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1 **Running title:** Biological relevance of *Bemisia tabaci* MED species cytotypes

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

4

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16

17

18

1 **ABSTRACT**

2 BACKGROUND: The taxonomy of the species complex *Bemisia tabaci* is still an unresolved issue.
3 Recently, phylogenetic analysis based on *mtCOI* identified 31 cryptic species. However,
4 mitochondrial diversity is observed within these species associated with distinct symbiotic bacterial
5 communities forming associations called here cytotypes. We investigated the biological significance
6 of two cytotypes (Q1 and Q2) belonging to the Mediterranean species, which were only found in
7 allopatry in the Western Mediterranean to date. We sampled over few years in Western Europe
8 (France and Spain) and found sympatric situations which allowed testing their reproductive
9 compatibility in the field by using microsatellite and insecticide resistance loci.

10 RESULTS: Field survey indicated that, despite its recent introduction, Q2 is well established in
11 France and Spain where it coexists with Q1. Microsatellite data showed that, in allopatry, Q1 and
12 Q2 are highly differentiated while there is few or no genetic differentiation when they coexist in
13 sympatry suggesting high rate of hybridization. Crossing experiments in the lab confirmed their
14 interfertility.

15 CONCLUSION: Q1 and Q2 hybridize which confirm that they belong to the same species despite
16 high degree of genetic differentiation at cytoplasmic and nuclear levels and also suggest that their
17 symbiotic bacteria are not involved in reproductive incompatibility.

1 INTRODUCTION

2 *Bemisia tabaci* is one of the most devastating pests of the world, directly through plant sap feeding
3 and indirectly by virus transmission.¹ Historically, based on significant variations between
4 populations, biotypes had been identified using ecological traits like host plant range, virus
5 transmission abilities, insecticide resistance, enzymatic variability and genetic markers such as
6 mitochondrial cytochrome oxidase gene (*mtCOI*).²⁻⁴ But, because some of these characters are
7 shared between genetic groups or show within-group variability higher than between group
8 variations, the term biotype is in fact inappropriate.⁵ Based on a 3.5% *mtDNA* sequence divergence
9 criterion, it has been proposed that *B. tabaci* is actually made up of at least 24 morphologically
10 indistinguishable species^{5,6} and, recently, this number has been increased to 31.⁷⁻¹⁰ Belonging to 11
11 major groups, these putative species regroup the previously described biotypes. This view is
12 consistent with some genetic data accumulated on reproductive incompatibilities within the
13 *B. tabaci* species complex.^{11,12} Beside the number of species in *B. tabaci* complex, several questions
14 remain open such as whether the *mtDNA* sequence divergence threshold used for species
15 boundaries identification (3.5%) is well founded. It has been suggested that this value is more or
16 less arbitrary and probably under-estimated. For example, Lee *et al.*¹⁰ proposed to increase this
17 threshold up to 4% because there is a lot of variability within species, particularly within the
18 Mediterranean (MED) species which appears to be genetically diverse, not only because it includes
19 the J, ASL, L and the highly damaging Q biotypes,⁵ but also because high genetic variability has
20 been observed within the Q biotype leading to the recognition of 4 *mtCOI* haplotypes (Q1 to
21 Q4).^{13,14} This variability could be explained by the fact that MED species may regroup several
22 entities with high genetic differentiation and possible separate species, challenging MED species
23 status. We thus clearly need more information to determine what are the true interbreeding limits of
24 the putative species within the *B. tabaci* complex, and whether some reproductive incompatibilities
25 occurs within groups recently described as putative species.

1 In addition to the uncertainty of the *mtDNA* divergence threshold proposed for species recognition,
2 another challenging point concerns mitochondrial DNA marker, which is probably not the best
3 marker to identify the limit of species. As in a number of other arthropods, *mtDNA* undergoes
4 indirect selection due to vertically transmitted microorganisms invading host populations.¹⁵ Linkage
5 disequilibrium between *mtDNA* haplotypes and bacterial endosymbionts may blur the clade history
6 and the history of infection. Moreover, horizontal transfers of bacterial endosymbionts followed by
7 their invasion in the host population can result to rapid spread of *mtDNA* lineages without any
8 reproductive barriers. This is achieved by their ability to manipulate host reproduction through
9 different ways notably by inducing cytoplasmic incompatibility (for review see Engelstädter &
10 Hurst,¹⁶). In addition to their effect on host reproduction, symbiotic bacteria may also play an
11 important role in host ecology, host evolution and speciation via a wide range of effects (review in
12 Ferrari & Vavre,¹⁷) such as resistance to natural enemies,¹⁸ resistance to insecticides and host-plant
13 specialization.^{19,20} They can also increase their host fitness in some contexts.²¹ Moreover, they can
14 be involved in the emergence of nuclear variation which can lead to speciation.²²

15 *B. tabaci*, like most phloem-feeding insects, harbours an obligatory maternally transmitted
16 endosymbiont, *Portiera aleyrodidarum*, which provides necessary nutrients. *B. tabaci* also hosts a
17 wide diversity of facultative bacteria since seven secondary endosymbionts, all maternally
18 transmitted, were reported.^{23,24} This symbiotic community appears species specific but also show
19 variations among species.^{13,25,26} For example, the 4 haplotypes identified within the MED species
20 harbour distinct bacterial communities.^{13,25,26} Q1 harbours *Hamiltonella* and *Cardinium* whereas
21 Q2 contains *Rickettsia* and *Arsenophonus*. *Wolbachia* can be found in both Q1 and Q2 but was
22 never observed in Q3, which hosts a separate *Arsenophonus* strain and *Rickettsia*. Q4 is a rare
23 haplotype only detected in Croatia and for which bacterial infection remains unknown. In this
24 paper, we will use the term 'cytotype' to designate a *mtCOI* haplotype associated with a specific
25 combination of symbiotic bacteria. These cytotypes have different geographic localizations since

1 Q3 was recorded only in Africa whereas Q1 is localized in the large west part of the Mediterranean
2 basin: Morocco, Tunisia, Portugal, France, Greece and Spain.^{13,27-29} All data available at the time on
3 the *B. tabaci* MED species in France and Spain indicated the mere presence of the Q1 cytotype.^{27,30-}
4 ³³ This cytotype has been found in several parts of the world like in China and America, while Q2 is
5 restricted to the Middle East countries of Mediterranean basin: Israel and Cyprus where Q1 is
6 absent.^{26,34,35} Q1 and Q2 cytotypes overlap in neighboring countries such as Turkey, Egypt and
7 Syria (Genbank records).^{6,31} As these two cytotypes harbour very different bacterial communities,
8 MED species provides a good framework to test and understand the role of these communities in
9 the *B. tabaci* species complex ecology and evolution. This particular situation also gives good
10 opportunities to test whether the 3.5% *mtCOI* threshold reflect the true limit of species within the
11 *B. tabaci* species complex in which bacterial endosymbiont infection is pervasive. This is
12 particularly important for pest management perspectives.

13 In this study, we report the presence of the Q2 cytotype in Western Europe in sympatry with Q1. We
14 followed the dynamic of colonization of the Q2 cytotype in France and Spain, which coexist locally
15 with Q1 cytotype. These sympatric situations give us the opportunity to test the biological limit of
16 Q1 and Q2 cytotypes by the use of microsatellite markers (expected to be neutrals) and nuclear
17 genetic differentiation analysis. We also analyzed insecticide resistance genes (expected to be under
18 selection) because of extensive use of insecticides to control *B. tabaci* populations.³⁶ Moreover, we
19 tested whether symbionts are involved in any nuclear differentiation and reproductive barriers.

1 **2 MATERIAL AND METHODS**

2 **2.1 *Bemisia tabaci* field sampling**

3 531 individuals from 10 localities were collected in France and Spain from 2006 to 2010 for a
4 temporal survey. Adults were collected in greenhouses on different hosts including ornamental
5 plants and vegetables (Table 1). Sampled adults were placed alive in ethanol (96%) and stored at
6 -20°C until DNA extraction. Besides the populations sampled in France and Spain in order to have
7 Q1 and Q2 “reference” cytotypes, we used samples from Greece and Israel where only one Q
8 cytype had been reported to date, Q1 in Greece and Q2 in Israel. This is confirmed by the recent
9 study of Gauthier et al (this issue).

10 **2.2 Insect lines and rearing**

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

18

19 **2.3 Crossing experiments**

20 Q1 and Q2 *B. tabaci* pupae were isolated and sexed from one to four hours after emergence. All
21 four possible crosses were performed (crosses between Q1 males and females, crosses between Q2
22 males and females, crosses between Q1 males and Q2 females and reciprocal crosses) with 10
23 replicates each. Five females and males were placed on 30mm diameter excised *Hibiscus* leaf disks
24 on 1% agar medium in a 50mm Petri dish for 6 days. All laid eggs and hatched eggs were counted

1 as well as emerging adults, which were also sexed to determine the offspring sex-ratio.

2

3 **2.4 Molecular Biology**

4 *2.4.1 DNA extraction*

5 For each individual total DNA was extracted in 26 μ L of a buffer containing 50mM KCl, 10mM
6 Tris-base pH 8, 0.45% Nonidet P-40, 0.45% Tween 20, and 50mg/mL Proteinase K. After 3h at
7 65°C samples were incubated at 100°C for 15min. A volume of 35 μ L of pure water was added to
8 this extract which was then stored at -20°C until use.

9

10 *2.4.2 mtCOI haplotype determination*

11 Cytotypes were identified by using a PCR-RFLP diagnostics tool as described in Henri *et al.*,
12 (submitted),³⁷ which allows the discrimination of all cytotypes within the MED species. Briefly,
13 amplification was performed on a part of the mitochondrial cytochrome oxidase 1 gene (*mtCOI*)
14 using the primers C1-J-2195 (5'-TTGATTTTTTGGTCATCCAGAAGT-3') and L2-N-3014 (5'-
15 TCCAATGCACTAATCTGCCATAATA-3').³⁸ PCR reactions were performed in 25 μ L volumes
16 containing 2.5 μ L Buffer, 200 μ M dNTP, 200nM primers, 0.5U DNA polymerase (*DreamTaq*,
17 Fermentas) and 2 μ L of DNA template. The cycling conditions consisted of an initial denaturation at
18 95°C for 5min followed by 30 cycles of 1min at 95°C, 1min at 50°C, 1min at 72°C and a final
19 extension at 72°C for 10min. Some of the PCR products (2 to 20 individuals per population) were
20 also sequenced for confirmation.

21

22 *2.4.3 Detection of endosymbiotic bacteria*

23 The six most frequent secondary symbiotic bacteria found in *B. tabaci* (*Wolbachia*, *Cardinium*,
24 *Hamiltonella*, *Rickettsia*, *Arsenophonus* and *Fritschea*) were screened for each individual using

1 specific PCR primers, as described in Gnankiné et al.³⁹ All samples were tested for the presence of
2 the obligatory symbiont *Portiera aleyrodidarum* to test for the quality of the DNA extraction.
3 Primers and PCR conditions are indicated in table 2.

4

5 2.4.4 Insecticide-resistant alleles detection

6 Resistant and susceptible alleles were identified in two genes associated with insecticide resistance
7 in *B. tabaci*: the *para*-type voltage gated sodium channel and the *ace1* genes. Two mutations in the
8 *para*-type voltage gated sodium channel gene, L925I and T929V (r1 and r2 alleles) were sought,
9 and one mutation in the acetylcholinesterase enzyme *ace1* (F331W, R allele). These mutations
10 confer resistance to pyrethroids (Pyr) and organophosphates (OPs) respectively. L925I and F331W
11 resistant mutations were identified by using the PCR-RFLP tool developed by Tsagkarakou *et al.*⁴⁰
12 The T929V resistant mutation was detected by PCR using primer pairs that amplified only one of
13 the two alleles.⁴⁰ Screening was performed on 7 to 29 individuals per population (Table 3). Twelve
14 ambiguous PCR products were sequenced for verification.

15

16 2.4.5 Microsatellite genotyping

17 7 microsatellites loci were used as neutral nuclear markers to study genetic differentiation among
18 populations and sympatric cytotypes (Table 4). *B. tabaci* is a haplo-diploid species, *i.e.* males hatch
19 from unfertilized eggs and thus are haploids, and therefore genotyping was done on females only.
20 Since in Greece and Israel, only one Q cytotype has been described yet (Q1 and Q2 respectively),
21 we expected that they have never been hybridized. In the present study, we have considered
22 populations located in these countries as reference for Q1 and Q2 cytotypes. Around 30 individuals
23 were genotyped from each (Table 1). Among the 531 individuals collected in France and Spain
24 between 2006 and 2010, 242 individuals were genotyped from four localities where Q1 and Q2

1 cytotypes were found in sympatry (2 sites per country).

2 PCR reactions were performed separately for each primer pair in 10 μ L volumes containing 200 μ M
3 of dNTP, 200nM of each primer (fluorescently labelled in 5') and 0.5IU Blue Taq DNA Polymerase
4 (Eurobio). Cycling conditions were initial denaturing at 94 $^{\circ}$ C for 2 min, followed by 35 cycles of
5 30 sec at 94 $^{\circ}$ C for denaturation, 30 sec at 52 or 57 $^{\circ}$ C depending on the primer set (Table 4) for
6 annealing and 1 min at 72 $^{\circ}$ C for elongation, with a final elongation at 72 $^{\circ}$ C for 10 min. Fluorescent
7 amplicons were loaded on an ABIPrism 3100-Avant Genetic Analyser (Applied Biosystem)
8 automated sequencer and allele sizes were calculated using GeneMapper software (Applied
9 Biosystems).

10

11 **2.5 Genetic data analyses**

12 The mean number of alleles per locus (A^{-}), the observed heterozygosity (HO) and the unbiased
13 expected heterozygosity (HE) were calculated using Genetix. Weir and Cockerham's estimators of
14 F_{IS} within populations and genetic groups were calculated using Genepop, as well as exact tests for
15 Hardy-Weinberg equilibrium.^{41,42} Linkage equilibrium between all pairs of loci and F estimates of
16 differentiation⁴¹ were calculated and tested with 5000 permutations using Genepop. The level of
17 population structure was assessed using the software STRUCTURE⁴³ which differentiates mixed
18 populations on the basis of allele frequency at each locus. Following options were used: 500 000
19 Markov Chain Monte Carlo with a burn-in period of 50 000, we allowed for admixture and
20 correlated allele frequencies; log-likelihood estimates were calculated for K=1 to 15 with 10
21 replicates each. STRUCTURE assumes Hardy-Weinberg equilibrium (HWE) and linkage
22 equilibrium within each group, that's why both hypotheses were tested subsequently using exact
23 tests implemented in Genepop 4.0. We also conducted a Principal Component Analysis (PCA),
24 which does not make any assumption such as HWE and linkage equilibrium, with R software,⁴⁴
25 *adeigenet* package.⁴⁵ In addition, a hierarchical analysis of molecular variance (AMOVA) was

1 performed, using *hierfstat* package,⁴⁶ implemented in R software, to test for geographical, host-
2 plant, cytotype and secondary symbionts effect. Significance was assessed with permutation tests (5
3 000 permutations). Resistant alleles frequencies were subjected to Fisher's exact test with simulated
4 p-values based on 1000 replicates using R statistical software,⁴⁷ while HWE was tested by X^2 with
5 R statistical software.⁴⁷

6

1 **3 RESULTS**

2 **3.1 Field survey in France and Spain**

3 From 2006 to 2010, a survey of *B. tabaci* populations was performed in France and Spain to follow
4 the geographical distribution of *B. tabaci* cytotypes. 511 individuals from 10 localities were
5 collected (Table 5). We also add data from Dalmon et al.,²⁷ and Gueguen et al.,¹³ collected from
6 2003 to 2006 (302 individuals from 14 localities) for reference situation (Figure 2A). Almost all
7 individuals belong to the MED species, except in two localities, where the MEAM1 species has
8 been observed, in a botanical garden in Nice (France) in 2004 and in Cabrils (Spain) in 2007.
9 However, as this situation is an exception and concern only very few individuals, MEAM1 species
10 will not be taken into account and discussed further in this study. From 2003 to 2006, all the
11 individuals belonged to the Q1 cytotype, as observed in all the western part of the Mediterranean
12 basin. Interestingly, in 2007, for the first time, Q2 cytotype was detected in several localities either
13 in sympatry with Q1 individuals in four locations in France and Spain, or not: only Q2 was found in
14 very high density in a greenhouse in Lyon (France). Co-existence of Q1 and Q2 cytotypes has also
15 been observed in 2010 in all the sampling sites (Figure 2). Proportion of Q2 individuals in the
16 situation of sympatry varied according to the collection from 6% to 83% in 2007. They
17 predominated in three (83%, 71% and 64%) of four localities in 2010 (22% of frequency in the site
18 where Q1 prevails).

19

20 **3.2 Analysis of reference Q1 and Q2 cytotypes**

21 To date, only Q1 cytotype has been detected in Greece and Q2 in Israel.^{26,48,49} In our sampling, the
22 determination of the *mtCOI* cytotype of individuals collected in three localities in Greece (10 per
23 locality) and one site in Israel (n=29) confirmed these data. We therefore used them as reference
24 samples for Q1 and Q2 cytotypes.

25

1 3.2.1 Endosymbiotic community

2 Individual detection of the seven main bacteria infecting *B. tabaci* revealed that these cytotypes
3 harbour a specific symbiotic community as already reported.^{13,26} The most common bacteria are
4 *Hamiltonella* for Q1 and the association *Arsenophonus/Rickettsia* for Q2 (Figure 1).

5

6 3.2.2 Detection of sodium channel and *ace1* resistant mutations

7 Resistance mutations in *ace1* (F331W) and *para-type voltage gated sodium* (L925I and T929V)
8 genes were identified by PCR-RFLP or PASA-PCR.⁴⁰ . The three mutations were detected in both
9 cytotypes: F331W (R allele) is fixed in Q1 and Q2 while L925I (r1 allele) has intermediate
10 frequencies in the two cytotypes (0.70 and 0.74 respectively). Q1 and Q2 cytotypes significantly
11 differ only in the frequencies of the T929V mutation (r2 allele ; 0.02 and 0.29 respectively; Fisher's
12 exact test: $p < 10^{-4}$).

13

14 3.2.3 Microsatellite analysis

15 The genetic analysis of the 59 individuals genotyped with 7 microsatellites loci revealed a higher
16 allelic richness in the Q1 than in the Q2 cytotypes (4.3 *versus* 3.4 alleles; Table 5). Analysis carried
17 out using STRUCTURE revealed a very high genetic differentiation between Q1 and Q2 cytotypes,
18 which is confirmed by PCA (Figure 1). Pairwise F_{ST} value reaches 0.27, which is very high for two
19 groups that belong to the same putative species.

20

21 3.3 Nuclear and cytoplasmic differentiation of sympatric Q1 and Q2 cytotypes

22 3.3.1 Endosymbiotic community

23 *Fritschea* was never detected and will not be further discussed. The bacterial screening did not
24 reveal any new association between a mitochondrial haplotype and the symbiotic community
25 compared to reference populations (Israeli and Greek), meaning that the cytoplasmic associations

1 are stable (Figure 3). In Q1 cytotype, *Hamiltonella* is almost fixed (93% of individuals infected)
2 while *Wolbachia* and *Cardinium* are present at intermediate frequencies (respectively 28% and
3 38%), with a high frequency of bi-infections (62%). In Q2 cytotype, *Rickettsia* is also almost fixed
4 (95%) while *Wolbachia* and *Arsenophonus* are found at a lower frequency (respectively 69% and
5 62%). In Lyon *Wolbachia* is absent.

7 3.3.2 Detection of sodium channel and *ace1* resistant mutations

8 In the French and Spanish sympatric populations sampled in 2010 (242 females tested), all the three
9 resistant mutations were detected in both Q1 and Q2 cytotypes in all the localities with a high
10 variability in frequency (Table 3). All individuals were homozygous for F331W (R allele) of *ace1*
11 gene. L925I (r1 allele) was highly frequent (0.56 to 1) and frequencies of T929V (r2 allele) were
12 low (0 to 0.30). These frequencies didn't differ between Q1 and Q2 cytotypes within all the four
13 localities (Fisher's exact tests: $p > 0.05$). No significant deviations from Hardy-Weinberg
14 equilibrium were detected between genotype frequencies within three localities (regardless of the
15 cytotype involved) for both *kdr* mutations (Fréjus, Saint-Laurent du Var, and Viladecans, X^2 :
16 $p > 0.05$). Only the population from Cabrils deviates slightly from HWE for T929V (X^2 : $p = 0.01$).

18 3.3.3 Microsatellite analysis

19 The 242 females previously used for resistant mutation detection, were genotyped based on 7
20 microsatellites loci (Table 5). A significant deviation from HWE ($p < 0.001$) was observed within all
21 localities caused by a systematic heterozygous deficiency which explain the high F_{IS} values within
22 localities (0.243 to 0.357). This deficiency is not due to a particular locus, since all the observed
23 heterozygosity is inferior to the expected heterozygosity in all loci. Within the four localities, the F_{IS}
24 is not higher when all individuals are consider than when cytotypes are considered separately (Table
25 5) thus suggesting that high F_{IS} are not explained by a genetic structure that differ among cytotypes.

1 This result is confirmed by STRUCTURE software analysis, which include sympatric Q1 and Q2 as
2 well as the Q1 (Greek) and Q2 (Israeli) reference cytotypes since three clusters have been
3 identified. The first cluster regroups all the Q2 reference individuals (from Israel). All Greek Q1
4 individuals are assigned to a second cluster. Individuals of sympatric population whatever Q1 or Q2
5 cytotypes belong to a third cluster but for the majority of them they are assigned with different
6 probabilities to the Greek cluster and this third one. The probability of individual assignment to
7 each cluster is shown in figure 4. Sympatric Q1 and Q2 cytotypes thus show similar genetic
8 composition that probably results from the hybridization of two entities one being close to Greek
9 population. The principal components analysis (PCA, Figure 5) of allele frequencies confirms the
10 STRUCTURE software Bayesian analysis. The first axis clearly separates the three groups: mixed
11 Q1-Q2 (Western part), reference Q1 (Greece) and reference Q2 (Israel). The second axis weakly
12 discriminated between Greek Q1 and mixed populations. Pairwise comparisons between localities
13 of these geographic groups are very high ($0.09 < F_{ST(\text{Mixed-Greece})} < 0.14$; $0.29 < F_{ST(\text{Mixed-Israel})} < 0.33$). The
14 point is that Q1 and Q2 cytotypes are totally homogeneous when they are sympatric.

15

16 We have successively tested for the effects of plant and symbiotic compartment on genetic
17 structuring within the four localities. The AMOVA was not significant ($p > 0.1$) for all these factors.
18 F-statistics show little but significant differentiation between pairs of these four French and Spanish
19 localities (F_{ST} ranging from 0.01 to 0.02, $p < 0.01$), meaning a weak geographical differentiation as
20 the only structuring factor. Allelic richness is quite homogeneous among the four localities where
21 sympatric cytotypes were observed (values ranging from 4.3 to 4.9), which is not significantly
22 higher than allelic richness detected within localities from reference Q1 (Greek) and Q2 (Israeli)
23 cytotypes.

24

25 *3.3.4 Crossing experiments*

1 Viable offspring were obtained in all the crosses performed suggesting there is neither nuclear nor
2 cytoplasmic incompatibilities between Q1 and Q2 cytotypes. The mean number of eggs laid and the
3 percentage of non-hatched eggs are indicated in table 6. The $Q1_{\text{♀}} \times Q1_{\text{♂}}$ cross produces more eggs
4 than the three others (Tukey HSD test: $p < 0.001$), but the percentage of hatched eggs is lower
5 (24%, Tukey HSD test: $p < 0.001$). The number of offspring is slightly higher in the $Q1_{\text{♀}} \times Q2_{\text{♂}}$
6 cross than in the others. We have verified that F1 individuals (males and females) were fertile and
7 thus able to produce viable offspring.

8

9

1 **4 DISCUSSION**

2 **4.1 Cytotype distribution in Western Europe**

3 *B. tabaci* is widely distributed in France and Spain and putative MED species largely predominates
4 in these areas as in the most part of the Mediterranean basin. MED species has been found in all
5 Spanish and French localities where we performed our sampling from 2003 to 2006. In very few
6 areas, MEAM1 putative species had been observed in France (Nice) in 2004 and in Spain (Cabriils)
7 in 2007 but this presence is anecdotal and will not be discussed further. Until 2007, Q1 was the only
8 cytotype of the MED species found in France and Spain, and more generally in the large West part
9 of the Mediterranean basin.^{27,50} In 2007, we detected for the first time the Q2 cytotype in four out of
10 the six localities considered in France and Spain. In 2010, Q2 was found in four other localities,
11 always in sympatry with Q1. Because the initial situation in these countries were well known, we
12 interpret this observation as a recent introduction of Q2 cytotype, which has also been observed in
13 other localities in the Western Mediterranean basin (Gauthier et al., in this issue). This introduction
14 is probably due to trade, which occur with high intensity among Mediterranean countries. Indeed, in
15 France, ornamental plants grow up in greenhouses, but the sowing and cloning are mostly
16 performed in others countries such as Israel, Kenya, Ivory Coast, Germany, Spain, Italy (Terraz,
17 pers. Com). We can suppose that Q2 was introduced from the Eastern part of the Mediterranean
18 basin (middle east) since it has always been detected over there,^{13,31,34} and now is spreading in the
19 basin. These results raise two questions: how the Q2 cytotype had been able to settle in these
20 localities while Q1 was already installed and at which geographic scale this situation occurs in the
21 Mediterranean basin? Extensive surveys of *B. tabaci* populations at a larger geographical scale
22 would give important insights to answer to these questions.

23

24 **4.2 Q1 and Q2 differentiation and hybridization**

25 Cytotypes are identified on the basis of mitochondrial DNA and their symbiotic bacteria.¹³ Until

1 2007, Q1 and Q2 cytotypes were considered allopatric in the Mediterranean basin with Q2 only
2 known in Israel, but also possibly present in border countries, while Q1 had a larger distribution.
3 This separate distribution is consistent with the very high nuclear genetic differentiation observed
4 between Q1 reference cytotype (Greece) and Q2 reference cytotype (Israel), about 29% F_{ST} for
5 microsatellite markers. Such a value is comparable with those obtained between distinct *B. tabaci*
6 species such as MED and MEAM1.^{51,52} This divergence indicates that they probably not hybridized
7 recently, as they had not in contact, leading us to consider these as reference cytotype, even if the
8 nuclear compositions observed in these countries are probably not representative of all the diversity
9 that can exist in each cytotypes. Moreover, this small sampling could explain high differentiation
10 values and the detection of Israeli Q2 specific alleles (many alleles from Q2 reference cytotype are
11 not found anywhere else). Our Q2 reference is then certainly not the population source of the
12 Western Q2 cytotypes as suggested by microsatellites analysis comparison of allopatric and
13 sympatric populations. Populations experiencing strong bottleneck during colonisation process or
14 pesticide treatments inducing reduced population effective size could also explain this high
15 differentiation.

16 This new situation with Q1 and Q2 cytotypes observed in sympatry in France and Spain gives the
17 opportunity to test their interfertility and the limits of the MED species, within the framework of the
18 *mtCOI* phylogeny of the *B. tabaci* complex species.^{5,10} The microsatellite analysis revealed three
19 highly differentiated clusters: Israeli Q2 reference, Greek Q1 reference and the Western mixed
20 cytotypes. These results indicated that Q1 and Q2 are homogeneous in sympatry (*i.e.* in France and
21 Spain) and clearly demonstrates their full hybridization and the absence of any reproductive barrier
22 amongst them. They also do not differ in the insecticide resistant-alleles frequency for the two
23 genes we considered, *i. e.* the *ace1* (resistance is fixed) and the *para*-type voltage gated sodium
24 channel genes. Further studies could focus on other resistance mechanisms such as over-expression
25 of cytochromes.^{53,54} In addition to hybridization in the field, crossing experiments in the lab confirm

1 that Q1 and Q2 individuals can interbreed and produce fertile offspring, indicating that there is no
2 nuclear incompatibility between them and that, as indicated by phylogenetic,⁵ they belong to the
3 same species.

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6 **4.3 Biological relevance of Q1 and Q2 cytotypes**

7 *4.3.2. Cytotypes stability*

8 Q1 and Q2 freely interbreed but harbour different symbiont communities even when they coexist in
9 sympatry (Q1 is infected by *Hamiltonella* and *Cardinium*, while Q2 is infected by *Rickettsia* and
10 *Arsenophonus*). This means that CO1 haplotype and bacterial associations are relatively stable at
11 time scale of several years, thus giving biological relevance to cytotypes under the hypothesis of a
12 role of bacteria on *B. tabaci* phenotype. The absence of new mitochondrial haplotype/bacterial
13 community associations can be explained either by the absence or rare events of horizontal transfers
14 or the impossibility of these bacteria to colonize a new cytoplasmic and/or nuclear environment.
15 Symbiotic vertically-bacteria are known to be horizontally transferred. Horizontal transmission can
16 occur through the diet between individuals that feed on the same host plant as demonstrated in
17 aphids and fruit flies,^{55,56} or during mating.^{57,58} Transfers can also occur between hosts and
18 parasitoids.^{59,60} Phylogenetic analysis revealed the existence of such transfers in *B. tabaci*.⁶¹
19 Moreover, lab experiments demonstrated that *Rickettsia* can be horizontally transmitted between *B.*
20 *tabaci* individuals through the host plant.⁶² Therefore a total absence of horizontal transfer is
21 unlikely but since the contact of Q1 and Q2 cytotypes occurred recently, lateral transfer between
22 cytotypes didn't occur yet (at the time scale of this study). Another explanation could be that new
23 *mtCOI*/bacteria associations are unstable either because of a maladaptation of the transferred
24 bacteria in the new environment or exclusion by the recipient cytotype. This exclusion can be
25 achieved by the host nuclear genes or by the symbionts already present. As the two cytotypes

1 interbreed and that we didn't detect any new bacterial infection neither in Q1 nor in Q2, we can
2 suppose that their nuclear compartments are homogeneous. Therefore the existence of nucleo-
3 cytoplasmic incompatibilities when a bacteria integrates the other cytotype is unlikely; even if we
4 can't exclude that some nuclear genes specific to each cytotype could be involved in bacterial
5 exclusion. Within-host, multiple infections could select for competitive symbionts monopolizing
6 resources at the expense of the other, allowing their own vertical transmission. There are some
7 examples of such cytoplasmic competition between bacteria (Goto, 2006; Oliver, 2006).^{63,64} The
8 stability of the *mtCOI*/symbiotic community associations indicated that the concept of cytotype has
9 a real biological significance.

10 4.3.2 *Effects of bacteria*

11 Q1 and Q2 hybridize despite different symbiotic communities infection, suggesting that bacteria
12 don't induce cytoplasmic incompatibility in *B. tabaci*, or weakly, which is consistent with the study
13 of Dalmon et al.,²⁷ on *Wolbachia* and *Cardinium*. This is confirmed in our study by laboratory
14 reciprocal crosses. However, as symbionts necessarily induce a physiological cost on their host^{64,65},
15 they are probably involved in another phenotypic effects that can cause their spread and
16 maintenance into the host populations (review in Ferrari & Vavre,⁶⁶). Some symbiotic bacteria
17 manipulate host reproduction and increase their transmission but apparently it is not the case in
18 *B. tabaci*. Others have a mutualistic strategy and confer direct fitness benefit to their host such as
19 protection against natural enemies,^{18,67} thermal tolerance,^{68,69} or host plant specialisation.²⁰ In
20 *B. tabaci*, there is little information about the phenotypic effects of the symbionts. It has been
21 demonstrated that *Rickettsia* enhance host fitness by increasing fecundity and survival to adulthood,
22 ²¹ *Rickettsia* is also suspected to increase sensibility to insecticides,¹⁹ and *Wolbachia* could protect
23 *B. tabaci* from parasitoids.⁷⁰ In Q1 and in Q2, one symbiont is fixed in natural populations,
24 *Hamiltonella* and *Rickettsia* respectively, while another is present at intermediate frequencies
25 (around 38% for *Cardinium* in Q1 and 60% for *Arsenophonus* in Q2). It is possible that *Rickettsia*

1 increases host performance in the Q2 populations of France and Spain, however, this trait has only
2 been demonstrated in Q2 populations in USA, and has not been observed in Q2 populations in
3 Israel. Future studies should focus on the phenotypic effects of the bacteria present in Q1 and Q2 in
4 these mixed populations of France and Spain.

5

6 **4.4 Cytotypes coexistence**

7 The sympatry observed in France and Spain is relatively new (only since 2007): Q2 cytotpe has
8 spread in Western Europe without excluding Q1 until now. The question is thus how this situation
9 will evolve and whether these two cytotypes will coexist or if one will be excluded. Coexistence of
10 several *B. tabaci* cytotypes is uncommon and generally a transient situation which lead to the
11 displacement and/or exclusion of one of the competing cytotypes.^{50,71,72} Indeed, each greenhouse
12 can provide an environment that is more or less suitable to one cytotpe, that exclude the less
13 adapted cytotpe. In this case, we should have a mosaic of situations where only one cytotpe will
14 be present in a greenhouse but not necessarily the same cytotpe in the different greenhouses
15 located in the same locality. Situations evolve according to the effect of bacteria on host phenotype,
16 such as host-plant preference or resistance to insecticides. We didn't find any difference in term of
17 host plant preference between cytotypes. Most of resistance mechanisms are nuclear while the two
18 cytotypes freely interbreed and reference Q1 and Q2 population show similar composition of
19 resistance genes. However, *Rickettsia* (associated to Q2 cytotpe) could increase susceptibility to
20 some insecticides (Kontsedalov et al., 2008) and thus disadvantage Q2 cytotpe in such
21 environment.

22

23 However it is possible that these two cytotypes coexist in the long term. Mixed populations could
24 be maintained through plant rotation and recurrent introductions of cytotypes. This high flow
25 between glasshouses is supported by the fact that observed geographical differentiation is very low,

1 only 1% F_{ST} between localities within country, and 2% between countries. This low differentiation
2 is in agreement with previous results,²⁷ or more recently Gauthier et al., (2013, this volume). This is
3 probably due to commercial exchanges of vegetable and ornamental crops (see above and Byrne et
4 al.,⁷³). Clearly, temporal survey with analysis of the nuclear compartment is required to bring more
5 insights into the evolution of the Q1 and Q2 distribution in Europe.

7 **CONCLUSION**

8 We report here, for the first time, the detection of the Q2 cytotype of *B. tabaci* MED species in
9 Western Europe (France and Spain). A short survey over a period of 5 years indicated that Q2 seems
10 to increase in frequency but without exclusion of the Q1 cytotype. Both cytotypes are always found
11 in sympatry. Genetic analysis showed complete hybridization of the two cytotypes in the field
12 despite a very high differentiation observed among allopatric sampling. This interfertility is
13 confirmed by crossing experiments in the lab. These results are consistent with the phylogenies
14 based on *mtCOI*, which regroup Q1 and Q2 cytotypes in the same MED species and indicated that
15 symbionts are probably not involved in reproductive isolation. Our data also showed that the stable
16 associations of mitochondrial haplotypes with bacteria confer a biological reality to Q1 and Q2
17 cytotypes.

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24 Peterschmitt for sample collection. Gabriel Terraz is recipient of a PhD studentship from the Rhône-
25 Alpes region (“Program Cible” Grant).

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1 TABLES AND FIGURES

2 **Table 1.** Characteristic of the sample site locations;

3 N: number of sampled individuals.

4 *: populations used for microsatellite analysis

5 α: the first two or three letters of the acronyms correspond to the locality; the last capital letter indicates the
6 host plant.

7 **Table 2.:** Characteristics of primers used for endosymbiont screening

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

10 **Table 4.** Characteristics of the primers used for microsatellite genotyping

11 **Table 5.** Genetic characteristics of the populations. N. number of individuals; Ho. observed
12 heterozygosity; He. unbiased expected heterozygosity; A⁻. mean number of alleles per population
13 per locus

14 **Table 6.** Results of crossing experiments. Mean eggs laid per replicates (± SD) , mean hatched eggs
15 (± SD), mean percentage of hatched eggs (± SD).

16 * means significantly different from others (Tukey HSD tests, p < 0,01). The number of replicates is
17 ten in each cross.

18 **Figure 1.** Characteristics of Greek Q1 and Israel Q2 cytotypes. a) Factorial map of the principal
19 components analysis. b) Infection status of Q1 individuals c) Infection status of Q2 individuals.

20 C: *Cardinium*, W: *Wolbachia*, H: *Hamiltonella*, R: *Rickettsia*, A: *Arsenophonus*

21 **Figure 2.** Map of cytotype distribution in France and Spain by year of sampling. Red: Q1 cytotype,
22 blue: Q2 cytotype, yellow: MEAM1 species.

23 **Figure 3.** Infection status of *Bemisia tabaci* individuals

24 C: *Cardinium*, W : *Wolbachia*, H : *Hamiltonella*, R : *Rickettsia*, A : *Arsenophonus*

25 n. number of individuals

26 **Figure 4.** Clustering results from STRUCTURE for all samples.

27 **Figure 5.** Factorial map of the principal components analysis with the entire dataset.

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29

1 **Table 1.** Characteristics of sample site locations

<i>Country</i>	<i>Region</i>	<i>Locality</i>	<i>Acronym</i> ϖ	<i>Year</i>	<i>Host plant</i>	<i>N</i>
France	Midi-Pyrénées	Lavour	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	30
		Fréjus	SlvH *	2010	Hibiscus	15
	Rhône-Alpes	Lyon	FroH *	2010	Hibiscus	48
	Spain	Catalonia	Lyon	LyoV	2007	Various plants
Cabrils			CabV	2007	Various plants	91
			CabM *	2010	Melon	31
			CabT *	2010	Tomato	40
Viladecans			ViIT *	2010	Tomato	31
	ViIC *	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

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3 N: number of sampled individuals.

4 *: populations used for microsatellite analysis

5 ϖ : the first two or three letters of the acronyms correspond to the locality; the last capital letter indicates the
6 host plant.

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8

1 **Table 2.** Characteristics of primers used for endosymbiont screening

2

Endosymbiont	Targeted gene	Primers	Primer sequence	Tm	Ref.
<i>Portiera</i>			5'-TGCAAGTCGAGCGGCATCAT-3'		
<i>aleyrodidarum</i>	<i>rDNA 16S</i>	28F 1098R	5'-AAAGTTCCCGCCTTATGCGT-3'	58°C	²³
			5'-GCTCAGAACGAACGCTATC-3'		
<i>Rickettsia</i>	<i>rDNA16S</i>	Rb-F Rb-R	5'-GAAGGAAAGCATCTCTGC-3'	58°C	⁷⁴
			5'-TGAGTAAAGTCTGGGAATCTGG-3'		
<i>Hamiltonella</i>	<i>rDNA 16S</i>	Hb-F Hb-R	5'- AGTTCAAGACCGCAACCTC -3'	58°C	²³
			5'- GCGGTGTAATAAGTGAAGAAAC-3'		
<i>Cardinium</i>	<i>rDNA 16S</i>	CFB-F CFB-R	5'- ACCTMTTCTTAACTCAAGCCT-3'	56°C	⁷⁵
			5'- TGGTCCAATAAGTGAAGAAAC-3'		
<i>Wolbachia</i>	<i>wsp</i>	81F 691R	5'-AAAAATTAACGCTACTCCA-3'	56°C	⁷⁶
			5'-CGTTTGATGAATTCATAGTCAAA-3'		
<i>Arsenophonus</i>	<i>rDNA 23S</i>	Ars-23S1 Ars-23S2	5'-GGTCCTCCAGTTAGTGTTACCCAAC-3'	60°C	⁷⁷
			5'-GAGTTTGATCATGGCTCAGATTG-3'		
<i>Fritschea</i>	<i>rDNA 23S</i>	Frit-F Frit-R	5'-GCTCGCGTACCACTTTAAATGGCG-3'	62°C	⁷⁸

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

Country	Locality	Cytotype	Sodium channel										ace				
			r1 genotypes				r1 allele resistance frequencies	r2 genotypes				r2 allele resistance frequencies	Genotypes			R allele resistance frequencies	
			n	r1r1	r1s1	s1s1		n	r2r2	r2s2	s2s2		n	RR	RS		SS
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	Cabriils	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	

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1 **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'	49	50
	BtIs2.3R	5'- CAAAATTAATGGTATTGACTC-3'		
BtIS1.13	BtIs1.13F	5'- CTAAGACCGATTCCTCC-3'	49	52
	BtIs1.13R	5'- GAATACTACACCTTCAATTACC-3'		
Locus 11	11a.	5'- CCAGAAAAGTGGACTTAAGA-3'	79	57
	11b.	5'- GATCTGGGTGTTTTCTTCTA-3'		
BT83	BT83F	5'- GATGCCACAGGTTGTCTGG-3'	80	57
	BT83R	5'- GCTTGCCAGGCACTTTCTAG-3'		
BtIS1.1	BtIs1.1F	5'- CCCATAGAACACGCTCC-3'	49	57
	BtIs1.1R	5'- CATTGGAAGCCTCGAATAC-3'		
BtIS1.2	BtIs1.2F	5'- CTTACCTCCATTCACC-3'	49	57
	BtIs1.2R	5'- ATCCCGAGTCTTATGTTG-3'		
Locus 145	145a	5'- CCTACCCATGAGAGCGGTAA-3'	27	57
	145b	5'- TCAACAAACGCGTTCTTCAC-3'		

2

3

1 **Table 5.** Genetic characteristics of the populations.

Country	Locality	Plant	Q1					Q2					Q1 + Q2				
			N	Ho	He	F _{IS}	A ⁻	N	Ho	He	F _{IS}	A ⁻	N	Ho	He	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	-	-	-	-	-

2 N. number of individuals; Ho. observed heterozygosity; He. unbiased expected heterozygosity; A⁻. mean number of alleles per population per locus

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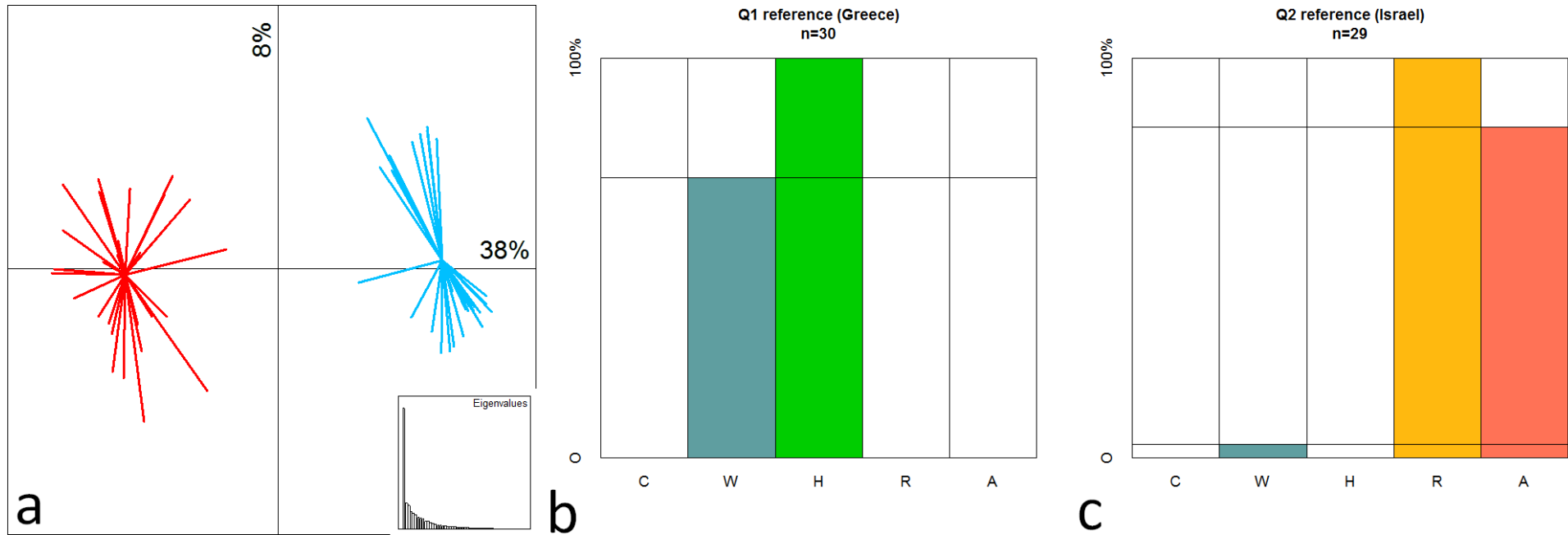
1 **Table 6.** Results of crossing experiments. Mean eggs laid per replicates (\pm SD) , mean hatched eggs (\pm SD), mean percentage of hatched eggs (\pm SD).

Cross	Q1♀XQ1♂	Q1♀XQ2♂	Q2♀XQ1♂	Q2♀XQ2♂
Eggs	127* \pm 43	75 \pm 13	40 \pm 13	65 \pm 9
Hatched eggs	32 \pm 15	48 \pm 9	32 \pm 12	39 \pm 8
Hatched eggs (%)	24* \pm 14	65 \pm 13	79 \pm 5	62 \pm 16

2 * means significantly different from others (Tukey HSD tests, $p < 0,01$). The number of replicates is ten in each cross.

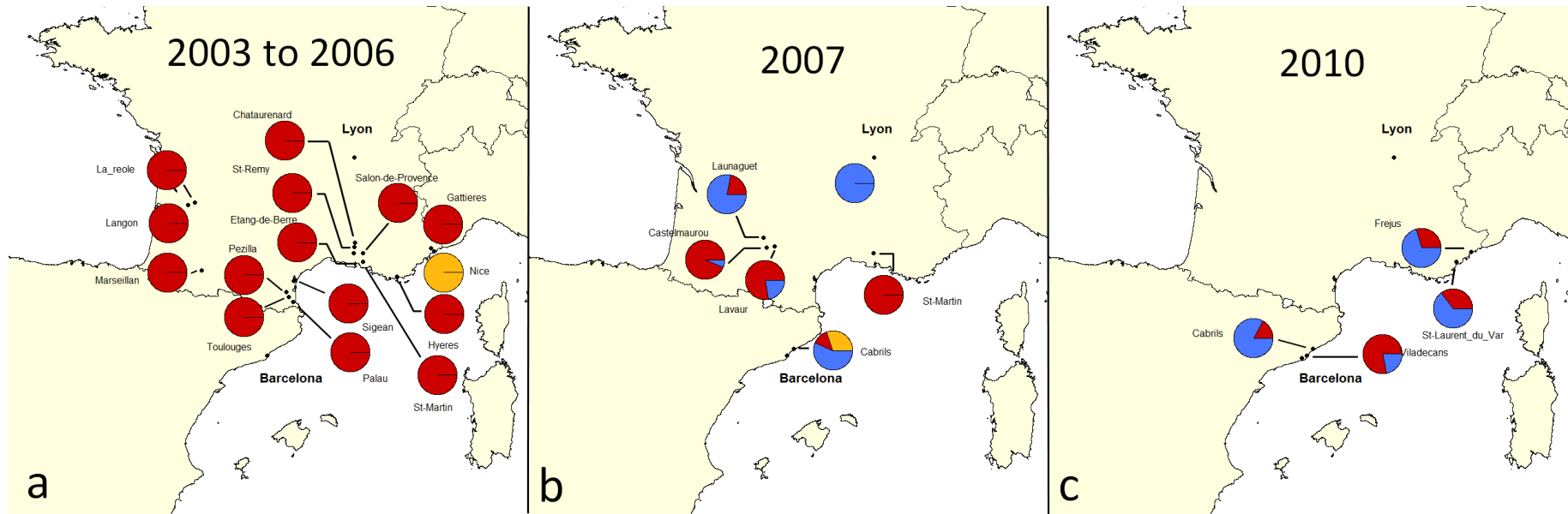
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- 1 **Figure 1.** Characteristics of Greek Q1 and Israel Q2 cytotypes. a) Factorial map of the principal components analysis. b) Infection status of Q1
 2 individuals c) Infection status of Q2 individuals



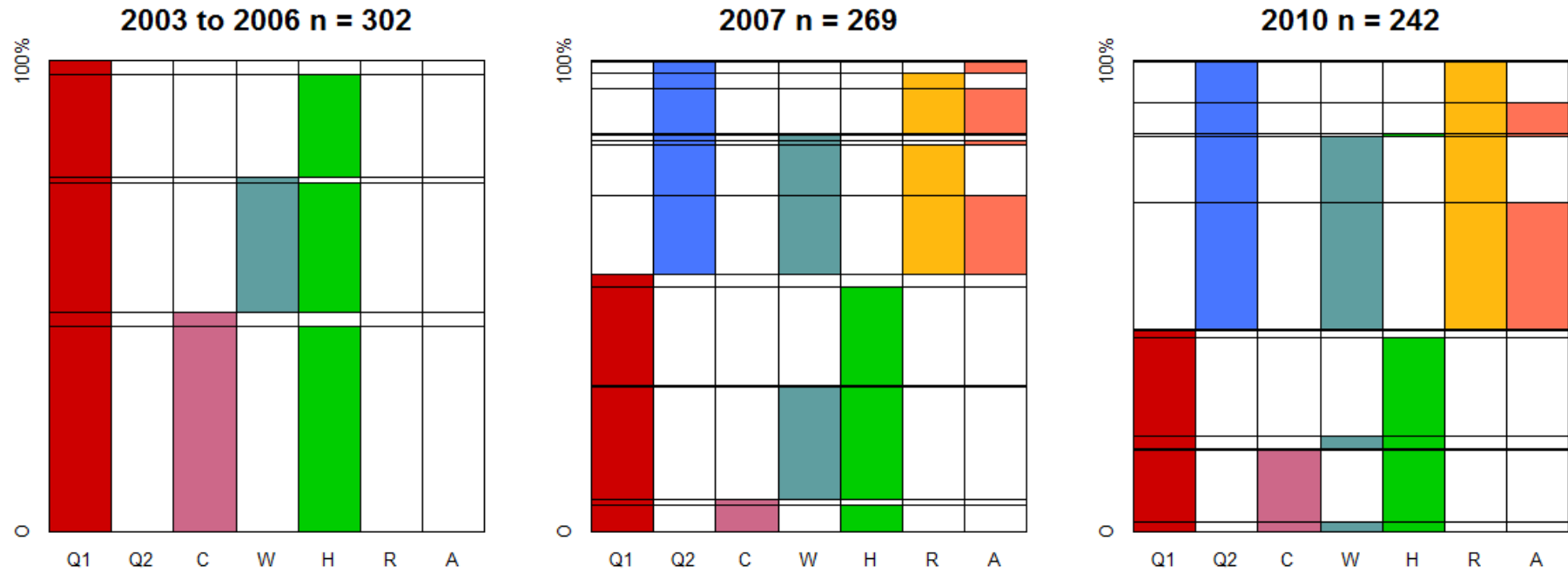
- 3 C: *Cardinium*, W: *Wolbachia*, H: *Hamiltonella*, R: *Rickettsia*, A: *Arsenophonus*

1 **Figure 2.** Map of cytotype distribution in France and Spain by year of sampling. Red: Q1 cytotype (MED species), blue: Q2 cytotype (MED
 2 species), yellow: MEAM1 species.



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1 **Figure 3.** Infection status of *Bemisia tabaci* individuals.



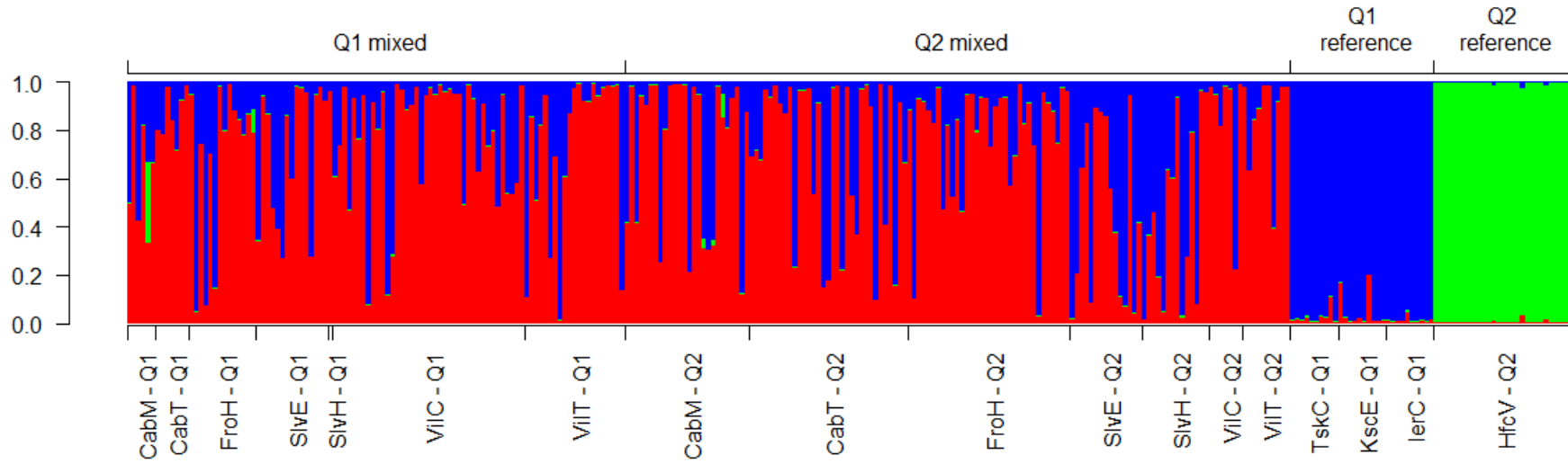
2
3 C: *Cardinium*, W : *Wolbachia*, H : *Hamiltonella*, R : *Rickettsia*, A : *Arsenophonus*

4

5

1 **Figure 4.** Clustering results from STRUCTURE for all samples.

2

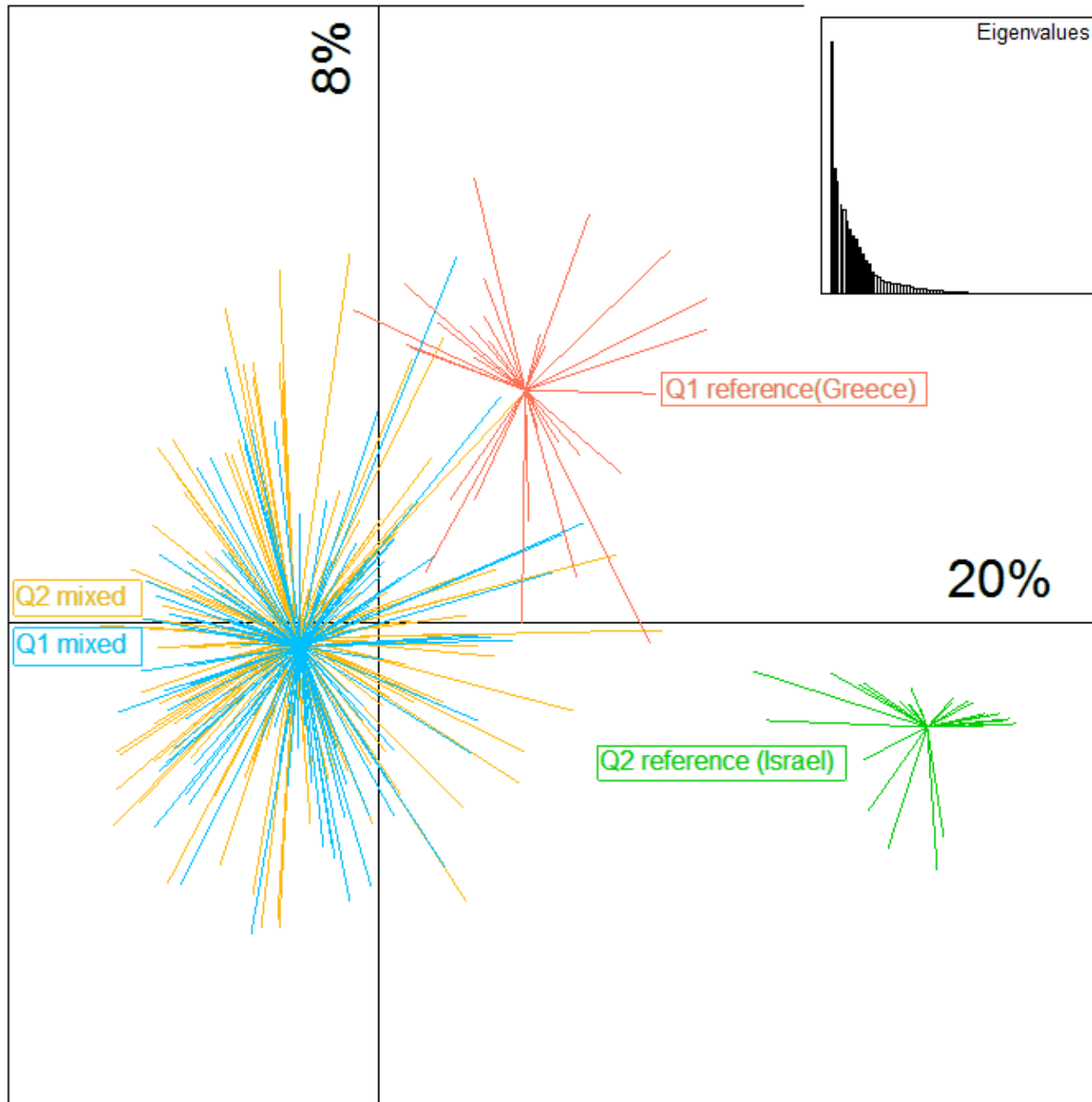


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1 **Figure 5.** Factorial map of the principal components analysis with the entire dataset.

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