5′-CAAAATTAATGGTATTGACTC-3′		
BtIS1.13	BtIs1.13F	5′-CTAAGACCGATTCCTCC-3′	50	52
	BtIs1.13R	5′-GAATACTACACCTTCAATTACC-3′		
Locus 11	11a	5′-CCAGAAAAGTGGACTTAAGA-3′	78	57
	11b	5′-GATCTGGGTGTTTTCTTCTA-3′		
BT83	BT83F	5′-GATGCCACAGGTTGTCTGG-3′	79	57
	BT83R	5′-GCTTGCCAGGCACTTTCTAG-3′		
BtIS1.1	BtIs1.1F	5′-CCCATAGAACACGCTCC-3′	50	57
	BtIs1.1R	5′-CATTGGAAGCCTCGAATAC-3′		
BtIS1.2	BtIs1.2F	5′-CTTACCTTCCATTCACC-3′	50	57
	BtIs1.2R	5′-ATCCCGAGTCTTATGTTG-3′		
Locus 145	145a	5′-CCTACCCATGAGAGCGGTAA-3′	29	57
	145b	5′-TCAACAAACGCGTTCTTCAC-3′		

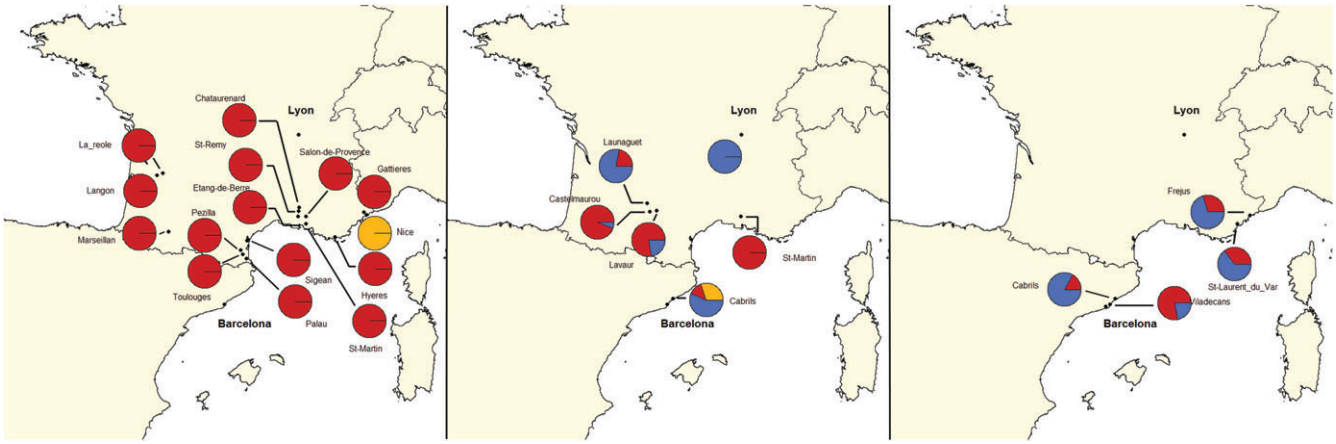

Figure 1. Map of cytotype distribution in France and Spain by year of sampling. Red: Q1 cytotype (MED species); blue: Q2 cytotype (MED species); yellow: MEAM1 species.

and *F*-estimates of differentiation[43] were calculated and tested with 5000 permutations using Genepop. The number of genetic groups was assessed using the software STRUCTURE,[45] which differentiates mixed populations on the basis of allele frequency at each locus. The following options were used: 500 000 Markov chain Monte Carlo with a burn-in period of 50 000, where admixture and correlated allele frequencies were allowed for; log-likelihood estimates were calculated for $K = 1–15$, with ten replicates each. STRUCTURE assumes Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium within each group, which is why both hypotheses were tested subsequently using exact tests implemented in Genepop 4.0. A principal component analysis (PCA) was also conducted, which did not make any assumption such as HWE and linkage disequilibrium, with R software,[46] *adegenet* package.[47] In addition, a hierarchical analysis of molecular variance (AMOVA) was performed, using the *hierfstat* package,[48] implemented in R software, to test for geographical, host-plant, cytotype and secondary symbiont effects. Significance was assessed with permutation tests (5000 permutations). Resistance allele frequencies were subjected to Fisher's exact test with simulated *P*-values based on 1000 replicates using R statistical software,[46] while HWE was tested by χ^2 with R statistical software.[46]

3 RESULTS

3.1 Field survey in France and Spain

From 2006 to 2010, a survey of *B. tabaci* populations was performed in France and Spain to follow the geographical distribution of *B. tabaci* cytotypes. A total of 531 individuals from ten localities were collected (Table 1) and compared with the data of Dalmon *et al.*[29] and Gueguen *et al.*,[10] which were used as the historical baseline situation in Western Europe (Fig. 1a). These previous studies were performed from 2003 to 2006 and included 14 localities; only the Q1 cytotype of MED species was observed. Here, almost all individuals collected belong to the MED species, except in two localities where the MEAM1 species has been previously observed, in a botanical garden in Nice (France) in 2004 and in Cabrils (Spain) in 2007. However, as this situation concerns very few individuals and is rather an exception, MEAM1 species will not be taken into account and discussed further in this study. In contrast to the situation in 2003–2006, with only the Q1 cytotype, in 2007 the Q2 cytotype was observed for the first time, in several localities, either in sympatry with Q1 individuals (four locations in France and Spain) or alone in a greenhouse in Lyon (France) with a very high population density. The coexistence of Q1 and Q2 cytotypes was also observed in 2010 at all of the sampling sites (Fig. 1). The proportion of Q2 individuals in sympatry with Q1 varied, according to the collection, from 6 to 83% in 2007. They predominated in three (83, 71 and 64%) of the four localities in 2010 (22% frequency at the site where Q1 prevails).

3.2 Analysis of reference Q1 and Q2 cytotypes

To date, only the Q1 cytotype has been detected in Greece and Q2 in Israel.[27,49,50] In the present sampling, the determination of the *mtCOI* cytotype of individuals collected in three localities in Greece (ten per locality) and one site in Israel ($n = 29$) confirmed these data. They were therefore used as reference populations for Q1 and Q2 cytotypes.

3.2.1 *Endosymbiotic community*

Individual detection of the seven main bacteria infecting *B. tabaci* revealed that Q1 cytotypes in Greece and Q2 cytotype in Israel harbour a specific symbiotic community, as already reported.[10,27] The most common bacteria are *Hamiltonella*/*Wolbachia* for Q1 and the association *Arsenophonus*/*Rickettsia* for Q2 (Fig. 2).

3.2.2 *Detection of sodium channel and ace1* resistance mutations

A resistance allele in *ace1* (F331W) and two resistance alleles of *para*-type voltage-gated sodium (L925I and T929V) genes were detected in both reference cytotypes Q1 and Q2: F331W (R allele) was fixed in Q1 and Q2, while L925I (r1 allele) showed intermediate frequencies in the two cytotypes (0.70 and 0.74 respectively). Q1 and Q2 cytotypes differed significantly only in the frequencies of the T929V mutation (r2 allele; 0.02 and 0.29 respectively; Fisher's exact test: $P < 10^{-4}$).

3.2.3 *Microsatellite analysis*

The genetic analysis of the 59 individuals genotyped (30 from Greece and 29 from Israel) with seven microsatellites revealed a higher allelic richness in the Q1 than in the Q2 cytotype (4.3 vs 3.4 alleles) (Table 5). Analysis carried out using STRUCTURE revealed a very high genetic differentiation between Q1 and Q2, which was confirmed by PCA (Fig. 2). The pairwise F_{ST} value reached 0.27, which is very high for two groups that belong to the same putative species.

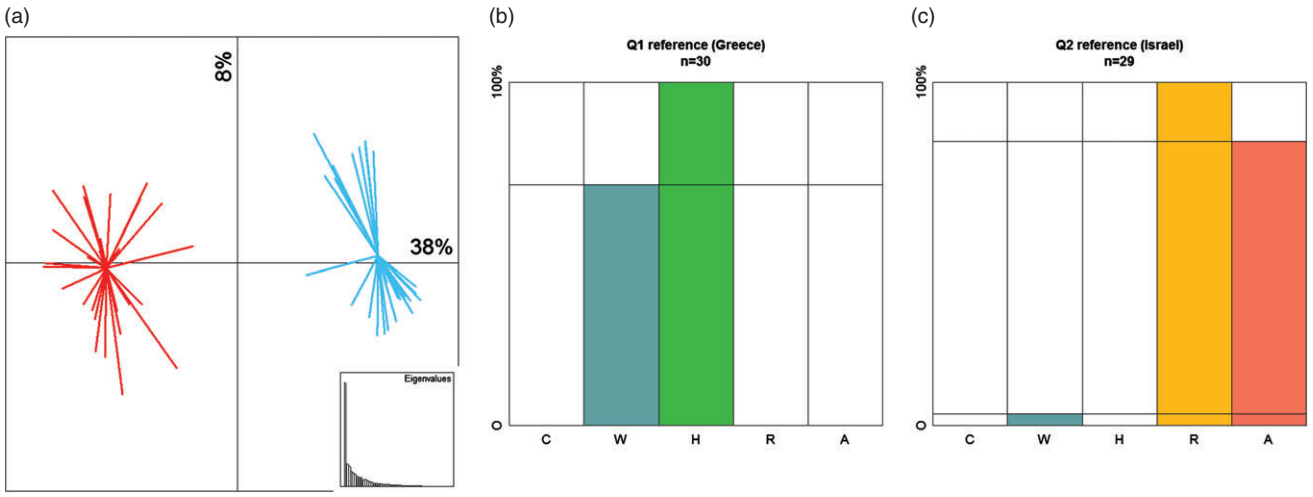

Figure 2. Characteristics of Greek Q1 and Israel Q2 cytotypes: (a) Factorial map of the principal component analysis; (b) infection status of Q1 individuals; (c) infection status of Q2 individuals. C: *Cardinium*; W: *Wolbachia*; H: *Hamiltonella*; R: *Rickettsia*; A: *Arsenophonus.*

Table 5. Microsatellite genetic diversity of *B. tabaci* populations[a]

			Q1					Q2					Q1 + Q2				
Country	Locality	Plant	*N*	H_o	H_e	F_{IS}	A^-	*N*	H_o	H_e	F_{IS}	A^-	*N*	H_o	H_e	F_{IS}	A^-
Spain	Cabrils	Melon	5	0.41	0.42	0.14	2.3	26	0.38	0.49	0.25	4.4	31	0.39	0.51	0.24	4.6
Spain	Cabrils	Tomato	7	0.33	0.43	0.31	3.3	33	0.35	0.49	0.30	4.3	40	0.35	0.50	0.30	4.6
Spain	Viladecans	Tomato	21	0.45	0.58	0.25	4.6	10	0.43	0.57	0.29	3.3	31	0.44	0.60	0.26	4.9
Spain	Viladecans	Cucumber	40	0.41	0.59	0.33	4.1	7	0.44	0.55	0.27	3.3	47	0.41	0.60	0.30	4.3
France	Fréjus	Hibiscus	14	0.42	0.52	0.23	3.4	34	0.37	0.52	0.31	3.6	48	0.38	0.53	0.28	4.9
France	St Laurent du Var	Eggplant	30	0.39	0.52	0.30	3.9	15	0.40	0.55	0.33	4.1	45	0.39	0.55	0.32	4.9
Greece	Thessaloniki	Cucumber	10	0.28	0.56	0.52	3.4	0	–	–	–	–	–	–	–	–	–
Greece	Kasteli-Chania	Eggplant	10	0.38	0.56	0.34	3.4	0	–	–	–	–	–	–	–	–	–
Greece	Ierapetra	Cucumber	10	0.43	0.54	0.21	3.4	0	–	–	–	–	–	–	–	–	–
Israel	Hof Carmel	Various	0	–	–	–	–	29	0.4	0.52	0.37	4.3	–	–	–	–	–

[a] N: number of individuals; H_o: observed heterozygosity; H_e: unbiased expected heterozygosity; F_{IS}: fixation indice from Weir and Cockerham; A^-: mean number of alleles per population per locus.

3.3 Nuclear and cytoplasmic differentiation of sympatric Q1 and Q2 cytotypes

3.3.1 Endosymbiotic community

Fritschea was never detected and will not be discussed further. The bacterial screening did not reveal any new association between a mitochondrial haplotype and a symbiotic community compared with the reference populations (Israeli and Greek), meaning that the cytoplasmic associations are stable (Fig. 3). In the Q1 cytotype, *Hamiltonella* was almost fixed (93% of individuals infected), while *Wolbachia* and *Cardinium* were present at intermediate frequencies (28 and 38% respectively), with a high frequency of bi-infections (62%). In the Q2 cytotype, *Rickettsia* was also almost fixed (95%), while *Wolbachia* and *Arsenophonus* were found at a lower frequency (69 and 62% respectively). In Lyon, *Wolbachia* was absent.

3.3.2 Detection of sodium channel and ace1 resistant mutations

In the French and Spanish sympatric populations sampled in 2010 (111 females tested), all three resistance mutations were detected in both Q1 and Q2 cytotypes in all of the localities investigated, with a high variability in frequency (Table 3). All individuals were homozygous for F331W (R allele) of the *ace1* gene. L925I (r1 allele) showed a high frequency (0.56 to 1), and the frequencies of T929V (r2 allele) were low (0–0.30). These frequencies did not differ between Q1 and Q2 cytotypes within all four localities (Fisher's exact tests: $P > 0.05$). No significant deviations from Hardy–Weinberg equilibrium were detected within three localities (regardless of the cytotype involved) for both *kdr* mutations (Fréjus, Saint-Laurent du Var and Viladecans, χ^2: $P > 0.05$). Only the population from Cabrils deviated slightly from HWE for T929V (χ^2: $P = 0.01$).

3.3.3 Microsatellite analysis

Field populations genotyped for seven microsatellite loci (Table 5) showed a significant deviation from HWE ($P < 0.001$). Within all localities, a systematic heterozygous deficiency was observed that revealed high F_{IS} values within localities (0.243–0.357). This deficiency was not due to a particular locus, as the observed heterozygosity was inferior to the expected heterozygosity in all loci. Within the four localities, the F_{IS} was not higher when all individuals were considered than when cytotypes were considered separately (Table 5), suggesting that high F_{IS} is not explained by a genetic structure that differs among cytotypes. This result was confirmed by STRUCTURE software analysis, as three clusters were

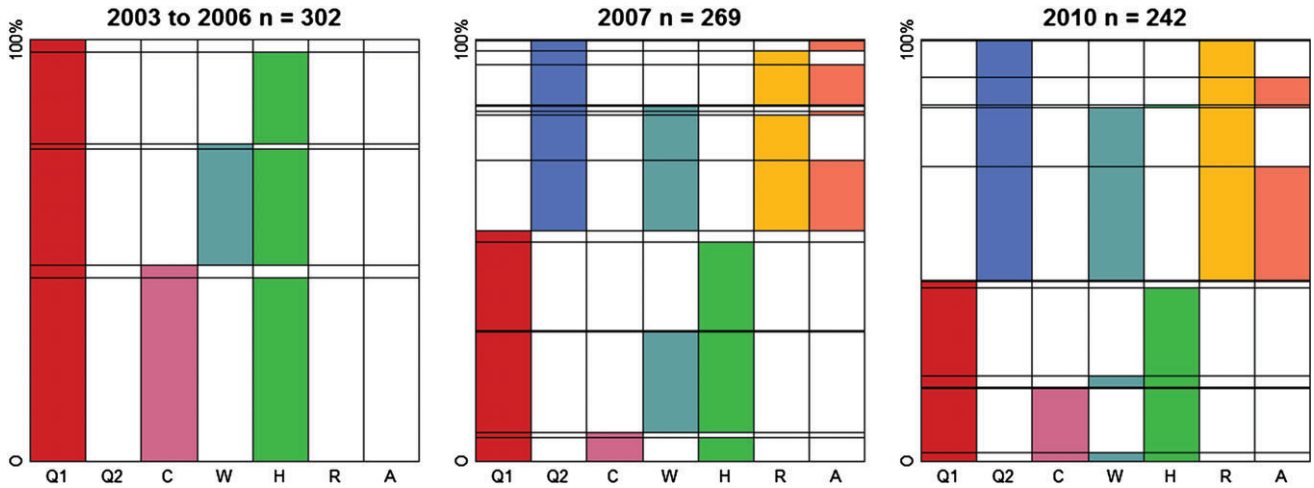

Figure 3. Infection status of females. C: *Cardinium*; W: *Wolbachia*; H: *Hamiltonella*; R: *Rickettsia*; A: *Arsenophonus*.

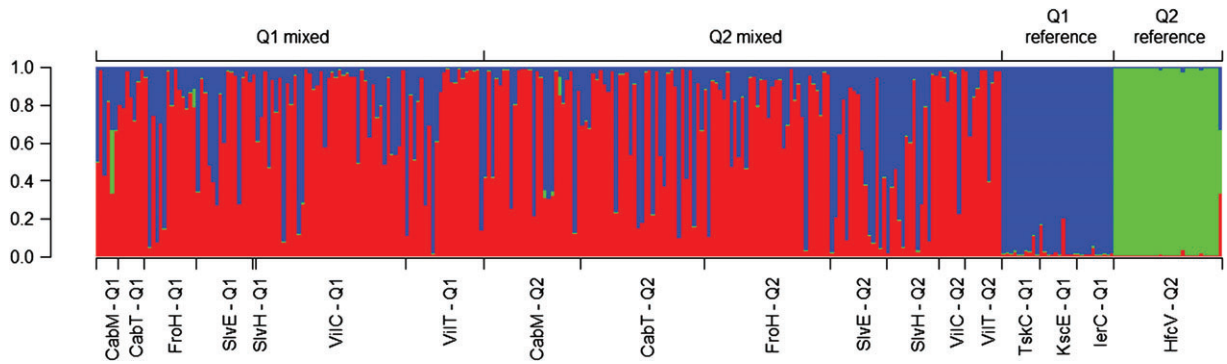

Figure 4. Clustering results from STRUCTURE for all samples.

identified when taking into account all sympatric and reference populations. The first cluster regrouped all of the Q2 reference individuals (from Israel). All Greek individuals (Q1 reference) were assigned to a second cluster. Q1 or Q2 cytotype individuals from sympatric populations were assigned, with probabilities more or less important, to the Greek Q1 reference cluster and a third cluster. Probabilities of sympatric individual assignment to each cluster are shown in Fig. 4. Sympatric Q1 and Q2 cytotypes thus showed a similar genetic composition that probably resulted from the hybridisation of two entities, one being close to the Greek population. The PCA (Fig. 5) of allele frequencies confirmed the STRUCTURE software Bayesian analysis. The first axis clearly separates the three groups: mixed Q1–Q2 (western part), reference Q1 (Greece) and reference Q2 (Israel). The second axis weakly discriminates between Greek Q1 and mixed populations. Pairwise comparisons between localities of these geographic groups are very high ($0.09 < F_{ST(\text{mixed}-\text{Greece})} < 0.14$; $0.29 < F_{ST(\text{mixed}-\text{Israel})} < 0.33$). The point is that Q1 and Q2 cytotypes are totally homogeneous when they are sympatric.

The authors have successively tested for the influence of plants and the symbiotic compartment on the genetic structure within the four localities. The AMOVA was not significant ($P > 0.1$) for all of these factors. The only structuring genetic factor was geographical differentiation: *F*-statistics showed little but significant differentiation between pairs of these four French and Spanish localities (F_{ST} ranging from 0.01 to 0.02, $P < 0.01$). Allelic richness was quite homogeneous among the four localities where sympatric cytotypes were observed (values ranging from 4.3 to 4.9), which is not significantly higher than the allelic richness detected within localities from reference Q1 (Greek) and Q2 (Israeli) cytotypes.

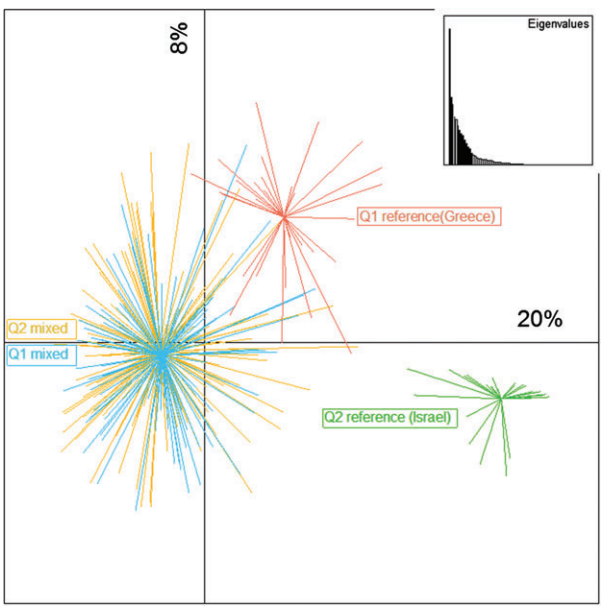

Figure 5. Factorial map of the principal component analysis with the entire dataset.

Table 6. Results of crossing experiments. Mean eggs laid per replicate (±SD), mean emerging adults (±SD) and mean percentage of emerging adults reported to the number of laid eggs (±SD)

	Cross[a]			
	Q1♀ × Q1♂	Q1♀ × Q2♂	Q2♀ × Q1♂	Q2♀ × Q2♂
Eggs	127* ± 43	75 ± 13	40 ± 13	65 ± 9
Emerging adults	32 ± 15	48* ± 9	32 ± 12	39 ± 8
Emerging adults (%)	24* ± 14	65 ± 13	79 ± 5	62 ± 16

[a] An asterisk (*) denotes a significant difference from the others (Tukey's HSD test, $P < 0.01$). The number of replicates in each cross was 10.

3.3.4 *Crossing experiments*

Viable offspring with both males and females were obtained in all of the crosses performed, suggesting that there is neither nuclear nor complete cytoplasmic incompatibilities between Q1 and Q2 cytotypes. The mean number of eggs laid and the percentage of non-hatched eggs are indicated in Table 6. The Q1♀ × Q1♂ cross produced more eggs than the other three (Tukey's HSD test: $P < 0.001$), but the percentage of hatched eggs was lower (24%, Tukey's HSD test: $P < 0.001$). The number of offspring was slightly higher in the Q1♀ × Q2♂ cross than in the others (Tukey's HSD test: $P = 0.01$). Fertility of F1 individuals (males and females) was verified by keeping them together for mating, and the presence of a high number of males and females confirmed hybridisation among both cytotypes.

4 DISCUSSION

4.1 Cytotype distribution in Western Europe

B. tabaci is widely distributed in France and Spain, and the putative MED species largely predominates in these areas, such as in most of the Mediterranean basin. The MED species has been found in all of the Spanish and French localities where sampling was performed from 2003 to 2006. In very few areas, the MEAM1 putative species was observed in France (Nice) in 2004 and in Spain (Cabrils) in 2007, but this presence is anecdotal. Until 2007, the Q1 cytotype, which associates a particular mitochondrial variant with *Hamiltonella* and *Cardium* bacteria, was the only cytotype belonging to the MED species found in France and Spain, and more generally in the large western part of the Mediterranean basin.[29,51] In 2007, the Q2 cytotype was detected for the first time in four out of the six localities studied in France and Spain. Q2 is another mitochondrial variant infected by *Rickettsia* and *Arsenophonus* symbionts previously reported in the Middle East (Israel and Turkey exclusively). In 2010, Q2 was found in four other localities in France and Spain, always in sympatry with Q1. Because the initial situation in these countries was well known, this observation was interpreted as a recent introduction of the Q2 cytotype, which has also been recently observed in other localities in the Western Mediterranean basin.[39,52] This introduction is probably due to trade, which occurs intensively among Mediterranean countries. Indeed, in France, ornamental plants grow up in greenhouses, but the sowing and cloning is mostly performed in other countries such as Israel, Kenya, the Ivory Coast, Germany, Spain and Italy (private communication). It can be supposed that Q2 was introduced from the Eastern part of the Mediterranean basin (Middle East), as it has always been detected over there,[10,33,36] and is now spreading in Western Mediterranean countries. Extensive surveys of *B. tabaci* populations on a larger geographical scale would make it possible to determine whether this situation is particular or whether it is occurring on a wider geographic scale in the Mediterranean basin. Nevertheless, these data raise the question of the ability of the Q2 cytotype to settle in these localities, whereas Q1 was already present, which is related to the question of cytotype coexistence discussed in Section 4.4. Some authors suggested a role of different interacting agroecological factors, such as insecticide use and high temperature (more suitable for Q2), as well as the extensive production of Solanaceae plants, in the invasion of Q2 in southern Italy.[52]

4.2 Q1 and Q2 differentiation and hybridisation

Until 2007, Q1 and Q2 cytotypes were known in allopatric conditions in the Mediterranean basin, with Q2 only reported in Israel, with its possible presence in bordering countries, while Q1 had a larger distribution from Greece to France and northern Africa. This separate distribution is consistent with the very high nuclear genetic differentiation that was measured in this study between the Q1 and the Q2 reference cytotypes (Greece and Israel respectively). The F_{ST} value (about 29%), measured with microsatellite markers, is comparable with those obtained between distinct *B. tabaci* species such as MED and MEAM1.[53,54] This divergence indicates that they probably did not hybridise recently, as they had not been in contact, leading the authors to consider these lines as reference cytotypes, even if the nuclear genetic compositions observed in these countries are probably not representative of all of the diversity that can exist in each cytotype. Moreover, this small sampling could explain the high differentiation values and the detection of Israeli Q2 specific alleles (many alleles from Q2 reference cytotype are not found anywhere else). The present Q2 reference is therefore certainly not the population source of the western Q2 cytotypes, as suggested by the microsatellite analysis comparison of allopatric and sympatric populations. Populations experiencing strong bottlenecks during colonisation processes or pesticide treatments inducing reduced population sizes could also explain this high differentiation.

This new situation with Q1 and Q2 cytotypes observed in sympatry in France and Spain provided the opportunity to test for their interfertility. The microsatellite analysis revealed three highly differentiated clusters: Israeli Q2 reference, Greek Q1 reference and the western mixed cytotypes. These results indicated that Q1 and Q2 are genetically homogeneous in sympatry (i.e. in France and Spain) and clearly demonstrate their full hybridisation and the absence of any reproductive barrier among them. They also do not differ for the insecticide-resistant allele frequency for the two genes considered here, i.e. the *ace1* (resistance is fixed) and the *para*-type voltage-gated sodium channel genes. Crossing experiments in the lab confirmed that Q1 and Q2 individuals can interbreed and produce fertile offspring, although care must be taken as the authors conducted crossing with Q2 and Q1 lines from Lyon and Sigean (France) respectively, the nuclear genetic backgrounds of which are unknown; therefore, a different result with Israeli Q2 and Greek Q1 reference cytotypes, which are strongly differentiated, cannot be excluded. All of these facts indicate that there is no nuclear incompatibility between them and that they belong to the same species, as suggested by phylogenetic analyses.[5]

4.3 Biological relevance of Q1 and Q2 cytotypes

4.3.1 *Cytotype stability*

Q1 and Q2 freely interbreed but harbour different symbiont communities, even when they coexist in sympatry (Q1 is infected by

Hamiltonella and *Cardinium,* while Q2 is infected by *Rickettsia* and *Arsenophonus*). This means that the *mtCOI* haplotype and bacterial associations are relatively stable on a timescale of several years, thus giving biological relevance to cytotypes under the hypothesis of a role for bacteria in the *B. tabaci* phenotype. The absence of new mitochondrial haplotype/bacterial community associations can be explained either by the absence or very rare events of horizontal transfers or the inability of these bacteria to colonise new cytoplasmic and/or nuclear environments. Symbiotic, vertically transmitted bacteria are also known to be horizontally transferred. Horizontal transmission can occur through the diet between individuals that feed on the same host plant, as demonstrated in aphids and fruit flies,[55,56] or during mating.[57,58] Transfers can also occur between hosts and parasitoids.[59,60] Phylogenetic analysis revealed the existence of such transfers in *B. tabaci*.[61] Laboratory experiments demonstrated indeed that *Rickettsia* can be horizontally transmitted between *B. tabaci* individuals through the host plant.[62] Therefore, a total absence of horizontal transfer is unlikely, but it is possible that lateral transfer between cytotypes has not yet occurred (at the time of this study) because the contact of Q1 and Q2 cytotypes is new. Another explanation could be that new *mtCOI*/bacteria associations are unstable either because of a maladaptation of the transferred bacteria in the new environment or exclusion by the recipient cytotype. This exclusion can be achieved by the host nuclear genes or by the bacterial endosymbionts that are already present. As the two cytotypes interbreed, it can be supposed that their nuclear compartments are homogeneous. Therefore, the existence of horizontal transfer is possible, even if it cannot be ruled out that some nuclear genes specific to each cytotype could be involved in bacterial exclusion. Within-host, multiple infections could select for competitive symbionts that monopolise resources at the expense of the other, allowing their own vertical transmission. There are some examples of such cytoplasmic competition between bacteria.[63,64] Nevertheless, the stability of the *mtCOI*/symbiotic community associations indicates that the concept of cytotype has a real biological significance.

4.3.2 Presence of bacteria

Q1 and Q2 hybridise, in spite of harbouring different symbiotic communities, suggesting that bacteria do not induce any cytoplasmic incompatibility in *B. tabaci* or low-level cytoplasmic incompatibility that was not detectable in the present experimental conditions. Clearly, laboratory experiments, such as precise measures of sex ratio, are required to affirm that these bacteria do not induce cytoplasmic incompatibility. However, it was shown in the present study that bacteria do not prevent hybridisation and gene flow between Q1 and Q2. As symbionts necessarily induce a physiological cost on their host,[64,65] they are probably involved in other phenotypic effects that allow them to spread and be maintained at high frequency in host populations (see the review in Ferrari and Vavre[16]). Some symbiotic bacteria manipulate host reproduction and increase their transmission, but this is apparently not the case in *B. tabaci*. Others have a mutualistic strategy and confer direct fitness benefits to their host, such as protection against natural enemies,[17,66] thermal tolerance[67,68] or host-plant specialisation.[19] For *B. tabaci*, there is little information about the phenotypic effects of the symbionts. It has been demonstrated that *Rickettsia* enhances host fitness by increasing fecundity and survival to adulthood,[20] and is also suspected to increase sensibility to insecticides.[18] *Wolbachia* could protect *B. tabaci* from parasitoids.[69] In Q1 and in Q2, one symbiont is fixed in natural populations, *Hamiltonella* and *Rickettsia* respectively, while others are present at intermediate frequencies (around 38% for *Cardinium* in Q1 and 60% for *Arsenophonus* in Q2). It is possible that *Rickettsia* increases host performance in the Q2 populations of France and Spain, which is suspected in Italy;[52] however, this trait has only been demonstrated in Q2 populations in the United States, and has not been observed in Q2 populations in Israel. Future studies should focus on the phenotypic effects of the bacteria present in Q1 and Q2 in these mixed populations of France and Spain.

4.4 Cytotype coexistence

The sympatry of Q1 and Q2 observed in France and Spain is relatively new (only since 2007), and the Q2 cytotype has spread throughout Western Europe without excluding Q1 until now. The question that arises is how this situation will evolve and whether these two cytotypes will still coexist or if one will be excluded. The coexistence of several *B. tabaci* cytotypes is uncommon and is generally a transient situation that leads to the displacement and/or exclusion of one of the competing cytotypes.[51,70,71] Each greenhouse can provide an environment (insecticide treatment, temperature, type of crop) that is more or less suitable to one cytotype, excluding the less adapted cytotypes. In this case there should be a mosaic of situations, even on a local scale, where cytotypes are allopatric from one greenhouse to another. Among the three previously cited factors, no data are available for temperature or for the use of insecticides. Moreover, most of the mechanisms known to confer resistance to insecticides are nuclear, while the two cytotypes freely interbreed and reference Q1 and Q2 populations show a similar composition of insecticide resistance alleles. However, *Rickettsia* (associated with the Q2 cytotype) could increase susceptibility to some insecticides[18] and thus disadvantage the Q2 cytotype in such an environment; however, a recent study proposed the opposite.[52] Parrella *et al.*[52] also suspect that host plants play a role in cytotype competition for the benefit of Q2, but the present authors did not find any difference in terms of host-plant preference between cytotypes.

However, it is possible that these two cytotypes coexist with a long-term equilibrium. Mixed populations could be maintained through plant rotation and the recurrent introductions of cytotypes. A high *B. tabaci* individual flow between glasshouses is supported by the fact that observed geographical differentiation is very low: only 1% F_{ST} between localities within a country, and 2% between countries. This low differentiation is in agreement with previous results.[29,39] This is probably due to the commercial exchanges of vegetable and ornamental crops (see above and Byrne *et al.*[72]). Clearly, a temporal survey coupled with an analysis of the nuclear compartment would provide more insight into the evolution of the Q1 and Q2 distribution in Europe.

5 CONCLUSION

The first detection of the Q2 cytotype of *B. tabaci* MED species in Western Europe (France and Spain) is reported here. A short survey over a period of 5 years indicated that Q2 seems to increase in frequency, but without the exclusion of the Q1 cytotype. Both cytotypes are always found in sympatry, except in one locality (Lyon, France). Genetic analysis showed hybridisation of the two cytotypes in the field, in spite of a very high differentiation observed among allopatric sampling. This interfertility is confirmed by crossing experiments in the lab. These results are consistent with the phylogenies based on *mtCOI*, which regroup Q1 and Q2 cytotypes in the same MED species, and indicate that symbionts do not prevent hybridisation between Q1 and Q2. The present data also show

that the stable associations of mitochondrial haplotypes with bacteria confer a biological reality to the Q1 and Q2 cytotypes.

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