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Correlation between the green-island phenotype and *Wolbachia* infections during the evolutionary diversification of Gracillariidae leaf-mining moths

Florence Gutzwiller[1], Franck Dedeine[1], Wilfried Kaiser[1], David Giron[1,*] & Carlos Lopez-Vaamonde[1,2,*]

[1]IRBI, UMR 7261, CNRS/Université François-Rabelais de Tours, 37200 Tours, France
[2]INRA, UR0633 Zoologie Forestière, F-45075 Orléans, France

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Correspondence
David Giron, Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS/ Université François-Rabelais de Tours, 37200 Tours, France.
Tel: +33 2 47 36 69 11;
Fax: +33 2 47 36 69 66;
E-mail: david.giron@univ-tours.fr

Present address
†Florence Gutzwiller, Faculty of Life Sciences, University of Manchester, Manchester M13 9PL, UK

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*These authors contributed equally to this work.

Abstract

Internally feeding herbivorous insects such as leaf miners have developed the ability to manipulate the physiology of their host plants in a way to best meet their metabolic needs and compensate for variation in food nutritional composition. For instance, some leaf miners can induce green-islands on yellow leaves in autumn, which are characterized by photosynthetically active green patches in otherwise senescing leaves. It has been shown that endosymbionts, and most likely bacteria of the genus *Wolbachia,* play an important role in green-island induction in the apple leaf-mining moth *Phyllonorycter blancardella*. However, it is currently not known how widespread is this moth-*Wolbachia*-plant interaction. Here, we studied the co-occurrence between *Wolbachia* and the green-island phenotype in 133 moth specimens belonging to 74 species of Lepidoptera including 60 Gracillariidae leaf miners. Using a combination of molecular phylogenies and ecological data (occurrence of green-islands), we show that the acquisitions of the green-island phenotype and *Wolbachia* infections have been associated through the evolutionary diversification of Gracillariidae. We also found intraspecific variability in both green-island formation and *Wolbachia* infection, with some species being able to form green-islands without being infected by *Wolbachia*. In addition, *Wolbachia* variants belonging to both A and B supergroups were found to be associated with green-island phenotype suggesting several independent origins of green-island induction. This study opens new prospects and raises new questions about the ecology and evolution of the tripartite association between *Wolbachia*, leaf miners, and their host plants.

Introduction

There is a huge diversity of insect–plant interactions, some of which involve an intimate and finely tuned association between partners. This is especially the case for internal feeders such as gall inducers and leaf miners (whose larvae develop inside leaves) whose intimate association with the host plant is expected to facilitate close interaction between independent genomes. Gall-inducing insects have long been known to alter the plant morphology and physiology for their own benefits (Stone and Schönrogge 2003), but data on the potential capacity of other feeding guilds to modify the plant have remained scarce. Some leaf-mining insects are, however, known to manipulate the plant physiology in a remarkable way. For instance in some aging leaves in autumn, the mined part

of the leaf stays green, creating a green patch on a yellowing leaf named a green-island (Kaiser et al. 2010). This allows leaf-mining larvae to best meet their metabolic needs and compensate for variation in food nutritional composition (Body et al. 2013).

Microorganisms have been shown to be important "hidden players" in insect–plant interactions (Frago et al. 2012; Biere and Bennett 2013; Sugio et al. 2015) and can affect, among other traits, insect host plant range (Hosokawa et al. 2007), insect feeding efficiency, or their ability to manipulate the plant physiology for their own benefit (Kaiser et al. 2010). Insect symbionts can indeed directly or indirectly affect the plant by interfering with plant signal transduction pathways, repressing the expression of plant defense-related genes, altering plant primary and secondary metabolism, or counteracting plant defenses (Body et al. 2013; Giron et al. 2013; Sugio et al. 2015; Zhu et al. 2014). The role of bacterial insect symbionts in the context of plant manipulation is an emerging area of research, and leaf miners provide an excellent model system to study such tripartite ecological interactions (Frago et al. 2012). Indeed, it has been recently demonstrated that the green-island phenotype can be symbiont-mediated (Kaiser et al. 2010; Body et al. 2013). For instance, in the apple leaf miner moth *Phyllonorycter blancardella* (Gracillariidae) system, when the leaf miners are cured of their symbionts with antibiotics, their offspring is not capable of inducing green-islands and larval mortality is significantly increased due to food nutritional imbalances (Kaiser et al. 2010). Based on a PCR screening using universal and specific primers, *Wolbachia* is the only endosymbiotic bacterium found so far in *P. blancardella*. It has therefore been concluded that the presence of *Wolbachia* in the leaf miner *P. blancardella* is essential for the induction of the green-island phenotype allowing the insect to deal with a food supply that is highly variable and nutritionally suboptimal, particularly under senescing autumnal conditions (Kaiser et al. 2010). Several cases now demonstrate that *Wolbachia* could act as a mutualistic symbiont (Dedeine et al. 2001; Hosokawa et al. 2010; Nikoh et al. 2014) including its role played in plant alterations (Barr et al. 2010; but see Robert et al. 2013 for contradictory results suggesting that the observed effect of symbionts can be context dependent) and more specifically in the induction of green-islands in *P. blancardella* (Kaiser et al. 2010; Body et al. 2013). It is, however, unknown whether other leaf miners show a similar strategy which would extend the role of *Wolbachia* as a nutritional mutualist (Nikoh et al. 2014) and key player in insect–plant interactions. It would also broaden our understanding of the evolution of plant–insect interactions and the possible mechanisms involved in the plant manipulation strategies (Frago et al. 2012; Andrew et al. 2013; Giron et al. 2013; Giron and Glevarec 2014; Harris et al. 2015); Sugio et al. 2015.

In this study, we address the following question: Do *Wolbachia* infection and green-island induction co-occur across the evolutionary diversification of leaf miners? To see whether there is positive correlation between the acquisition and persistence of the green-island phenotype and *Wolbachia* infection, we screened the presence of green-islands and *Wolbachia* in 74 species of Lepidoptera including 60 leaf-mining Gracillariidae moths. We also optimized both traits onto a molecular phylogeny of the moths using a comparative approach. If *Wolbachia* plays an important role in the formation of green-islands in Gracillariidae, then we would expect positive correlations between the acquisition and persistence of the green-island phenotype and the acquisition and persistence of *Wolbachia* infection during the evolutionary diversification of Gracillariidae.

Materials and Methods

Taxon sampling

We sampled 119 specimens from 60 Gracillariidae species (Table S1, Supporting information) and 1 specimen from each of the 14 outgroup taxa. Leaves with mines were collected and individually reared as described in Lopez-Vaamonde et al. (2003). The presence/absence of green-islands was recorded for leaf miners reared from yellow leaves, and the occurrence of green-islands was set as unknown for leaf miners reared from green leaves.

DNA extraction, PCR amplification, and sequencing

We extracted DNA from whole specimen of 60 immature stages and 73 adult moths kept at −20°C (most of them in 99.5% ethanol) using the Microkit XS Nucleospin (Machery Nagel, France). The kit protocol was followed except for the incubation time with proteinase K, which was set to overnight and the final elution, which was a succession of two elutions with 10 μL of buffer AE.

Leaf miners

Sequence data were collected for one mitochondrial gene: cytochrome oxidase I (*COI*) and the three nuclear genes: elongation factor-1 alpha (*Ef1-α*), histone 3 (*H3*), and wingless (*Wg*). *COI* was used as DNA barcode for species identification (Hebert et al. 2003), and the three nuclear genes were chosen because they have been identified as good markers to reconstruct the phylogeny of *Lepidoptera*

(Wahlberg and Wheat 2008) in general and Gracillariidae in particular (Kawahara et al. 2011).

The primers used in both amplification and sequencing were LEP-F1 (5′ ATT CAA CCA ATC ATA AAG ATA T) and LEP-R1 (5′ TAA ACT TCT GGA TGT CCA AAA A) for the *COI* gene (Hebert et al. 2004); H3 F (5′ ATG GCT CGT ACC AAG CAG ACG GC) and H3 R (5′ ATA TCC TTG GGC ATG ATG GTG AC) for the *H3* gene (Colgan et al. 1998); Ef1-F (5′ GGG AAA TGG CAA GCA AAA TGG) and Ef1-R (5′ CAT CGC ACT AAG ACC CAC C) for the *Ef1-α* gene (Serbielle et al. 2009); and LepWG1 (5′ GAR TGY AAR TGY CAY GGY ATG TCT GG) and LepWG2a (5′ ACT ICG CAR CAC CAR TGG AAT GTR CA) for the *Wg* gene (Brower and DeSalle 1998).

DNA of forty-one samples was extracted and barcoded at the Canadian Centre for DNA Barcoding (CCDB – Biodiversity Institute of Ontario, University of Guelph) using the standard high-throughput protocol described in Ivanova et al. (2006). The remaining samples were barcoded and sequenced for all the other three genes at the Institut de Recherche sur la Biologie de l'Insecte (IRBI – Centre National de la Recherche Scientifique & University F-Rabelais in Tours). PCR was performed in a 25 μL volume containing 1 unit of Goldstar polymerase (Eurogentec, France), 0.2 mmol/L of dNTP, 1.5 mmol/L of $MgCl_2$, and 50 pmol of each of the primers. The PCR conditions were 1 min at 94°C, 6 cycles at 94°C (1 min), 45°C (1 min and 30 sec), and 72°C (1 min and 15 sec), followed by 36 cycles at 94°C (1 min), 51°C (1 min 30 sec), and 72°C (1 min 15 sec), and a final extension at 72°C (5 min) for the *COI and Ef1-α* genes; 1 min at 94°C followed by 40 cycles at 94 94°C (1 min), 45°C (1 min), and 65°C (1 min) and a final extension of 65°C for 10 min for the *H3* gene; and 7 min at 95°C followed by 40 cycles of 95°C (1 min), 50°C (1 min), and 72°C (2 min) and a final extension at 72°C for 10 min for *Wg*.

Wolbachia screening and genotyping

The 16S *rRNA* gene was first used to detect the presence of *Wolbachia*. For the samples where no 16S *rRNA* was amplified, we used the fructose-bisphosphate aldolase (*fbpA*) gene, which is known to be more sensitive although less specific than 16S *rRNA* (Simões et al. 2011). The different *Wolbachia* supergroups were assigned based on the 16S *rRNA* sequence dataset. When enough DNA was available, we also collected sequence data from the outer surface protein (*wsp*) in order to distinguish closely related *Wolbachia* strains.

The primers used in both amplification and sequencing were W-Specf (5′ CAT ACC TAT TCG AAG GGA TAG) and W-Specr (AGC TTC GAG TGA AAC CAA TTC) for the 16S *rRNA* gene fragment (438 base pairs) (Werren and Windsor 2000); fbpA-F1 (5′ GCT GCT CCR CTT GGY WTG AT) and fbpA-R1 (5′ CCR CCA GAR AAA AYY ACT ATT C) for the *fbpA* gene (Baldo et al. 2006); and wsp81f (5′ TGG TCC AAT AAG TGA TGA AGA AAC) and wsp691r (5′ AAA AAT TAA ACG CTA CTC CA) for the *wsp* gene (Braig et al. 1998).

PCR conditions were 1 min at 95°C followed by 40 cycles 95°C (1 min), 50°C (1 min), and 72°C (1 min) and a final elongation at 72°C of 10 min for both 16S *rRNA* and *fbpA* genes; and 2 min at 95°C followed by 35 cycles of 95°C (30 sec), 52°C (30 sec), and 72°C (1 min and 30 sec) and a final elongation at 72°C of 10 min for the *wsp* gene.

Sequencing

All PCR products (both leaf miners and *Wolbachia*) were purified with the Nucleospin Extract II kit (Macherey-Nagel, France) following the kit protocol except for the elution incubation time being set to 15 min and elution centrifugation time being set at 2 min. The purified PCR products were sequenced directly with an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin–Elmer Biosystems) on an ABI PRISM 3100 automated sequencer. All products were sequenced in both directions.

COI (674 bp), H3 (328 bp), *EF1-α* (664 bp), Wg (406 bp), *fbpA* (524 bp), 16S *rRNA* (398 bp), and *wsp* (564 bp) sequences were read, edited, and aligned using Geneious pro 5.5.7. All sequences have been deposited in GenBank (accession numbers are available in Table S1, Supporting information). DNA barcodes have been deposited in the Published Projects section of the Barcode of Life Data systems (BOLD) project code: GRISL (www.barcodinglife.org) (Ratnasingham and Hebert 2013).

Species identification

Adult leaf miners were identified based on wing patterns and, in some cases, genitalia and host plant data. In addition, both adults and leaf-mining larvae were identified using COI sequences using the BOLD online database (http://v3.boldsystems.org/).

Phylogenetic analysis

All Lepidoptera and *Wolbachia* gene sequences were aligned using ClustalW algorithm within Geneious.

The reconstruction of the phylogenetic tree was based on H3, COI, Wg, and *EF1-α*. We performed Bayesian phylogenetic analyses on the concatenated host gene

alignments using MrBayes v. 3.2 (Ronquist et al. 2012) with branch support based on posterior probabilities (Pp). We used jModelTest v. 0.1.1 (Posada 2008) to select the least complex substitution model for each gene and a GTR substitution model with a gamma distributed rate of variation across sites, and a proportion of invariable sites was found to be optimal for all four genes. We used default priors, and each of the four host genes was allowed to have its own unlinked substitution model. We ran two parallel runs having three incrementally heated chains for 30 million generations, while sampling trees from the current cold chain every 10 000 generations. We discarded 10 trees sampled prior to reaching chain stationary as a burn-in from both runs, and the remaining 5982 trees were used to calculate a consensus tree.

The 24 *wsp* sequences obtained in this study were aligned to 22 relevant *wsp* sequences previously published (Baldo et al. 2006). We chose *wsp* sequences from *Wolbachia* variants that were assigned to one of two supergroups of *Wolbachia* (A and B) using multilocus sequence typing (MLST) approach (i.e., eight wsp sequences from A *Wolbachia* and 11 from B *Wolbachia*) (Baldo et al. 2006). We added the *wsp* sequence obtained from the *Wolbachia* infection previously detected in the leaf miner *P. blancardella* (Kaiser et al. 2010). Two additional wsp sequences from C and D *Wolbachia* supergroups (Bazzocchi et al. 2000) were used as outgroup in phylogenetic analyses. Similarly, the 36 sequences of the 16S gene obtained in this study were aligned to eight relevant sequences representative of A and B *Wolbachia* supergroups and one sequence of *Rickettsia* as outgroup. The two obtained alignments were individually used for phylogenetic analyses using both Bayesian and maximum-likelihood inferences. Maximum-likelihood inferences were performed using Seaview v. 4.4.1 (Gouy et al. 2010). The appropriate model of evolution was estimated with jModelTest v. 0.1.1 (Posada 2008). The models selected were GTR+I+G for both *wsp* and 16S rRNA genes.

Ancestral state reconstruction

The ancestral states of green-island formation and *Wolbachia* infection were traced using the ace function of "Analyses of Phylogenetics and Evolution" (APE) in R (http://ape-package.ird.fr/) to reconstruct ancestral character states using maximum likelihood and optimized on the moth Bayesian topology.

Correlation analysis

We tested the correlation between the occurrence/absence of green-islands and the occurrence/absence of *Wolbachia* using BayesTraits (available on http://www.evolution.rdg.ac.uk) (Pagel 1994). We used all 5982 sampled trees of leaf miners identified during the Mr Bayes search. As polytomies are not allowed by BayesTraits, they were removed using the multi2di function in the ape package version 3.0–7, which replaces the polytomies by several dichotomies with a zero branch length.

For the correlation analysis, we used the Bayes factor test that compares the log of the harmonic mean between two MCMC analyses of discrete character evolution (Barker et al. 2007). One analysis allows the character to evolve independently, and the other analysis assumes that their evolution is correlated. Prior to the MCMC, a ML analysis was performed for dependent and independent scenario, and for each, the distribution of the different transition rates was taken into account to set the MCMC parameters.

Given the results of the likelihood analysis, we used a MCMC prior as uniform distribution for all rates, with different ranges for each parameter. For the independent model, we used a range of parameters from 0 to 1000 for alpha-1 and beta-1 and from 0 to 100 for alpha-2 and beta-2. For the dependent model, we used a range from 0 to 1000 for q21, q24, and q31; from 0 to 500 for q42; and from 0 to 100 for q12, q13, q34, and q43.

For both MCMC analyses, we used 5 050 000 generations including a burn-in period of 50 000 generations. The sampling period was set at 1000 generations, and a rate deviation of two was used. The mean of the acceptance rate was, respectively, around 21.0% and 19.5% for dependent and independent models. Using less constrained parameters (uniform 0–1000 for every transition rates), the mean of the acceptance rates was higher, but this caused the distribution for the independent model to be bimodal. We decided to choose priors that made the acceptance rate distribution be most similar between the two analyses because the change in the range of the uniform distribution had little impact on the results of the correlation test.

A likelihood ratio test implemented in BayesTraits was finally used to test for a correlation between the acquisition of green-island phenotype and the acquisition of *Wolbachia* through the phylogeny of moths.

Results

DNA barcoding and species identification

Of the 163 DNA extractions performed (75 immature stages and 88 adult moths), 135 were successfully barcoded. Among these 135 barcodes, we found two cases (1.5%) of unexpected DNA amplification. In a first case, a *Wolbachia* was sequenced rather than the insect, (Smith et al. 2012) and in a second case, a parasitoid was

barcoded from a leaf-mining larva. Both sequences were excluded from phylogenetic analysis. In total, 133 specimens representative of 74 species were DNA barcoded. All specimens were identified down to species level except six specimens that belong to five new undescribed species (no species name available): Two of them were from Japan and three from French Guiana (Table S1).

Lepidoptera phylogeny

Sequence data were obtained for 116 specimens (68 species) for *H3* gene, 25 specimens (23 species) for *Wg,* and 23 specimens (22 species) for *Ef1-α* (Table S1, Supporting information). The total concatenated alignment consisted of 2072 base pairs. The highest proportion of variable characters was found in *COI* (50%), followed by similar values for *Wg* (40%) and *H3* (35%), and slightly lower proportions for *EF1-α* (25%).

The Bayesian tree (Fig. 1) revealed well-supported relationships between genera and species within subfamilies Gracillariidae and Lithocolletinae. Conspecific individuals collected from different locations and/or hosts also form strongly supported monophyletic clades. *Lithocolletinae* forms a monophyletic group (Pp = 1.00) with *Cameraria* as sister taxa to *Phyllonorycter* (Pp = 0.91).

Green-island phenotype

Of the 107 samples for which mines were observed on yellow leaves, 30 specimens (28%) showed green-islands. Among these 30 moths, 28 belonged to the Gracillariidae family. (Fig. 1; Table S1, Supporting information). The ML reconstruction suggests that ancestral Gracillariidae did not form green-islands (Fig. 2) and were not infected with *Wolbachia* (Fig. 3). Of the 23 species for which the presence/absence of green-islands was scored in more than one specimen, three species (*P. pyrifoliella, P. joannisi, and P. comparella*) showed between-individuals variability for green-island formation (i.e., some individuals produced green-islands and others did not).

Pattern of *Wolbachia* infection

We successfully amplified the rRNA 16S gene for 39 of the 133 samples. Of the 94 samples that did not amplify, 93 were screened for *fbpA* gene. For one sample (*Ectoedemia heringella* (FG19)), there was not enough DNA to perform PCR amplification, so its *Wolbachia* infection status was set as unknown. Of the 93 samples that did not amplify 16S, only two amplified *fbpA* gene (*Anthophila fabriciana* (FG10) and *Phyllonorycter oxyacanthae* (FloG157)). Of these two samples, the latter was successfully sequenced and identified as a *Wolbachia* sequence. The infection status of the other sample remained unknown (Table S2, Supporting information).

Of 119 Gracillariidae leaf miners analyzed, 35 specimens (30%) were infected with *Wolbachia* (Table S1, Supporting information). Seven Gracillariidae species showed intraspecific variability for *Wolbachia* infection (*P. acerifoliella*, *P. comparella*, *P. issikii, P. joannisi, P. oxyacanthae, P. platani,* and *P. rajella*). No obvious phylogenetic separation between infected and uninfected clades was observed (Figs 1 and 3).

From 36 of the 39 amplified 16S, we obtained the sequences of the region 16S (Fig. 4). We aligned them with 8 other *Wolbachia* sequences from GenBank for which the supergroup was known and one sequence from *Rickettsia*. Phylogenetic trees reconstructed using both Bayesian and maximum-likelihood inferences were congruent and revealed that our sequences clustered into two main groups (Fig. 4; Table S2, Supporting information). According to the imported sequences, the two lineages most likely correspond to the two most common *Wolbachia* supergroups infecting arthropods, named A and B (Fig. 4; Table S2, Supporting information). In addition, we obtained the *wsp* sequence from 24 infected individuals that we aligned with *wsp* sequences belonging to *Wolbachia* A and B supergroups (Baldo et al. 2006). The Bayesian tree (Fig. 5) shows that samples infected with *Wolbachia* A form a well-supported monophyletic group. *Wolbachia* that most probably belong to the supergroup B based on their 16S sequences formed two main lineages (named B1 and B2) in the Bayesian *wsp* phylogenetic tree (Fig. 5; Table S2, Supporting information). ML analyses showed the same result for both 16S and *wsp* genes (data not shown).

No case of multiple *Wolbachia* variants infecting same individuals was detected. In particular, attentive observation of sequence chromatograms revealed no case of double peak. However, some individuals collected in same populations were sometimes found to harbor different *Wolbachia* strains belonging to either A or B *Wolbachia* supergroup (e.g., *P. cerasicolella*) (Tables S1 and S2, Supporting information). As observed in many other arthropod–*Wolbachia* associations (e.g., see Frost et al. 2010), Lepidoptera specimens infected with the same *Wolbachia* variant do not form monophyletic groups (Fig. 1). Although *wsp* gene is not suitable for phylogenetic reconstruction and *Wolbachia* supergroup assignment (Baldo et al. 2005), it is worth noting that both *wsp* and 16S sequence datasets generally gave congruent information on the two *Wolbachia* supergroups (Tables S1 and S2, Supporting information), with the exception of three samples (143, 151, and 66).

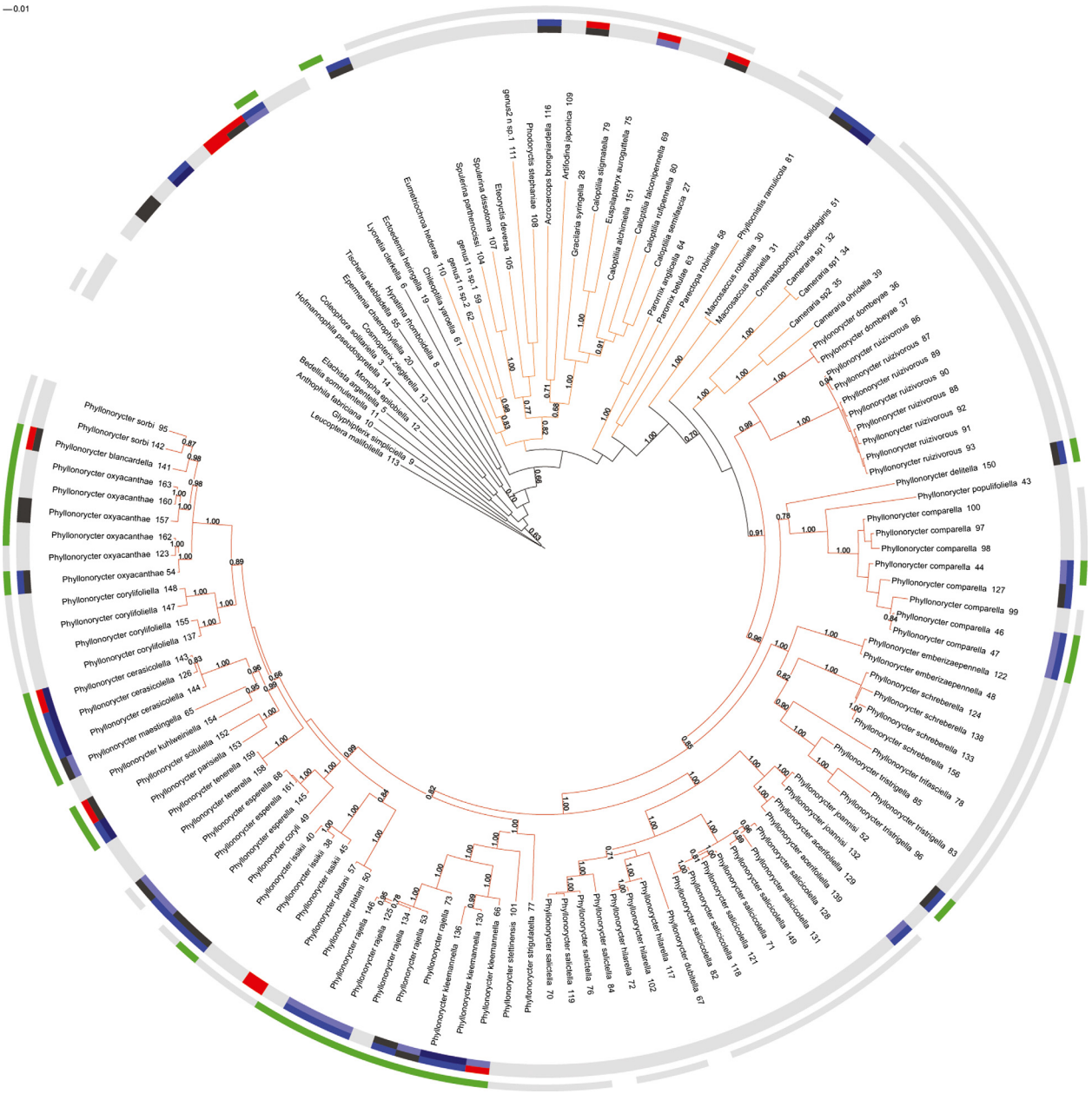

Figure 1. Bayesian phylogeny of studied Lepidoptera and distribution of green-island formation and *Wolbachia* infection based on COI, H3, Wg, and *EF1-α* genes. The PP support values above 0.6 are shown above branches. Colored branches indicate species belonging to the family Gracillariidae (red = *Phyllonorycter* species). For all the color strips, light gray indicates the absence of the character and a color its presence. White indicates the absence of data. The two first rows (starting from inside) of color strips represent *Wolbachia* groups according, respectively, to wsp and 16S. The red represent the A group and blue the B group; dark gray is for the infected strain for which the group is unknown. The two tones of blue in the wsp band represent two subgroups of the B group. The last band shows the green-island presence. Each taxa name is the species name followed by the sample name.

Correlation between *Wolbachia* infection and green-island formation

Of 28 Gracillariidae specimens that were found capable of green-island formation, 22 (78.6%) were associated with the presence of *Wolbachia* (Fig. 1; Table S1, Supporting information). However, of 76 Gracillariidae specimens for which no green-island was observed, only eight specimens (10.8%) were infected with *Wolbachia*. Interestingly, six specimens (four specimens of *P. oxyacanthae*, one

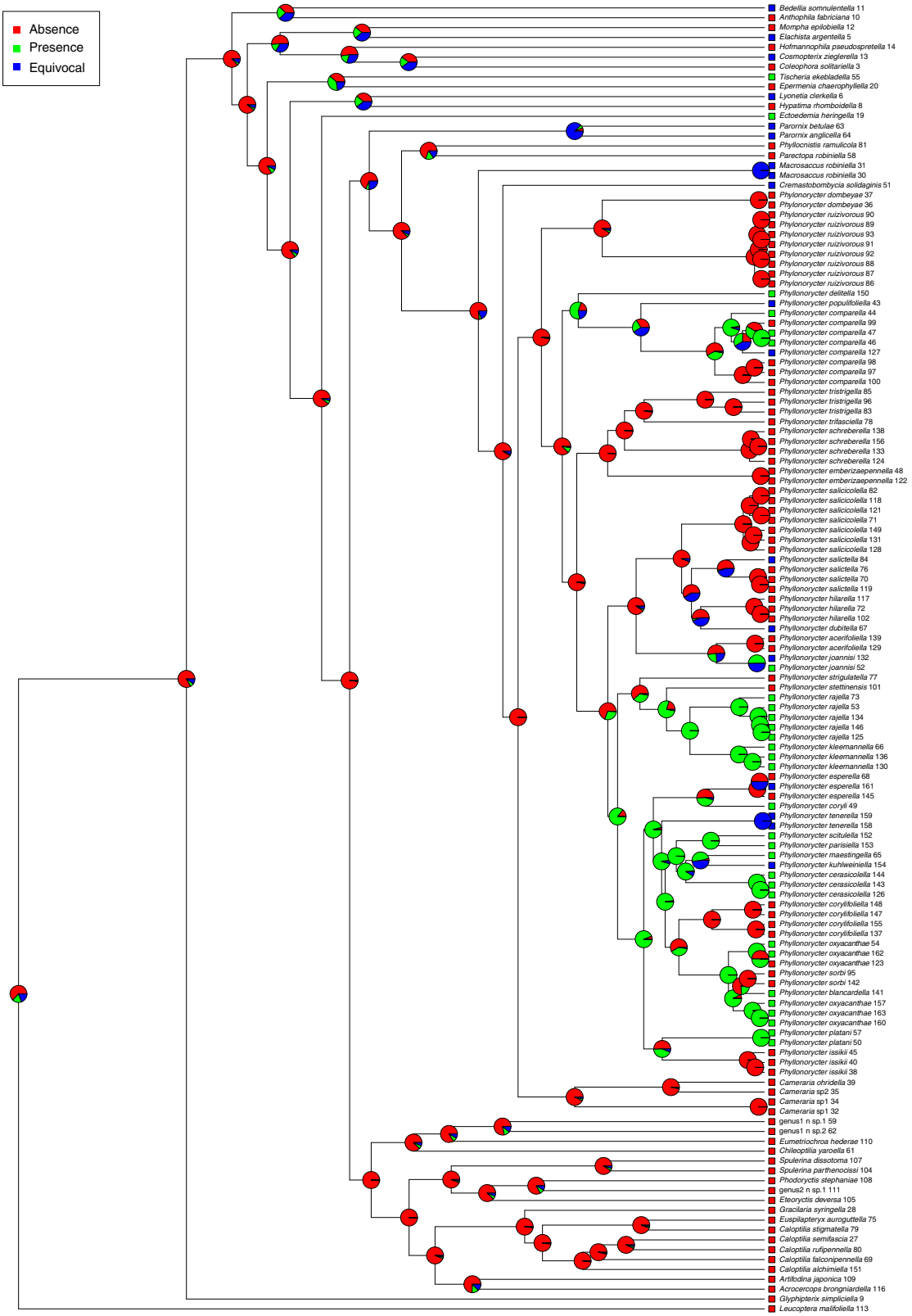

Figure 2. ML reconstruction of the green-island ancestral state on the leaf miner's phylogeny. Red color shows the absence of green-island, green color the presence of green-island, and blue color when green-island presence or absence was unknown.

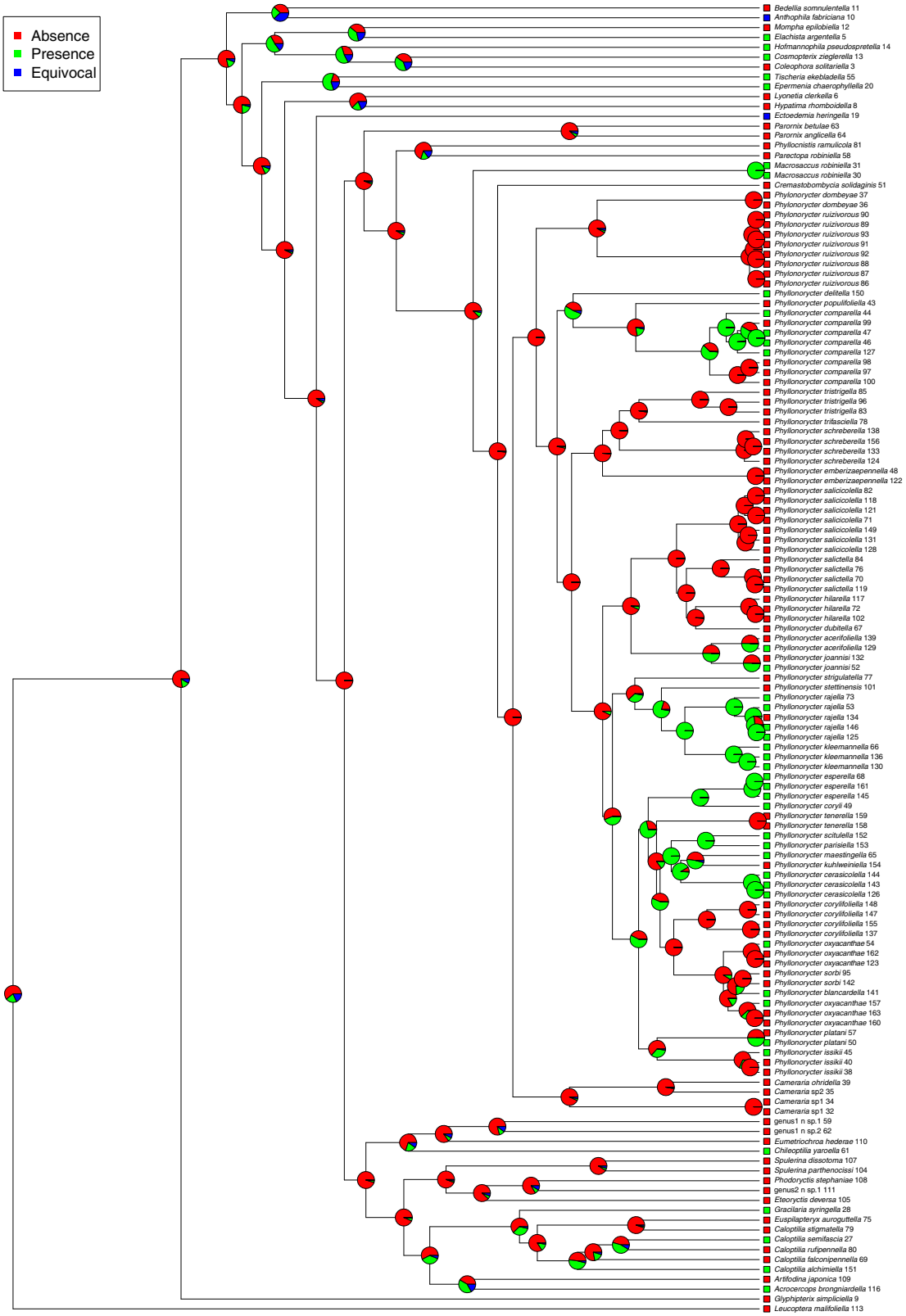

Figure 3. Reconstruction of the ancestral state of *Wolbachia* infection on the leaf miners phylogeny. Red color shows the absence of *Wolbachia* infection, green color the presence of *Wolbachia,* and blue color when *Wolbachia* presence or absence was unknown.

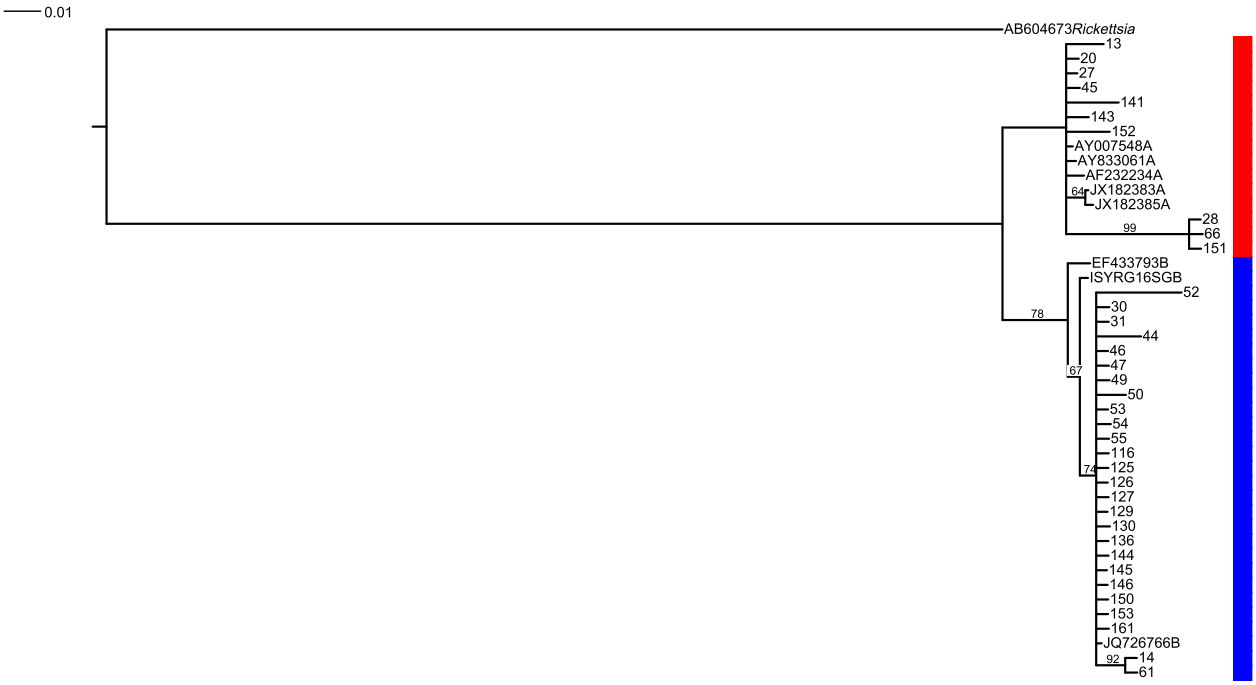

Figure 4. Bayesian phylogeny of *Wolbachia* based on 16S where the two groups A and B correspond, respectively, to red and blue. Pp supporting values over 70% are displayed above branches. Terminal taxa are named with either the DNA extract code of their Lepidoptera hosts (Table S1) or with accession numbers for sequences imported from GenBank.

specimen of *P. platani*, and one specimen of *P. rajella*) were able to induce green-island without the presence of *Wolbachia* (Fig. 1; Table S1, Supporting information).

Wolbachia variants belonging to both A and B supergroups were found to be associated with green-island formation.

The likelihood ratio test shows that green-island formation and *Wolbachia* infection are highly correlated (LR = 2(−116.440786–(−137.681566)) = 42.48156; df = 4 *P*-value <0.0005).

Discussion

This study aimed to (1) examine the *Wolbachia*/leaf miner/green-island interaction by screening the co-occurrence of green-island phenotype and *Wolbachia* infection in 60 species of leaf-mining Gracillariidae moths, and (2) test the hypothesis that the presence of *Wolbachia* and the presence of green-island phenotype are traits that were acquired and persisted independently across the Gracillariidae phylogeny.

DNA barcoding as a tool for identification of immature stages

DNA barcoding (Hebert et al. 2003) has been shown to be an efficient tool for species identification in Lepidoptera in general (Hebert et al. 2004; Dinca et al. 2011; Mutanen et al. 2013; Rougerie et al. 2014) and Gracillariidae in particular (Lees et al. 2013; Kirichenko et al. 2015). However, some groups present problems such as high intraspecific variability (Meier et al. 2006) – sometimes caused by *Wolbachia* (Kodandaramaiah et al. 2013); low interspecific divergence (Wiemers and Fiedler 2007); and pseudogenes (Song et al. 2008). In our study, DNA barcoding allowed us to identify immature stages of leaf miners to species level. Therefore, we used larvae directly in our *Wolbachia* screening without the need to rear them to adulthood. Using larvae for *Wolbachia* detection is particularly relevant in the context of this study because only larvae are in intimate interaction with the plant and therefore are expected to directly benefit from the presence of the green-island phenotype. Without the help of DNA barcodes, the identification of those larvae would have been most challenging and based entirely on host plant data, which may have introduced ID errors, despite the high degree of host specialization of most Gracillariidae species (Lopez-Vaamonde et al. 2003, 2006).

Correlation between the presence of Wolbachia and a green-island phenotype: a functional link?

A significant correlation was found between the acquisition of the green-island phenotype and *Wolbachia*

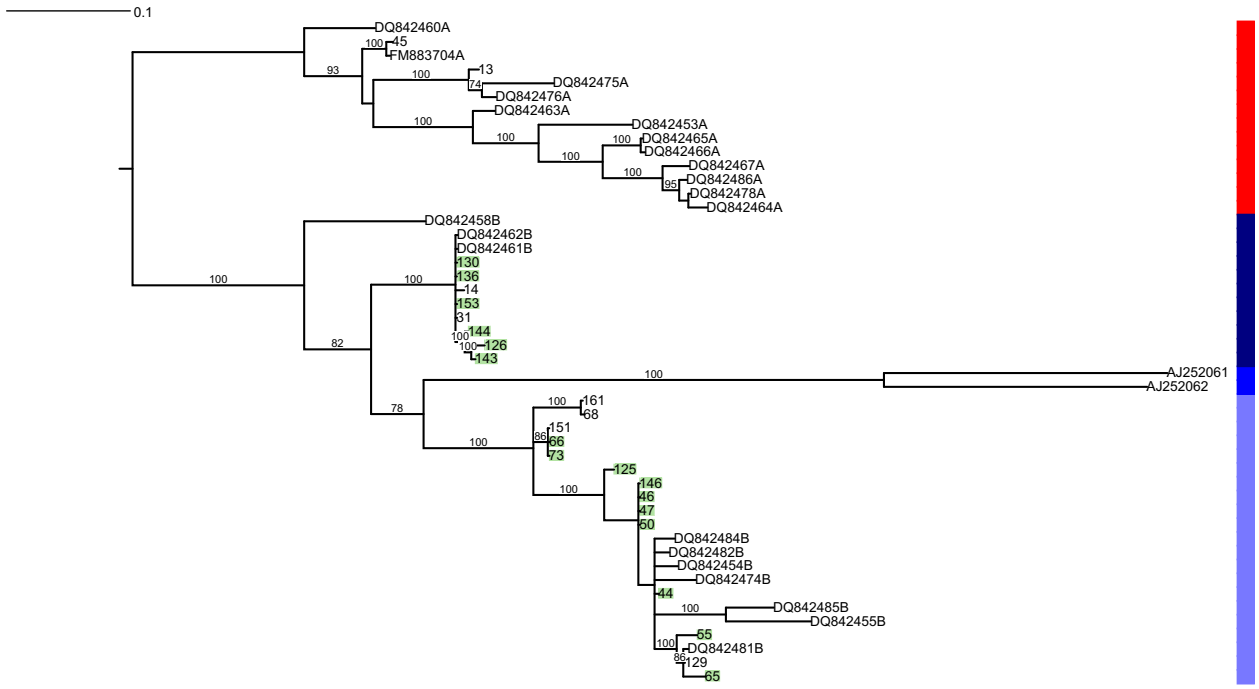

Figure 5. Bayesian phylogeny of *Wolbachia* based on *wsp* gene where the two groups A and B correspond, respectively, to red and blue. The two subgroups inside the B group are represented by two different blue shades. Pp supporting values over 70% are displayed above branches. Terminal taxa are named with either the DNA extract code of their Lepidoptera hosts (Table S1) or with accession numbers for sequences imported from GenBank. Samples highlighted in green indicate Green-island associated *Wolbachia*.

infection through the evolutionary diversification of Gracillariidae. Therefore, *Wolbachia* is likely to have played an important role in the evolution of the green-island phenotype, not only in the apple leaf miner (Kaiser et al. 2010) but also in Gracillariidae leaf-mining moths in general.

We also found evidence for infection by different *Wolbachia* strains among the Gracillariidae suggesting that horizontal transfers of *Wolbachia* play a role in dynamics of the green-island phenotype. The several independent gains and losses of *Wolbachia* infections over the Gracillariidae phylogeny are consistent with the results of a previous study on the genus *Phyllonorycter* (West et al. 1998). Horizontal transfers and losses of *Wolbachia* have also been shown to be common in other groups (Dedeine et al. 2005; Frost et al. 2010). The formation of green-islands is also characterized by a dynamic scenario of gains and losses through Gracillariidae evolution. Therefore, the co-occurrence of both traits is most probably the result of a functional link between bacteria and the insect. For instance, *Wolbachia* could have a direct role in the plant manipulation by producing cytokinins or give the leaf miner the ability to produce these phytohormones leading to the green-island phenotype (Kaiser et al. 2010; Body et al. 2013; Giron and Glevarec 2014).

Our results suggest that Gracillariidae are most likely infected by two different groups of *Wolbachia* and that both seem to be associated with green-island formation. Several explanations could account for this result: (1) The green-island function have several origins in different parasitic *Wolbachia* clades (convergence?), (2) the genetic mechanism enabling this function is present in all *Wolbachia* strains with an incomplete expression (conserved function in *Wolbachia* genus), or (3) a gene transfer could have occurred between the different *Wolbachia* strains, for instance, through prophage infection that can be shared during a coinfection (Kent et al. 2011; Boto et al. 2010).

Intraspecific variability of the interaction between *Wolbachia* infection and green-island formation

We failed to detect *Wolbachia* infection in six specimens from three *Phyllonorycter* species that were yet collected from leaf mines with green-islands. We cannot totally exclude the possibility that these individuals were in fact infected, but that our PCR-based diagnostics failed to detect the infection because the bacterial density in these individuals was below the detection threshold (Arthofer et al. 2009; Schneider et al. 2013). Another explanation is

that other mechanisms allowed the induction of green-island phenotype in the plant. In this case, several hypotheses can be suggested: (1) Another symbiont such as a bacterium, virus, or fungi might be responsible for green-island formation (Kent et al. 2011; Giron et al. 2013; Giron and Glevarec 2014); (2) the leaf miner itself could also have evolved the ability to form green-islands without any associated symbiont; and (3) a gene transfer may have occurred from *Wolbachia* to another bacterium during a coinfection or directly into the insect genome followed by a secondary loss of *Wolbachia*. Indeed, gene transfers from symbiotic bacteria, including *Wolbachia,* to their insect host are known in several species (Boto 2010; Sugio et al. 2015). These bacterial genes transferred to the host can be functional inside the insect, and the position of *Wolbachia*, infecting the germinal cells, makes the heritability of the gene transfer more likely (Walters et al. 2008). Frequent gene transfers between *Wolbachia* together with dynamic infection could explain the presence of the green-island function among phylogenetically distant *Wolbachia* clades. Once acquired, the function is inherited and the newly transferred bacteria can spread the extended phenotype to other leaf miner species. This could account for part of the variability of the green-island phenotype among the Gracillariidae. Despite no evidence of coinfection with different *Wolbachia* strains in our study, it could also explain the gene transfer to another symbiont or the insect with a smaller rate followed by the loss of the *Wolbachia* due to its cost/virulence.

Additionally, of the 37 leaf miners infected with *Wolbachia* (including 35 leaf-mining Gracillariidae*)*, eight showed an absence of green-island. The absence of green-island despite a *Wolbachia* infection can be due to different factors. A *Wolbachia* strain could lack the ability to induce green-islands although in our case both groups of *Wolbachia* A and B (B1 & B2) were found to be associated with green-island formation. Alternatively, either the host plant or the insect or both might be unable to produce green-islands. The phytohormones cytokinins have been found to play a key role in the green-island induction as well as in several plant–insect–bacteria interactions (Giron et al. 2013; Giron and Glevarec 2014; Schäfer et al. 2014) and may constitute key components of these "incompatible" interactions. Failure to alter the plant metabolism – resulting in an induced extended phenotype – is, however, largely under-investigated both in ecological and in laboratory settings. Ecological surveys of herbivore failure/success and molecular investigations of compatible/incompatible interactions will undoubtedly help to unravel mechanisms involved in plant manipulation by herbivorous insects (such as leaf miners and gall inducers) and the role played by insect symbionts (Fernandes et al. 2003; Harris et al. 2015). It will also open new promises for the understanding of natural community structure and the development of new plant resistance strategies in agriculture (Harris et al. 2015).

Conclusion

Our results show that the acquisitions of the green-island phenotype and *Wolbachia* infections are associated in Gracillariidae moths. Two *Wolbachia* strains are associated with the green-island formation in Gracillariidae suggesting several independent origins of green-island induction. In some species, the green-island phenotype is not dependent on the presence of *Wolbachia* raising the question of whether other symbionts could potentially be associated with green-island phenotype. Although *Wolbachia* endosymbionts are ubiquitously found in diverse insects and primarily known for their influence on the reproductive biology of their hosts, our study strongly support the view that *Wolbachia* can also be considered as a nutritional mutualist and reinforce the idea that *Wolbachia* played a key role in the adaptation of leaf miner insects to their host plant and in the evolution of the plant–insect interactions.

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Conflict of Interest

None declared.

Data Accessibility

DNA sequences: Leaf-Miners : GenBank accessions KF367636-KF367728; *Wolbachia*: GenBank accessions KR698117-KR698176 (available in Supplementary data Table S1).

Specimen voucher data, sequences, and images for all specimens can be found online within the BOLD dataset DS-GRISL1 accessed at http://dx.doi.org/10.5883/DS-GRISL1We have deposited the concatenated alignment to build the phylogenetic trees (Figs 1-3) in the Dryad repository (http://dx.doi.org/10.5061/dryad.q4747).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Lepidoptera specimen information.
Table S2. *Wolbachia* specimen information.