Wolbachia Infections and Mitochondrial Diversity of Two Chestnut Feeding Cydia Species

Dimitrios N. Avtzis1*, Vangelis Doudoumis2*, Kostas Bourtzis2,3

1 Laboratory of Forest Entomology, Forest Research Institute, Hellenic Agricultural Organization “Demeter”, Vassilika Thessaloniki, Greece, 2 Department of Environmental and Natural Resources Management, University of Patras, Agrinio, Greece, 3 Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria

Abstract

Cydia splendana and C. fagiglandana are two closely related chestnut feeding lepidopteran moth species. In this study, we surveyed the bacterial endosymbiont Wolbachia in these two species. Infection rates were 31% in C. splendana and 77% in C. fagiglandana. MLST analysis showed that these two species are infected with two quite diverse Wolbachia strains. C. splendana with Sequence Type (ST) 409 from the A-supergroup and C. fagiglandana with ST 150 from the B-supergroup. One individual of C. splendana was infected with ST 150, indicating horizontal transfer between these sister species. The mitochondrial DNA of the two Cydia species showed a significantly different mtDNA diversity, which was inversely proportional to their infection rates.

Introduction

Wolbachia pipientis are gram-negative Alphaproteobacteria. They are members of the family Rickettsiales, which are considered the most ubiquitous obligate intracellular symbionts reported so far in Arthropods [1]. Current estimates suggest that about 40% of all arthropod species may be infected with Wolbachia [2]. By establishing both somatic and gonadal infections, Wolbachia is able to manipulate many aspects of the biology, physiology, ecology and evolution of its hosts, including their reproductive properties [3–4]. In insects, Wolbachia has been reported to induce thelytokous parthenogenesis, feminization of genetic males, male killing, while the most abundant phenotype is cytoplasmic incompatibility (CI); however, Wolbachia infections with no obvious reproductive effect have also been detected [3–4]. All the above reproductive alterations favor an increase in infected females in host populations and thus the spread of Wolbachia, since the predominant mode of transmission of this symbiont is maternal [5].

Several studies suggest that Wolbachia infections can be transferred horizontally between different hosts. This is supported by the lack of congruence between host and symbiont phylogenetic trees [6–7]. Experimental evidence has been provided implicating parasitism, cannibalism and predation as potential routes for horizontal Wolbachia transfers in different systems [8–10]. Hybridization and introgression may have played a pivotal role in the movement of Wolbachia between closely related species [11–14]. In addition, ecological niche sharing could also be a driving force of horizontal transmission events [15].

During the last few years, many cases of mito-nuclear discordance have been reported in insect systems and, in most of them, Wolbachia has been identified as the driving factor. Being both maternally transmitted, mitochondrial DNA (mtDNA) and Wolbachia are in linkage disequilibrium. The spreading of a given Wolbachia strain will also result to the spreading of the associated mtDNA haplotypes (selective sweep), thus changing the frequency of the mtDNA haplotypes in a host population. Such selective sweeps have a significant impact on mtDNA, but not on nuclear DNA evolution and have to be considered in population, phylogenetic and phylogeographic studies [16–23].

The presence of Wolbachia in insect pests has implications for the management and control of these insects [24–25]. Population control of agricultural pests could be achieved with the Incompatible Insect Technique (IIT), which is based on the mechanism of Wolbachia-induced CI. The application of IIT requires, however, a perfect sexing system for male-only releases. In the absence of such a system, a combination of IIT with the Sterile Insect Technique (SIT) is recommended [26].

The chestnut feeding Cydia moths, C. splendana and C. fagiglandana comprise two of the most abundant and dangerous insect pests of sweet chestnut in European countries [27]. As a result, most pertinent studies so far aimed mainly at mapping their spatial distribution and refining control measures to reduce the damage caused by these pests [28–30]. However, trapping the two Cydia species using pheromones has been proven to be rather difficult [31]. Even the disentanglement of their distributions has been less than satisfactory, as these sympatrically occurring species not only resemble each other morphologically but also share...
similar life cycles [32]. DNA barcoding seems to provide the most precise approach to define their geographical distributions with accuracy [33].

Given that Wolbachia affects many aspects of the biology of its hosts and that it is a potential tool for pest control, we investigate here the prevalence of this symbiont in Greek populations of the two Cydia moth species. In addition, we genotype the detected bacterial strains via MLST and wsp gene-based approaches. We finally discuss the influence of Wolbachia infections on mitochondrial evolution and host population structure.

Materials and Methods

Sample collection, mtDNA barcoding and Wolbachia genotyping

Chestnuts suspected of moth infestation were collected from 15 chestnut-producing regions of Greece (Figure 1 and Table S1) and sent to the Forest Research Institute (Vasilika, Thessaloniki, Greece). For the collection of populations no specific permission was required while it did not involve any endangered, protected or threatened species. Chestnuts were manually opened and live larvae were placed individually into vials with 100% ethanol.

Figure 1. Distribution map of the sampled Greek populations (taken from NASA Earth Observatory–public domain). Underlined acronyms indicate those populations that participated in the MLST analysis.

doi:10.1371/journal.pone.0112795.g001
From each of the 243 larvae, DNA was extracted using the GenElute Kit (Sigma) and processed following the manufacturer’s protocol. Amplification of an approximately 800 bp long locus from the 3’ end of mitochondrial cytochrome oxidase I (COI) gene was carried out with primers “Jerry” and “Pat” [34] in reactions containing 0.6 µl of MyTaq (BioLine, GmBH, Germany), 5 µl of the 5 × MyTaq Red Reaction Buffer (BioLine, GmBH, Germany), 20 µl of each primer, 8 µl of DNA extract and ddH2O to a final volume of 25 µl. PCR conditions were: an initial denaturation step at 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 s, 45°C for 30 s and 72°C for 1 minute, followed by a final extension step of 3 minutes at 72°C. PCR products were purified with the PureLink PCR Purification Kit (Invitrogen) and sequenced with an ABI 3730XL at CEMIA SA (Larissa, Greece) using both PCR primers. A specific 16S rRNA gene-based PCR assay was used for detection of Wolbachia with primers WspecF and WspecR resulting in an amplicon of 438 bp [35]. PCR amplifications were performed in 20 µl reaction mixtures containing 4 µl 5 × reaction buffer (Promega), 25 mM MgCl2, deoxynucleotide triphosphate mixture (25 mM each), 25 µM of each primer, 0.1 U of Taq polymerase (Promega), 12.2 µl water and 1 µl of template DNA. The PCR protocol was: 35 cycles of 30 sec at 95°C, 30 sec at 54°C and 1 min at 72°C. The Wolbachia strains were genotyped with MLST- and wsp gene-based approaches. The wsp and MLST genes (gatB, coxA, hcpA, ftsZ and fbpA) were amplified using the respective primers reported in Baldo et al. [36] (Table S2). PCR amplifications were performed in 20 µl reaction mixtures containing 1× reaction buffer (Promega), 25 mM MgCl2, deoxynucleotide triphosphate mixture (25 mM each), 25 µM of each primer, 0.1 U of Taq polymerase (Promega), 12.2 µl water and 1 µl of template. PCR reactions were performed as follows: 5 min of denaturation at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at the appropriate temperature for each primer pair (52°C for ftsZ, 54°C for gatB, 53°C for coxA, 56°C for hcpA, 58°C for fbpA and wsp) and 1 min at 72°C. All reactions were concluded by a final extension step of 10 min at 72°C. For all PCR reactions described the appropriate negative (no DNA) and positive controls were included. All PCR were performed in triplicates. The PCR products were purified using a PEG (polyethylene glycol) - NaCl method [37]. Both strands of the products were sequenced using the corresponding forward and reverse primers. A dye terminator-labelled cycle sequencing reaction was carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems). Reaction products were analyzed using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems).

Phylogenetic analysis

Wolbachia sequences were manually edited with SeqManII by DNASTar and aligned using MUSCLE [38] and ClustalW [39], as implemented in Geneious 6.1.6 [40]. A final adjustment was done by eye. Phylogenetic analyses were performed using Maximum-Likelihood (ML) estimation and Bayesian Inference (BI) for a concatenated data set of the protein coding genes (gatB, coxA, hcpA, ftsZ and fbpA), as well as for the wsp gene. For the Maximum-Likelihood phylogeny, PAUP version 4.0b10 [41], as implemented in Geneious 6.1.6 [40], was used to select the optimal evolution model by critically evaluating the selected parameters, using the Akaike Information Criterion (AIC) [42]. For the concatenated data set and the wsp sequences, the submodels GTR+I+G and TVM+I+G were selected, respectively. The ML tree was constructed with 1000 bootstrap replications. Bayesian analyses were performed as implemented in MrBayes 3.1 [43]. The selected options were: random starting trees, four separate runs, each composed of four chains which were run for 6,000,000 generations; the first 20,000 generations were discarded and the cold chain was sampled every 100 generations. Posterior probabilities were computed for the remaining trees. All phylogenetic analyses were done with Geneious, version 6.1.6 [40].

MtDNA sequences were visualized using CHROMAS v. 1.43 and then aligned using CLUSTAL X [44] with the default settings.

### Table 1. Wolbachia infection status in Greek C. splendana and C. fagiglandana populations. (n.d.: not detected)

| Population | C. splendana | C. fagiglandana |
|------------|--------------|-----------------|
| 1 AO-HAL   | 4/4 (100%)   | 12/16 (75%)     |
| 2 KA-LAR   | 2/3 (67%)    | 9/10 (90%)      |
| 3 ME-DRA   | 2/7 (28,6%)  | 2/7 (22,2%)     |
| 4 EA-CRE   | n.d.         | 4/5 (80%)       |
| 5 HO-THE   | 1/1 (100%)   | 13/15 (86,67)   |
| 6 ME-LAR   | 3/20 (15%)   | 16/19 (84,2)    |
| 7 KA-TRI   | 5/40 (12,5%) | n.d.            |
| 8 AG-LES   | 4/16 (25%)   | n.d.            |
| 9 SPE-FTH  | 3/7 (42,9%)  | 4/4 (100%)      |
| 10 HA-EVI  | 2/7 (28,6%)  | n.d.            |
| 11 WE-CRE  | 1/2 (50%)    | 3/6 (50%)       |
| 12 AN-KAR  | 0/5 (0%)     | 3/4 (75%)       |
| 13 KA-LAK  | 16/28 (57,1%)| n.d.            |
| 14 AR-HAL  | 0/4 (0%)     | 2/2 (100%)      |
| 15 PA-PEL  | 3/3 (100%)   | 6/6 (100%)      |

Total: 46/147 (31%) n.d. 74/96 (77%)

doi:10.1371/journal.pone.0112795.t001
Table 2. MLST and wsp allele profiles of Wolbachia strains in Greek Cydia populations.

| Sample code | Host | Haplotype | Super group | ST | MLST genes | wsp codon | coxA | hcpA | ftsZ | fbpA | hapV | fspB | fspA |
|-------------|------|-----------|-------------|----|------------|-----------|------|------|------|------|------|------|------|
| 7H.1AK      | Fagi  | 8         | 150 16 14   | 40 | 36 4 4 10 10 | 10 10 8 10 8 |
| 6A.1AK      | Fagi  | 8         | 150 16 14   | 40 | 36 4 4 10 10 | 10 10 8 10 8 |
| 5E.1AK      | Fagi  | 6         | 150 16 14   | 40 | 36 4 4 10 10 | 10 10 8 10 8 |
| 2C.1AK      | Fagi  | 5         | 150 16 14   | 40 | 36 4 4 10 10 | 10 10 8 10 8 |
| 8E.1BK      | Splenda12 | A        | 409 175 206 | 241 | 241 140 396 674 212 | 7 26 26 26 | 26 26 |
| 4E.1BK      | Splenda12 | A        | 409 175 206 | 241 | 241 140 396 674 212 | 7 26 26 26 | 26 26 |
| 3G.1BK      | Splenda12 | A        | 409 175 206 | 241 | 241 140 396 674 212 | 7 26 26 26 | 26 26 |
| 9F.2AK      | Splenda12 | A        | 409 175 206 | 241 | 241 140 396 674 212 | 7 26 26 26 | 26 26 |

Identical nucleotide sequences at a given locus for different strains were assigned the same arbitrary allele number. Each strain was identified by the combination of the five MLST allelic numbers, representing its allelic profile. Each unique allelic profile was assigned an ST, which ultimately characterizes a strain [53]. Wsp profiles are shown in the last left column, respectively. (HVR: Hyper Variable Region).

Verified haplotypes were deposited in NCBI GenBank and referenced by haplotype designations provided below. Patterns of molecular diversity based on the mtDNA sequences were assessed by estimating haplotype (Ho), nucleotide diversity (π) [45] and the average number of nucleotide differences (k) [46] for every population using MEGA v.5 [47]. MEGA v.5 was also used in constructing the phylogenetic tree containing the C. splendana and C. faggiglandana haplotypes. For that, we employed the Neighbor Joining algorithm on the pair-wise Tamura-Nei [48] distances while the statistical support was assessed by 500 bootstrap replicates. Additionally, all populations were tested for the neutral mutation hypothesis with Tajima’s D and Fu’s F statistics [46,49–50] using DNAsr version 5 [51]. All these parameters were also calculated for each species separately, complemented with the mismatch distribution plot estimated with DNAsr version 5 [51]. Finally, the correlation between genetic diversity (haplotype and nucleotide diversity) and Wolbachia prevalence was estimated with GenStat 12th Edition (supplied by VSN International).

Nucleotide sequence accession numbers

All MLST, wsp and COI gene sequences generated in this study have been deposited into NCBI GenBank under accession numbers KJ139995–KJ140073 and KJ398246–KJ398313 respectively. The MLST and wsp gene sequences have been also deposited in the Wolbachia MLST database.

Results

Wolbachia in chestnut-feeding Cydia populations

A total of 147 field-collected adult insects from 14 populations of C. splendana and 96 field-collected adult insects from 11 populations of C. faggiglandana were tested. A significant difference was observed in the prevalence of Wolbachia between the two species (Table 1). Wolbachia infections were more prevalent in C. faggiglandana (77%) than in C. splendana (31%). This difference was not associated with the origin of the insects: cultivation or forest populations (data not shown). All populations of C. faggiglandana were infected while Wolbachia infection appeared to be absent from 2 out of 11 populations of C. splendana (Table 1). The prevalence of infection varied in the populations of both species ranging from 12.5 to 100% (Table 1).

The Wolbachia strains present in nine adult insects originating from different C. faggiglandana populations (one forest- and two plantation-derived) and C. splendana populations (one forest- and two plantation-derived) were genotyped using MLST analysis. These samples were collected from different regions of Greece, as illustrated in Figure 1. As shown in Table 2, all C. faggiglandana specimens were found to be infected with the same B-supergroup Wolbachia strain with MLST gene alleles and Sequence Type (ST150) belonging to the clonal complex Sequence Type Complex 41 (STC-41). In addition, all these specimens carried Wolbachia strains with an identical COI gene profile (allele 10; Table 2). Four out of the five C. splendana specimens studied were infected with the same A-supergroup Wolbachia exhibiting new alleles for the MLST genes coxA (206), hecA (241) and fhpA (396) and consequently a new sequence type (ST 409). In addition, a new wsp gene allele (674) was identified, closely related to allele 597, with new Hyper Variable Region (HVR) profile and HVR3 (262) and HVR4 (299) haplotypes (Table 2). The fifth C. splendana specimen (sample code 9E.1BK, originating from Paiko Pella or PA-PEL) carried the same B-supergroup Wolbachia strain detected in the C. faggiglandana samples (Table 2).
Phylogenetic analysis based on the concatenated dataset of all MLST loci revealed that the Wolbachia strain infecting all C. fagiglandana specimens and one of C. splendana (8E.1BK, PA-PEL population) belong to supergroup B, while the Wolbachia strain infecting the other four C. splendana specimens is a member of supergroup A, as shown in the Maximum Likelihood tree presented in Figure 2. The MLST analysis also showed that the Cydia Wolbachia strain of supergroup B is member of the common clonal complex STC-41 while the A supergroup Wolbachia strain infecting C. splendana (excluding the population PA-PEL) clusters with ST 99 and ST 264 strains (Figure 2). Phylogenetic analysis based on the wsp gene confirmed the MLST-based data (data not shown). MLST- and wsp gene-based Bayesian phylogenetic analysis provided identical results.

Mitochondrial DNA analysis

A 792 bp locus of the mtDNA COI gene was analyzed for each of the 243 Cydia samples tested for Wolbachia infection. Phylogenetic analysis indicated the presence of two distinct clades (Figure 3). The first clade included all samples of C. fagiglandana, while all C. splendana samples were assigned to the second clade, in complete agreement with the morphological identification. Forty-eight haplotypes were retrieved from the 147 C. splendana larvae (Hdiv. = 0.3265), whereas only 17 haplotypes were detected in the 96 C. fagiglandana larvae (Hdiv. = 0.1770). Despite the significantly lower haplotype diversity estimated for C. fagiglandana, the mean number of single nucleotide polymorphisms (k) was threefold higher compared to the value for C. splendana (9.765 versus 3.199 single nucleotide polymorphisms). As a consequence, the mean nucleotide diversity (θ) was also threefold higher (0.0123 and 0.0040 for C. fagiglandana and C. splendana, respectively) (Table 3). Neutral evolution was tested for each population separately and the results are presented in Table 3. In general, estimates of Tajima’s D and Fu & Li’s F statistics were not statistically significant. While Tajima’s D and Fu & Li’s F values were statistically supported when calculated for C. splendana samples (D = −2.48522, P < 0.01 and F = −4.51468, P < 0.02), indicating an excess of rare variants, they were not for C. fagiglandana samples (D = 0.00147, P > 0.1 and F = −0.21269 P > 0.1) (Table 3). Finally, the mismatch distribution plots of the two species were considerably different, with that for C. splendana exhibiting a unimodal curve (Figure S1) whereas the one for C. fagiglandana appears ragged (Figure S1).

The influence of Wolbachia on mitochondrial DNA diversity was also investigated. As shown in Figure 3, Wolbachia was detected in 24 out of 51 and in 15 out of 17 haplotypes of C. splendana and C. fagiglandana, respectively. The infection was fixed in 15 C. splendana and 9 C. fagiglandana haplotypes, while 6 haplotypes of C. splendana and 6 of C. fagiglandana included both infected and uninfected specimens. As shown in Table 3, a strong positive correlation was revealed between haplotype diversity (Hd) and Wolbachia infection for C. splendana (γ = 0.3947x+50.97, R² = 0.2935) while for C. fagiglandana, this correlation is negative (γ = −0.0182x+48.461, R² = 0.0015). The
Table 3. Nucleotide polymorphisms of mtDNA COI gene and Wolbachia infection status of Greek *C. splendana* and *C. fagiglandana* populations.

| Haplotypes | Cydia splendana | Molecular Diversity | Neutrality tests | Cydia fagiglandana | Molecular Diversity | Neutrality tests |
|------------|-----------------|---------------------|-----------------|------------------|---------------------|-----------------|
|            |                 | Wolbachia inf | Ht  | Hd  | π    | k   | Tajima's D | Fu & Li' s F | Wolbachia inf | Ht  | Hd  | π    | k   | Tajima's D | Fu & Li' s F |
| 1          | AO-HAL          | 4/4 (100%)       | 4   | 1,0000 | 0,0032  | 2,5000 | −0,79684 NS | −0,75299 NS | 12/16 (75%) | 7   | 0,4375 | 0,0125 | 9,0498 | −0,37846 NS | −0,50512 NS |
| 2          | KA-LAR          | 2/3 (67%)        | 2   | 0,6667 | 0,0038  | 3,0000 | Cannot be calculated |         | 9/10 (90%) | 5   | 0,5000 | 0,0119 | 9,4000 | −0,49951 NS | −0,53560 NS |
| 3          | ME-DRA          | 2/7 (28,6%)      | 5   | 0,7143 | 0,0025  | 2,0000 | 0,27345 NS | 0,27834 NS | 2/7 (22,2%) | 5   | 0,5556 | 0,0146 | 11,6000 | 0,73029 NS | 0,78365 NS |
| 4          | EA-CRE          | n.d.              |     |      |      |      |            |            |               |     |        |        |        |            |            |
| 5          | HO-THE          | 1/1 (100%)       | 1   | 1,0000 | 0,0000  | 0,0000 | Cannot be calculated |         | 13/15 (86,67) | 5   | 0,3571 | 0,0134 | 10,6000 | −0,29593 NS | −0,31777 NS |
| 6          | ME-LAR          | 3/20 (15%)       | 11  | 0,5500 | 0,0045  | 3,6000 | −1,86291* | −2,50525* | 16/19 (84,2) | 9   | 0,4737 | 0,0119 | 9,4444 | 0,57495 NS | 1,21707 NS |
| 7          | KA-TRI          | 5/40 (12,5%)     | 13  | 0,3250 | 0,0028  | 2,2564 | −1,90007* | −2,34303 NS | n.d.         |     |        |        |        |            |            |
| 8          | AG-LES          | 4/16 (25%)       | 6   | 0,3750 | 0,0042  | 3,3300 | −1,43477 NS | −1,57979 NS | n.d.         |     |        |        |        |            |            |
| 9          | SPE-FTH         | 3/7 (42,9%)      | 5   | 0,7143 | 0,0023  | 1,8000 | −0,41017 NS | −0,41751 NS | 4/4 (100%) | 1   | 0,2500 | 0,0000 | 0,0000 | Cannot be calculated |         |
| 10         | HA-EVI          | 2/7 (28,6%)      | 3   | 0,4286 | 0,0017  | 1,3300 | Cannot be calculated |           | n.d.          |     |        |        |        |            |            |
| 11         | WE-CRE          | 1/2 (50%)        | 2   | 1,0000 | 0,0025  | 2,0000 | Cannot be calculated |         | 3/6 (50%) | 2   | 0,3333 | 0,0202 | 16,0000 | Cannot be calculated |         |
| 12         | AN-KAR          | 0/5 (0%)         | 4   | 0,8000 | 0,0019  | 1,5000 | −0,75445 NS | −0,67466 NS | 3/4 (75%) | 2   | 0,5000 | 0,0139 | 11,0000 | Cannot be calculated |         |
| 13         | KA-LAK          | 16/28 (57,1%)    | 8   | 0,2857 | 0,0032  | 2,5357 | −1,32001 NS | −1,62334 NS | n.d.         |     |        |        |        |            |            |
| 14         | AR-HAL          | 0/4 (0%)         | 3   | 0,7500 | 0,0017  | 1,3300 | Cannot be calculated |         | 2/2 (100%) | 1   | 0,5000 | 0,0000 | 0,0000 | Cannot be calculated |         |
| 15         | PA-PEL          | 3/3 (100%)       | 3   | 1,0000 | 0,0025  | 2,0000 | Cannot be calculated |         | 6/6 (100%) | 4   | 0,6667 | 0,0116 | 9,1667 | −0,67840 NS | −0,69994 NS |
| Total      |                 | 46/147 (31%)     | 48  | 0,3265 | 0,0040  | 3,1990 | −2,48322*** | −4,51468*** | 74/96 (77%) | 17  | 0,1770 | 0,0123 | 9,7647 | 0,00147 NS | −0,21269 NS |

Haplotypes (Ht) and Haplotype diversity (Hd) were retrieved from the analysis of a 792 bp long locus of mtDNA COI gene. Nucleotide diversity (π) and average number of nucleotide differences (k) were based on the number of segregating sites. Neutrality tests were performed only in populations that contained more than 3 individuals, with the following levels of significance: NS: not significant, *P<0.05, **P<0.01, ***P<0.001 (n.d.: not detected).

doi:10.1371/journal.pone.0112795.t003
results for the correlation between nucleotide diversity (π) and *Wolbachia* infection were similar. In *C. splendana*, the correlation is still positive but weaker \((y = 0.0005x + 0.2638, R^2 = 0.0311)\), whereas in *C. fagiglandana*, it is strongly negative \((y = -0.007x + 1.8884, R^2 = 0.3948)\).

**Discussion**

The detection of *Wolbachia* in *Cydia splendana* and *C. fagiglandana* species infesting chestnuts in Greece, in concert with the pronounced differences in the levels of mtDNA genetic
diversity, suggest that the presence of the symbiont might have shaped the population structure of these two species in Greece. Our findings clearly suggest that the species exhibit different Wolbachia-infection patterns associated with contrasting mtDNA diversity levels. MLST genotyping also allows a robust separation of Wolbachia strains infecting these two species, and indicates horizontal Wolbachia transfer between these sister species.

Wolbachia infection status and mtDNA diversity

Our research adds both chestnut feeding Cydia species to the long list of insect species that are infected with Wolbachia [52]. Cydia splendana as well as C. faggiglandana were carrying Wolbachia infections; yet the infection status varied significantly between the two species. While Wolbachia was found in more than ⅓ of C. faggiglandana individuals analyzed (77%), the rate was considerably lower for C. splendana (only 31%). The high level of Wolbachia infection of C. faggiglandana is coupled with a reduced haplotype diversity index \( H_s = 0.1770 \), compared to that of C. splendana \( H_s = 0.3265 \). C. faggiglandana, in which Wolbachia infection is more prevalent, shows a lower mtDNA diversity than the less frequently infected C. splendana. Lower mtDNA diversity is attributed to many different factors that range from recent population expansions after bottleneck [46] to selective sweeps [18] and selection against deleterious mutations [53]. The fact that, for both species, no mtDNA haplotype was found to be consistently associated with a specific Wolbachia strain for both species [54], along with the fact that several haplotypes contain infected and uninfected individuals, argue against a Wolbachia-driven selective sweep.

The strong negative correlation between Wolbachia-infection and mtDNA diversity supports the notion that Wolbachia has influenced intraspecific divergence, thus reducing the genetic diversity of Greek C. faggiglandana populations. This is congruent with previous studies demonstrating a similar effect of Wolbachia [18,55–59]. It is interesting to note, however, that the C. faggiglandana-clade shows stronger intraspecific divergence, despite harbouring fewer haplotypes. According to Ritter et al. [23], deep intraspecific divergences in DNA barcode studies can be due to both Wolbachia infection and phylogeographic structure. However, as for the C. faggiglandana individuals, no significant effect of phylogeography could be inferred (both neutrality tests exhibited values with \( P>0.1 \) while the mismatch distribution plot was "ragged", indicating a steady-state population), it can thus be assumed that the dominance of Wolbachia rather than other phylogeographic events shaped the intraspecific divergence. For the C. splendana individuals in contrast, molecular mtDNA indices (statistically significant negative neutrality tests coupled with a unimodal mismatch distribution plot) argue for population expansion after a recent bottleneck [60–61].

Horizontal transfer

In addition to differentiation at infection level, MLST revealed a further difference in the identity of Wolbachia strains infecting the sister species. A strain that belongs to Supergroup B and is identical to the one found in Colotis amata (Pieridae, Lepidoptera) from India, was identified in samples of C. faggiglandana regardless of geographic origin and genetic assignment. This indicates that Wolbachia infection of C. faggiglandana was not determined by any other agent, such as environment or intraspecific divergence, something that was already reported before [62–63]. On the other hand, most of the C. splendana samples were infected with a Supergroup A strain. However, the population PA-PEL was, based on MLST and wsp-based analysis, infected with the same strain present in C. faggiglandana samples suggesting a possible horizontal transmission event. Several studies have provided evidence that Wolbachia strains can be horizontally transferred not only between sister species, but even between distantly related taxa [64–65]. By enhancing vertical transmission through horizontal transfer, Wolbachia strains increase their potential to spread rapidly and to overcome evolutionary dead-ends that could threaten their survival. Even though it seems to be a rare phenomenon (given the similarities in life cycles and their sympatric distributions), there are still some indications that argue for the scenario of horizontal transfer. The same Wolbachia strain infects individuals of different haplotypes, while different Wolbachia strains are detected in individuals of the same haplotype. In conclusion, haplotype assignment does not seem to be correlated with the prevalence of Wolbachia, which argues for multiple, independent infections [66].

Distribution of Wolbachia infections

At the population level, Wolbachia infection varies greatly in both species. The occurrence of Wolbachia in C. faggiglandana populations ranges from 22.2% to 100%, while in C. splendana populations the range was even wider (0–100%). Such strong differences of the infection status between populations have also been recorded in several other arthropod species [63,67]. The non-uniform infection level among populations is thought to be associated with geographic origin and attributed to local differences in environmental conditions [68]. In our case, the simultaneous study of two sister species from the same sites allows an evaluation of correlation between geographic origin and Wolbachia infection status. This approach revealed indeed a weak yet positive correlation (0.1561) between geographic origin of each population and its infection level. As demonstrated in previous studies, the occurrence and prevalence of Wolbachia in a given population can depend on environmental conditions, temperature being the stronger effector [69–70]. That both species occur broadly in sympathy, and thus are subject to the same environmental conditions, suggests that the Wolbachia strains differ in their interactions with these hosts.

Conclusions

In summary, our investigation reveals the presence of Wolbachia in two of the most harmful pests of chestnut production. Screening of several populations of these pests in Greece showed that the prevalence of Wolbachia-infection between these species differed significantly, being inversely proportional correlated with mtDNA diversity. The varying Wolbachia infection levels among populations of both species suggest an influence of local environmental conditions.

Supporting Information

Figure S1 Mismatch Distribution diagrams inferred from the haplotypes of Greek C. splendana (A) and C. faggiglandana (B). The expected frequency is represented by a continuous line, while the observed frequency is shown by a dotted line. (TIF)

Table S1 Coordinates of the sampled locations. (DOCX)

Table S2 PCR primers used for Wolbachia genotyping. (DOCX)
References

1. LePage D, Bordenstein SR (2013) Wolbachia, can we save lives with a great pandemic? Trend Parasitol 29(8): 385–393.

2. Züg R, Hamerstein P (2012) Still a host of hosts for Wolbachia: Analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLOS One 7(6): e38543.

3. Werren JH, Baldo L, Clark ME (2008) Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751.

4. Sainudi A, Bourzis K (2003) Parasites more than just a bug in insects’ genitalia. Curr Opin Microbiol 13: 67–72.

5. Funkhouser LJ, Bordenstein SR (2013) Mom Knows Best: The University of: Maternal Microbial Transmission. PLoS Biol 11(6): e1001631.

6. Werren JH, Zhang W, Guo LR (1995) Evolution and phylogeny of Wolbachia: reproductive parasites of arthropods. Proc R Soc Lond [Biol] 261: 56–63.

7. Cordaux R, Pichon S, Hatira HBA, Doublet V, Greve P, et al. (2012) Widespread Wolbachia infection in terrestrial isopods and other crustaceans. In: Srinivas T, Taint S, Sournia S, editors. Advances in Terrestrial Isopod Biology. ZooKeys: 125–131.

8. Heath BD, Butcher RD, Whitleff WG, Hubbard SF (1999) Horizontal transfer of Wolbachia between phylogenetically distant insect species by a naturally occurring mechanism. Curr Biol 9: 313–318.

9. Huigens ME, Luck RF, Klaassen RH, Maas MF, Timmermans MJ, et al. (2000) Infections pathenogenensis. Nature 405: 178–179.

10. Le Clec’h W, Chavellier FD, Genty L, Bertaux J, Bouchon D, et al. (2013) Wolbachia: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751.

11. Xiao J, Wang N, Murphy RW, Cook J, Jia LY, et al. (2012) Wolbachia infections mimic cryptic speciation in two parasitic butterfly species, Ecto-American. PLoS One 9: e112795.

12. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

13. Ritter S, Michalski SG, Settele J, Wiemers M, Fric ZF, et al. (2013) Wolbachia: reproductive parasites of arthropods. Proc R Soc Lond [Biol] 261: 56–63.

14. Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, et al. (2004) Wolbachia infection more than just a bug in insects’ genitalia. Curr Opin Microbiol 13: 67–72.

15. Xiao J, Wang N, Murphy RW, Cook J, Jia LY, et al. (2012) Wolbachia infection and dramatic intra-specific mitochondrial DNA divergence in a Fig. wasp. Evol 66: 1907–1916.

16. Morrow JL, Frommer M, Shearman DCA, Riegler M (2014) Tropical tephrillid fruit fly community with high incidence of shared Wolbachia strains as platform for horizontal transmission of endosymbionts. Environ Microbiol. [Epub ahead of print] DOI: 10.1111/1462-2920.12382.

17. Charlat S, Duperouzy A, Hornet EA, Dyson EA, Davies N, et al. (2009) The joint evolutionary histories of Wolbachia and mitochondria in Hylomimus bellina. PLoS One 4(11): e7793.

18. Heath BD, Butcher RD, Whitleff WG, Hubbard SF, (1999) Horizontal transfer of Wolbachia between phylogenetically distant insect species by a naturally occurring mechanism. Curr Biol 9: 313–318.

19. Ilinsky Y (2013) Coevolution of Wolbachia and mosquitoes to control dengue. PLoS Biol 9: 64.

20. Ritter S, Michalski SG, Settele J, Wiemers M, Fric ZF, et al. (2013) Wolbachia: reproductive parasites of arthropods. Proc R Soc Lond [Biol] 261: 56–63.

21. Ritter S, Michalski SG, Settele J, Wiemers M, Fric ZF, et al. (2013) Wolbachia: reproductive parasites of arthropods. Proc R Soc Lond [Biol] 261: 56–63.

22. Ilinsky Y (2013) Coevolution of Wolbachia and mosquitoes to control dengue. PLoS Biol 9: 64.

23. Ilinsky Y (2013) Coevolution of Wolbachia and mosquitoes to control dengue. PLoS Biol 9: 64.

24. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

25. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

26. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

27. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

28. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

29. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

30. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

31. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

32. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.

33. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to control dengue transmission. PLoS One 6: 1907–1916.
55. Johnstone RA, Hurst GDD (1996) Maternally inherited male-killing microorganisms may confound interpretation of mtDNA variation in insects. Biol J Linn Soc 53: 453–470.
56. Shoemaker DD, Vaishali K, Jaenike J (1999) Wolbachia and the evolution of reproductive isolation between Drosophila recens and Drosophila subquinaria. Evol 53: 1157–1164.
57. Ballard JWO (2000) When one is not enough: introgression in Drosophila. Mol Biol Evol 17: 1126–1136.
58. Ballard JWO, Chernoff B, James AC (2002) Divergence of mitochondrial DNA is not corroborated by nuclear DNA, morphology or behavior in Drosophila simulans. Evol 56: 527–545.
59. Sun X-J, Xiao J-H, Cook JM, Feng G, Huang D-W (2011) Comparisons of host mitochondrial, nuclear and endosymbiont bacterial genes reveal cryptic fir wasp species and the effects of Wolbachia on host mtDNA evolution and diversity. BMC Evol Biol 11: 86.
60. Rodgers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol 9: 552–569.
61. Aris-Brosou S, Excoffier L (1996) The impact of population expansion and mutation rate heterogeneity on DNA sequence polymorphism. Mol Biol Evol 13: 494–504.
62. Stahlhut JK, Desjardins CA, Clarck ME, Baldo L, Russel JA, et al. (2010) The mushroom habitat an an ecological arena for glbal exchange of Wolbachia. Mol Ecol 19: 1940–1952.
63. Doudoumis V, Tsiamis G, Wannwiri P, Brebfoard C, Alam U, et al. (2012) Detection and characterization of Wolbachia infections in laboratory and natural populations of different species of tsetse (genus Glossina). BMC Microbiol 12 (Suppl 1): S3.
64. Baldo L, Ayoub NA, Hayashi CY, Russell JA, Stahlhut JK, et al. (2008) Insight into the routes of Wolbachia invasion: high levels of horizontal transfer in the spider genus Agelenopsis revealed by Wolbachia strain and mitochondrial DNA diversity. Mol Ecol 17: 557–569.
65. Nos VI, Fleming VM, Feil EJ, Breeuwer JA (2009) How diverse is the genus Wolbachia? Multiple-gene sequencing reveals a putatively new Wolbachia supergroup recovered from spider mites (Acarí: Tetranychidae). Appl Environ Microbiol 75: 1036–1043.
66. Symula RE, Alam U, Breifoard C, Wu Y, Echodu R, et al. (2013) Wolbachia association with the tsetse fly, Glossina fuscipes fuscipes, reveals high levels of genetic diversity and complex evolutionary dynamics. BMC Evol Biol 13: 31–42.
67. Cheng Q, Ruel TD, Zhou W, Moloo SK, Majwa P, et al. (2000) Tissue distribution and prevalence of Wolbachia infections in tsetse flies, Glossina spp. Med Vet Entomol 14: 44–50.
68. Yun Y, Lei C, Peng Y, Liu F, Chen J, et al. (2010) Wolbachia strains typing in different geographic population spider, Hylyphantes graminicola (Linyphiidae). Curr Microbiol 62: 139–145.
69. Mouton L, Henri H, Bouletreau M, Vavre F (2006) Effect of temperature on Wolbachia density and impact on cytoplasmic incompatibility. Parasitol 132: 49–56.
70. Toju H, Fukatsu T (2011) Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. Mol Ecol 20: 853–868.