Nonrandom Wolbachia Infection Status of *Drosophila melanogaster* Strains with Different mtDNA Haplotypes

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Wolbachia are maternally inherited bacteria, which typically spread in the host population by inducing cytoplasmic incompatibility (CI). In *Drosophila melanogaster*, Wolbachia is quite common but CI is variable, with most of the studies reporting low levels of CI. Surveying mitochondrial DNA (mtDNA) variation and infection status in a worldwide *D. melanogaster* collection, we found that the Wolbachia infection was not randomly distributed among flies with different mtDNA haplotypes. This preferential infection of some mtDNA haplotypes could be caused by a recent spread of mtDNA haplotypes associated with the infection. The comparison of contemporary *D. melanogaster* samples with lines collected more than 50 years ago shows that indeed one haplotype with a high incidence of Wolbachia infection has increased in frequency. Consistent with this observation, we found that the acquisition of a Wolbachia infection in a population from Crete was accompanied with an almost complete mtDNA replacement, with the Wolbachia-associated haplotype becoming abundant. Although it is difficult to identify the evolutionary forces causing the global increase of wMel, the parallel sweep of Wolbachia and an mtDNA haplotype suggests a fitness advantage of the Wolbachia infection.

Introduction

Wolbachia are intracellular α-Protobacteria that are maternally transmitted in their invertebrate hosts. They affect the biology of their host in many ways, ranging from mutualistic effects to the establishment of reproductive isolation and thus speciation (Werren 1997; Bordenstein et al. 2001; Telschow et al. 2005). The best-studied effect of Wolbachia infection is cytoplasmic incompatibility (CI), which describes, in its simplest form, the effect of reduced fertility in crosses between an infected male and an uninfected female. Levels of incompatibility depend on different factors, such as Wolbachia strain, host genotype, developmental time and age of the males, and environmental conditions (Olsen et al. 2001; Reynolds and Hoffmann 2002; Reynolds et al. 2003; Fry et al. 2004; Yamada et al. 2007).

Models for the population dynamics of Wolbachia showed that infections are expected to spread rapidly in natural populations (Caspari and Watson 1959). One example is the case of the spread of a Wolbachia infection in natural populations of *Drosophila simulans*. In California, the infection was spreading at a rate of more than 100 km/year, and populations with a low level of infection were found to be almost completely infected within 3 years (Turelli and Hoffmann 1991).

In contrast to the consistently strong CI observed in *D. simulans*, the CI observed in its close relative *Drosophila melanogaster* is much more variable (Bourtzis et al. 1996; Hoffmann et al. 1998; Poinsot et al. 1998; McGraw et al. 2002; Reynolds and Hoffmann 2002). One possible source of the heterogeneity was identified by Yamada et al. (2007), who showed that the ability to induce CI decreases with the increase in male developmental time. This "younger brother" effect is independent of Wolbachia density and distribution pattern, maternal effects, and male age. CI expression decreases both with male developmental time

and age, but their effect is independent from each other (Yamada et al. 2007). Nevertheless, this effect is less pronounced in *w*MelCS than in *w*Mel, two different Wolbachia strains (Yamada et al. 2007). In the light of the recently uncovered diversity of Wolbachia-infecting *D. melanogaster* (Riegler et al. 2005), this raises the possibility that levels of CI vary among genetically very similar Wolbachia strains.

Given the apparent differences in CI among *D. melanogaster* and *D. simulans*, Hoffmann et al. (1998) proposed that infection with Wolbachia could provide fitness benefits for the host in order to persist in the population. Nevertheless, several studies (Hoffmann et al. 1998; Harcombe and Hoffmann 2004; Montenegro et al. 2006) failed to find any beneficial effects of Wolbachia in *D. melanogaster*. In contrast, Fry et al. (2004) found both positive and negative fitness effects associated with Wolbachia bacteria, whereas Olsen et al. (2001) reported a host-dependent increase of fecundity associated with Wolbachia infection in Australian *D. melanogaster*. Also Aleksandrov et al. (2007) found a longer life span and higher competitiveness in females infected with Wolbachia than in unintended females with the same genetic background. Further experimental support for fitness benefits of Wolbachia infection comes from a study in *D. simulans*, which showed that after some time of coevolution with the host, 30% increase in fecundity (this corresponds to a 10% higher fecundity than uninfected females) was observed (Weeks et al. 2007).

An interesting facet of the population dynamics of Wolbachia in *D. melanogaster* was added by the discovery of distinct genetic Wolbachia lineages. Using temporally spaced samples, Riegler et al. (2005) showed that one Wolbachia genotype (*w*Mel) replaced others within less than 100 years. In the light of the highly variable levels of CI in *D. melanogaster*, this observation was viewed as further indication for fitness benefits of Wolbachia infection.

In Drosophila, Wolbachia is almost exclusively maternally transmitted (Hoffmann et al. 1990). Thus, the spread of one Wolbachia genotype, either by CI or by fitness benefits to the host will affect the distribution of mitochondrial DNA (mtDNA) haplotypes in the host species. The infection status of individuals in natural populations in combination with their mtDNA haplotype could provide more insight into the population dynamics of the Wolbachia

infection. In this study, we analyzed a worldwide *D. melanogaster* collection and found a nonrandom infection status among *D. melanogaster* strains with different mtDNA haplotypes. A temporal analysis on the global and local scale shows the spread of the mtDNA haplotype with the highest frequency of Wolbachia infection.

Materials and Methods
Mitochondrial Data

Previously, we collected sequence data for a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene from 35 natural populations of *D. melanogaster* (Nunes et al. forthcoming). Details about the origin of the flies are provided in supplementary table S1 (Supplementary Material online). The data were deposited in GenBank with accession numbers EF153514–153618. In this work, we extended that data set by including 19 additional natural populations from the European/Mediterranean region, following the same protocol as in Nunes et al. (forthcoming). Three individuals per population were sequenced, each corresponding to different isofemale lines.

To investigate the change in frequency of mtDNA haplotypes over time, we also sequenced ten long-term isofemale stocks and all individuals available from the three temporally spaced Cretan collections (11 from Kr, 16 from KRE, and 14 from VVC). Additionally, we sequenced a 511-bp fragment of the ND1 gene for all VVC individuals (primers: Fw 5'-AAAGGTCCTAATAAAGTTGG-3' and Rv 5'-AAATTCTCGCATATTCAGC-3').

In all cases, a single individual was sequenced per line.

New sequences were deposited in GenBank with accession numbers FJ158857–158973.

Wolbachia Screening

The recent availability of the *Wolbachia pipientis* full genome allowed the discovery of at least five different variants of Wolbachia that can be discriminated based on several polymorphic markers. To test for an association between Wolbachia variants and mtDNA haplotypes, we screened all individuals sequenced for the COI gene using polymorphic markers for the presence of the insertion sequence IS5 in two loci (IS5-WD0516/7 and IS5-WD1310) and the copy number of two minisatellite repeats (VNTR-141 and VNTR-105), following the procedure of Riegler et al. (2005). These four markers are sufficient to discriminate among the five different strains of Wolbachia. The polymerase chain reaction (PCR) products were separated by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. Due to heterogeneous amplification efficiency of the primers used to amplify the different markers, when the infection titer was too low, some loci yielded no visible amplification. Therefore, in some cases, it was not possible to unambiguously determine the Wolbachia strain. Wolbachia infection was scored as follows: no infection, 0; *w*Mel, 1; *w*MelCS, 2; not unambiguously determined but not *w*MelCS, *w*MelCS2, or *w*Mel2, 3; new Wolbachia genotype, 4; not unambiguously determined, 5; and *w*Mel2, 6. In cases when the Wolbachia variant could

not be unambiguously determined, but *w*MelCS, *w*MelCS2, or *w*Mel2 could be excluded, we scored this 3. Most likely, variant 3 will be wMel because the other alternative, wMel3, is very rare (Riegler et al. 2005). Details on the scoring procedure can be found in supplementary table S2 (Supplementary Material online). In supplementary table S3 (Supplementary Material online), the results from the Wolbachia screening can be found for all individuals analyzed, together with the correspondent mtDNA haplotype.

Homogeneity and Fisher's exact tests were performed using the categorical statistics package (Engels 1988).

Nuclear Data

The three Cretan populations were genotyped for 12 microsatellite loci, 5 X-linked, and 7 autosomal (Caracristi and Schlötterer 2003; Schlötterer et al. 2006). The loci were amplified in four sets of triplex PCRs using fluorescent-labeled forward primers (Metabion, Martinsried, Germany). A total of 10 µl PCRs were carried out with 50 ng genomic DNA, 1 µM of each primer, 2.5 mM MgCl$_2$, 200 µM deoxynucleoside triphosphates, and 1.0 unit *Taq* polymerase. Following an initial denaturation of 3 min at 94 °C, the cycling profile consisted of 34 cycles with 30 s at 94 °C, 30 s at 50–54 °C (depending on the primer pair), and 50 s at 72 °C. Final extension was done for 45 min at 72 °C to allow for quantitative terminal transferase activity of the *Taq* polymerase. Details on the loci, fluorescent labels and multiplex combinations used are given in supplementary table S4 (Supplementary Material online).

Fragment length was determined with the internal size standard (ET-ROX 400, GE Healthcare, Bucks, UK) on a MegaBace 500 Sequencer (GE Healthcare) and analyzed with Genetic Profiler v.2.0 (GE Healthcare).

Genetic Differentiation over Time of *D. melanogaster* Cretan Populations
Differentiation Based on mtDNA

Estimates of pairwise F_{ST} between the three populations from Crete were calculated using ARLEQUIN version3 (Excoffier et al. 2005), and their significances were obtained by 10,000 permutations.

Differentiation Based on ncDNA

Pairwise Θ values between populations were calculated as an unbiased estimator of F_{ST} (Weir and Cockerham 1984) implemented in the MSA software (Dieringer and Schlötterer 2003). As inbreeding in isofemale lines does not affect the F_{ST} calculation, we used the nondiscarded data set. We used the Bonferroni correction to account for multiple testing (Sokal and Rohlf 1995).

Comparison between Both Estimates

As the effective population size of mtDNA is smaller than the population size of nuclear markers, we extrapolated

the genetic differentiation observed for the microsatellites to the expected differentiation assuming a reduced population size.

Under the assumption of an infinite-island model, Wright's fixation index for the nuclear DNA is $F_{ST}nc = 1/(1 + 4N_e m)$, where N_e is the effective population size and m is the migration rate (Wright 1950). If the sex ratio is even and there is no preferential migration of one of the sexes, then $F_{ST}mt = 4F_{ST}nc/(1 + 3F_{ST}nc)$. The observed global F_{ST} obtained for the mtDNA (0.78, $P < 0.0003$) was almost four times larger than the highest microsatellite locus global F_{ST} (0.062, $P > 0.1$; 0.21 after correction for the different population size) and more than 20 times larger than the median among all microsatellite loci (0.0084; 0.03 after correction).

Modeling of Wolbachia Infection Spread in Crete

To model the infection spread of Wolbachia in Crete, we applied the same discrete-generation model, which was previously used to explain the spread of Wolbachia in *D. simulans* (Hoffmann and Turelli 1988; Hoffmann et al. 1990; Turelli et al. 1992). This model uses three parameters: μ, an estimate of maternal leakage; H, the relative hatch rate of incompatible crosses; and F, the fecundity of infected females relative to that of uninfected ones. Setting F to 1, which implies no fitness benefit or cost, the frequency of infection among adults in generation t follows $p_{t+1} = \frac{p_t(1-\mu)}{1-(1-H)p_t(1-p_t)-\mu(1-H)p_t^2}$ (Hoffmann et al. 1990).

Using this equation, we modeled the change in infection frequency, for a wide range of H values, within 40 generations, which roughly corresponds to 4 years (assuming the ten generations per year for wild *D. melanogaster*). The initial frequency of Wolbachia-infected individuals (i.e., invading infected individuals) was set to a conservative value of 5%. Lower initial frequencies would result in higher CI estimates. Our value of μ corresponds to 2.6% rate of maternal leakage in *D. melanogaster* (Hoffmann et al. 1998).

Results and Discussion

We expanded a recent collection of *D. melanogaster* COI haplotypes and added further populations from the Mediterranean and Europe as this geographic region revealed most mitochondrial diversity (Nunes et al. forthcoming). In total, we analyzed three individuals from 54 populations for their COI sequence and Wolbachia infection status. Seventy-one percent of the lines were infected (table 1). Among the lines for which the Wolbachia strain could be unambiguously determined (see Materials and Methods), wMel was the most frequent one, occurring in 60% of the cases (supplementary table S3, Supplementary Material online). Figure 1 shows the distribution of the Wolbachia genotypes identified among the mtDNA haplotypes. The wMel genotype is associated with five different COI haplotypes, but a joint analysis of all *D. melanogaster* population samples showed that Wolbachia infection is not randomly distributed among the mtDNA haplotypes (homogeneity test, $P < 0.0001$, table 1). This heterogeneity

Table 1
Distribution of Wolbachia Infection among Haplotypes

COI Haplotype	Infected Flies	Noninfected Flies
2	93	24
3	1	0
4	1	0
5	0	1
6	0	1
7	0	1
8	2	2
9	0	1
12	0	1
13	3	0
1	1	9
10	11	1
11	2	4
15	0	2
18	1	0
Total	115	47

in infection status remained significant after excluding those mtDNA haplotypes for which no infected flies were detected (homogeneity test, $P < 0.0001$). Only haplotypes closely related to haplotype 2 are associated with wMel infection. Thus, horizontal transmission is rare and the wMel infection is old, probably predating the emergence of several mtDNA haplotypes. Interestingly, haplotype 2 is not only associated with wMel infection but also with two other Wolbachia genotypes. This suggests that additional Wolbachia genotypes may exist, which could not be distinguished by the markers available.

The observed heterogeneity in infection status is expected under two different scenarios. 1) The probability of Wolbachia infection is independent of the mtDNA genotype, but the maternal transmission rate varies among strains with different mtDNA haplotypes. Thus, Wolbachia
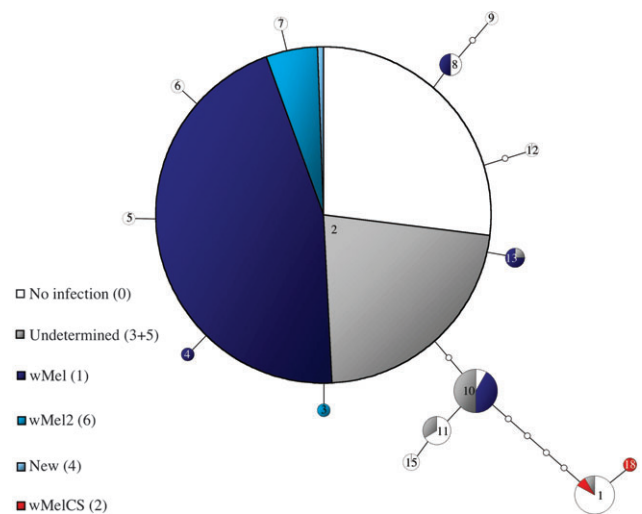

FIG. 1.—Wolbachia strain–mtDNA association shown in a haplotype network of COI haplotypes. The number inside the circles refers to the mtDNA haplotype (supplementary table S3, Supplementary Material online). Colors refer to the Wolbachia genotype (see Materials and Methods). Circle size and partition are proportional to sample size. Node connection length is not scaled. Small circles in the branches refer to number of mutation steps.

Table 2
Number of Flies with the Different COI Haplotypes in Old (long-term stocks) and Recently Collected Populations of *Drosophila melanogaster*

COI Haplotype	Old Collections	Recent Collections
2	3	117
3	0	1
4	0	1
5	0	1
6	2	1
7	0	1
8	0	4
9	0	1
12	1	1
13	0	3
1	2	10
10	0	12
11	0	6
15	0	1
18	0	1
19	2	0

Table 3
Wolbachia Genotypes and COI Haplotypes for the Three Temporally Spaced Collections from Crete

	Kr (1998)	KRE (2002)	VVC (2006)
COI haplotype			
2	0	0	12
1	11	15	1
10	0	0	1
11	0	1	0
Wolbachia infection status			
0	11	16	0
1	0	0	6
2	0	0	1
3	0	0	1
5	0	0	6

NOTE.—Wolbachia infection scoring: no infection, 0; wMel, 1; wMelCS, 2; not unambiguously determined but not wMelCS, wMelCS2, or wMel2, 3; new Wolbachia genotype, 4; not unambiguously determined, 5; and wMel2, 6.

infection is lost at unequal rates. 2) The population is characterized by a change in Wolbachia infection resulting from either active or passive Wolbachia spread. An active spread of Wolbachia infection could occur through infection in a previously Wolbachia-free population or the replacement of one Wolbachia strain with another one. Alternatively, Wolbachia infection status may be passively influenced by the spread of a beneficial mutation in the mtDNA. Through the maternal transmission of Wolbachia, the infection status of individuals with the selectively favored mtDNA is expected to differ from the remainder of the population.

Both scenarios are extremes and different combinations are also conceivable, but for simplicity, we focus on the two extremes for the remainder of the manuscript. Each scenario leads to different predictions for the dynamics of Wolbachia infection and mtDNA haplotype frequency changes. As the first scenario does not invoke selection, changes in mtDNA haplotype frequencies (and thus Wolbachia infection rate) depend on genetic drift. The second scenario predicts directional changes in both mtDNA haplotype frequencies as well as Wolbachia infection rate. Hence, the analysis of temporally spaced samples should provide insight into which of the two scenarios is more likely to explain the heterogeneity in Wolbachia infection status among *D. melanogaster* lines with different mtDNA haplotypes.

We analyzed ten long-term isofemale stocks, which were collected before 1955, and found that the frequency of the haplotypes was significantly different from the one found in recent collections (homogeneity test, $P < 0.0005$; table 2). In particular, haplotype 2 was significantly less frequent than in the more recent collections (two-tailed Fisher's exact test, $P < 0.05$; table 2). We also noted a lower, though not significant, infection status in the long-term isofemale stocks, but it is not clear to what extent past antibiotic treatments influenced the infection status of the lines tested. The small sample size of the long-term isofemale stocks and the heterogeneity in collection sites prevents testing of whether this heterogeneity can be explained by genetic drift alone or if it is due to directional forces.

We further studied the change in mtDNA frequency in a temporal sample collected over 8 years in Crete. Again, we noted a marked change in mtDNA haplotype composition between the samples, with haplotype 2 becoming abundant in sample VVC, collected in 2006. In the other two samples, Kr (1998) and KRE (2002), haplotype 1 predominates and no significant change in haplotype composition was apparent (table 3). We compared the genetic differentiation of mtDNA and nuclear microsatellites to distinguish between genetic drift and directional forces. The highly significant differentiation of mtDNA contrasted sharply with the low and nonsignificant differentiation for microsatellites (table 4). As the effective population size of mtDNA is smaller than the population size of nuclear markers, we extrapolated the genetic differentiation observed for the microsatellites to the expected differentiation assuming a reduced population size. After this correction, we still found the mtDNA samples to be more differentiated than expected based on the microsatellite data. Even the most highly differentiated microsatellite locus had an almost 4-fold lower F_{ST} than the mtDNA (0.21 vs. 0.78, respectively). Hence, our data suggest a directional change in mtDNA haplotype composition in the Cretan population. Interestingly, the marked change in mtDNA haplotype frequency is accompanied by a change in Wolbachia infection frequency. Whereas the first two population samples from Crete did not contain a single line infected with Wolbachia, the third sample contained 100% infected lines. wMel was the predominant Wolbachia strain in this sample (table 3). Remarkably, the only individual with mtDNA haplotype 1

Table 4
Mitochondrial and Nuclear Genetic Differentiation between the Three Temporally Spaced Collections from Crete

	Kr	KRE	VVC
Kr	—	0.014	0.010
KRE	0.0252	—	0.005
VVC	0.835***	0.790***	—

NOTE.—Below diagonals are the F_{ST} values obtained based on mtDNA, and above diagonals are the F_{ST} values obtained based on ncDNA.
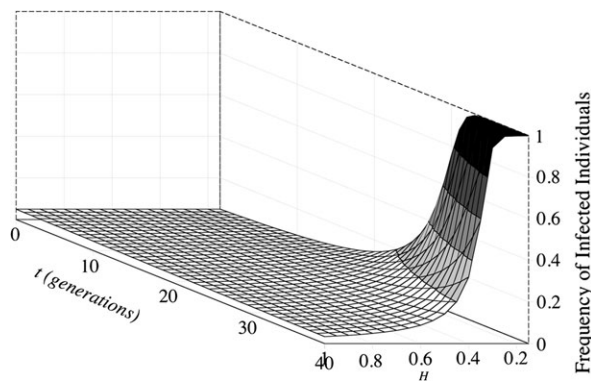***$P < 0.001$.

F_IG_. 2.—Change in infection frequency over 40 generations with different values of H (relative hatch rate from incompatible crosses). With an initial frequency of 5% of Wolbachia-infected individuals, a high CI expression ($H \leq 0.3$) is necessary to lead to the observed increase of the infection frequency.

was also infected, but with *w*MelCS, the Wolbachia strain that has been replaced by the *w*Mel strain (Riegler et al. 2005).

To distinguish between a CI and positive fitness effects mediated increase in Wolbachia infection in the Crete population, we evaluated the parameter range for which the frequency of Wolbachia could increase from 5% to 100%. Interestingly, we found that a relative hatch rate 0.3 or less would be required to capture the increase in Wolbachia infection seen in Crete (fig. 2). Fast-developing males of a given family could in principle express CI levels as high as this (Yamada et al. 2007). However, as the high CI is limited to a very short time interval, a natural population with males of different age classes will have a substantially lower mean CI. Furthermore, in *D. simulans*, the mating success of older males seems to be higher (Hoffmann et al. 1990; Turelli and Hoffmann 1995). If the same holds true for *D. melanogaster*, the impact of the "younger male" effect would be even smaller. Consequently, the parallel increase of haplotype 2 with the *w*Mel Wolbachia infection in the Cretan population is most likely the result of a fitness benefit, mediated either through mtDNA or Wolbachia.

To distinguish between a novel beneficial mutation in an mtDNA haplotype or the presence of a Wolbachia fitness benefit to its hosts as the cause of the increase of Wolbachia frequency in Crete, we sequenced an additional mtDNA gene (ND1) to obtain a higher haplotype resolution. In the case of an mtDNA-driven selective sweep, only a single mtDNA haplotype should have spread in Crete. Instead, a Wolbachia infection could have been associated with multiple haplotypes. Interestingly, we found two different ND1 haplotypes associated with COI haplotype 2 (supplementary table S3, Supplementary Material online). Although it is possible that both mtDNA haplotypes are selectively favored in the Crete population (rather than a new mutation), we favor the alternative explanation that the Wolbachia infection may have provided a fitness advantage to the Crete population. Nevertheless, further experiments, including functional tests separating mtDNA haplotypes from the Wolbachia infection, are required.

It is important to note that although a fitness advantage conferred by the bacteria seems to be the best explanation

for the increase of haplotype 2 in Crete, it cannot be directly extrapolated to the worldwide data. Whereas in Crete, haplotype 2 and the Wolbachia infection reached almost fixation within 4 years, the worldwide increase of haplotype 2 is less pronounced. One possibility for this discrepancy is that Crete populations were almost Wolbachia free, whereas in the global *D. melanogaster* population, *w*MelCS is being replaced by *w*Mel (Riegler et al. 2005). Hence, a considerable number of factors, such as heterogeneity in CI, maternal transmission, and fitness benefit to the host need to be considered jointly. Even if the host genome does not affect the Wolbachia dynamics in natural *D. melanogaster* populations, the recently discovered heterogeneity in Wolbachia genotypes (Riegler et al. 2005) raises the interesting question of a different behavior of the already described and further cryptic Wolbachia genotypes.

Supplementary Material

Supplementary tables S1–S4 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

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