***Wolbachia* (Alphaproteobacteria: Rickettsiales) Infections in Isolated Aphid Populations from Oceanic Islands of the Azores Archipelago: Revisiting the Supergroups M and N**

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Abstract

Aphids (Hemiptera: Aphididae) have provided a suitable model to study endosymbionts, their community, and dynamics since the discovery of the obligate endosymbiont *Buchnera aphidicola* in these organisms. In previous studies, *Wolbachia* was found in some aphid species. In the present study, we report the prevalence of *Wolbachia* in aphids sampled from a geographically isolated region (Azores Islands), aiming at a better understanding and characterization of the two newly reported supergroups, M and N. The description of the supergroup M was based on *16S rRNA* as well as some protein-coding genes. However, the assignment of the supergroup N was according to *16S rRNA* gene sequences of a very few samples. We collected aphid samples and performed phylogenetic analysis of *16S rRNA* gene as well as four protein-coding genes (*gatB*, *ftsZ*, *coxA*, and *hcpA*). The results demonstrate that the *16S rRNA* gene data can unambiguously assign the strain supergroup and that the two supergroups, N and M, are equally prevalent in Azorean aphids. The available sequence data for the protein-coding markers can identify supergroup M but the status of supergroup N is inconclusive, requiring further studies. The data suggest that horizontal transmission of *Wolbachia* (Hertig and Wolbach) between two phylogenetically distant aphid species cohabiting the same plant host.

Key words: endosymbiosis, phylogenetic analysis, protein-coding genes and *16S rRNA* genes

Many arthropods have developed close relationships with plants which they feed upon; therefore, they can cause direct damage to their hosts by depleting nutrients or/and can be harmful by transmitting disease to their hosts (Perilla-Henao and Casteel 2016). On the other hand, arthropods have also developed a variety of symbiotic associations, with their intracellular microorganisms (endosymbionts) that can provide an effective system to control pest and disease vector populations (Saridaki and Bourtzis 2010, Bourtzis et al. 2014).

The endosymbionts that provide essential nutrients for the hosts, thus indispensable for their survival are referred as primary or obligate endosymbionts. Additionally, may harbor secondary facultative endosymbionts that can be dispensable, although under certain circumstances they can provide some benefits to their hosts and/or modify their biology (Engelstädter and Hurst 2006, Oliver et al. 2010, Oliver et al. 2014). The secondary endosymbionts, in some cases, can modify and turn into obligate ones (reviewed in Moya et al.

2008). The best studied facultative endosymbionts are *Wolbachia*; these Alphaproteobacteria are widely spread in diverse invertebrate groups (Zug and Hammerstein 2012). They are primarily transmitted vertically from mother to offspring, although horizontal mode of transmission has also been reported (Stouthamer et al. 1999, Russell et al. 2003, Ahmed et al. 2015). Based on phylogenetic studies, *Wolbachia* have been divided into distinct monophyletic lineages known as supergroups (Zhou et al. 1998, Baldo et al. 2006, Lo et al. 2007) and so far, 16 *Wolbachia* supergroups are described: A–Q (except G that is, in fact, a combination of A and B) (Augustinos et al. 2011, Bing et al. 2014, Glowska et al. 2015). A new supergroup (R) was proposed by Wang et al. (2016) in cave spider species, however, this designation was challenged by another study after reanalyzing the data (Gerth 2016). Depending on the bacterial type and the host–*Wolbachia* symbiotic association, the symbiont can influence their host's biology in numerous manners including the induction

of several reproductive anomalies (cytoplasmic incompatibility, male killing, feminization, etc.) (Breeuwer and Werren 1990, Stouthamer et al. 1990, Werren et al. 2008), but also positive effects, including nutritional role, are found (Nikoh et al. 2014, Akoundi et al. 2016). Thus, strain typing is essential in understanding the genetic structure, evolution, and biology of the bacteria.

Aphids are a highly diversified and widely distributed group of insects (Blackman and Eastop 1984); these plant sap-feeding insects can cause severe damage to plants. Aphids have evolved very close associations with bacterial endosymbionts. Their success may have been related to their ability to use a large variety of plants or/and acquiring endosymbionts. The primary endosymbionts of aphids, *Buchnera aphidicola*, provide some essential nutrients that are lacking in their unbalanced diet (Hansen and Moran 2011). Until now, up to nine secondary or facultative symbionts have been reported in aphids (reviewed in Guo et al. 2017). Depending on their type, the bacteria may benefit the host in diverse ways such as heat shock resistance, parasitoid wasp resistance, fungi resistance, enhancement of host plant fitness, and favoring the supply of essential nutrients with another co-obligatory symbiont (Chen et al. 2000, Oliver et al. 2003, Gómez-Valero et al. 2004, Oliver et al. 2005, Scarborough et al. 2005, Lukasik et al. 2013a, De Clerck et al. 2015). They can also have negative impact in the host by reducing the host's longevity, host plant fitness, etc., (Chen et al. 2000, Simon et al. 2007, Polin et al. 2014). Some secondary endosymbionts, such as *Serratia symbiotica* and *Wolbachia*, can potentially provide nutrition to the host (Koga et al. 2003, Pérez-Brocail et al. 2006, Koga et al. 2007, De Clerck et al. 2015). A single symbiont might influence the host in diverse manners and a single host might carry several symbiont species interacting with each other and influencing the host biology (Tsuchida et al. 2004, Oliver et al. 2010, Russell et al. 2013, Lukasik et al. 2013b, Oliver et al. 2014, Heyworth and Ferrari 2015, Leclair et al. 2016a). Moreover, change in symbiotic status (secondary or facultative to primary or obligate) in some aphid species has also been reported (Lamelas et al. 2008, Lamelas et al. 2011, Manzano-Marín and Latorre 2014, Latorre and Manzano-Marín 2017). The first report on the presence of a *Wolbachia* strain in an aphid species, *Cinara cedri*, was in 2004 (Gómez-Valero et al. 2004). However, until recently *Wolbachia* infections were considered rare in aphids, but a different picture was emerged by two recent studies of European and Chinese populations (Augustinos et al. 2011, Wang et al. 2014). Augustinos et al. (2011) identified the two already described supergroups (A and B) and characterized two new ones (M and N) in 425 European samples representing 144 species, while Wang et al. (2014) detected three supergroups (A, B, and M) in 109 samples representing 73 species. The infection rate was unprecedentedly high in Chinese populations (100%), while only 8.7% (37 of 425 samples) of the European populations harbored *Wolbachia*. In both studies, the supergroup M was the most prevalent in aphids. Wang et al. (2014) hypothesized that this supergroup spread recently and rapidly in aphids from China. Despite the high rate of infection and elevated number of samples studied, supergroup N was not detected in Chinese populations. On the other hand, in European populations N supergroup was only detected in three samples, representing two species (*Toxoptera aurantii* and *Neophyllaxis podocarpus*), collected from two of the Azorean Islands (Terceira and São Miguel in April 2009 and September 2010, respectively). Thus, so far, Azores is the only geographical area where supergroup N has been found. Supergroup M was the only other supergroup detected in four of Azorean samples representing one species of Aphidinae.

Due to geographical isolation and predicted population size reduction of the founders, we expect to detect less strain types in

island compared to mainland, but also the occurrence of new isolates in island is quite possibly due to the novel ecological conditions that the host is subjected to. To better understand the prevalence and types of *Wolbachia* in aphid populations from the Azorean Archipelago, in this study, we characterize the bacteria strains using gene fragments of the *coxA*, *ftsZ*, *gatB*, and *hcpA* genes in 115 aphid samples collected in three Azorean islands (São Miguel, São Jorge, and Pico).

Materials and Methods

Aphids Collection

Aphids were collected in June 2012 from three of the nine islands of the Azores Archipelago, in the Atlantic Ocean (Supp Fig. S1). The nearest island to the mainland is about 1.600 km west of the coast of Portugal. The collection sites, aphid family, species, and their host plants are presented in Supp Table S1. Aphids were kept in 100% ethanol at -20°C until their DNA extraction. The aphids' identification was based on the dichotomous keys provided by Blackman and Eastop (1989, 1994). A total of 115 aphid samples representing 18 species were collected. From these samples, 89 belonged to Aphidinae subfamily (16 species); 21 to Lachninae subfamily (3 species); and 5 to Drepanosiphinae subfamily (1 species). *Aphis fabae* was the most abundant species (20 samples) followed by *Aphis spiraeicola* (16 samples), *Toxoptera aurantii* (15 samples), *Aphis gossypii* (8 samples), *N. podocarpus* (5 samples), and *Aphis hederarum* (4 samples) (Table 1). Other species were represented only by one or two samples. Eleven samples of the Aphidinae subfamily and 14 of the Lachninae subfamily were not identified at species level (Supp Table S1). Two samples collected in Barcelona from two adjacent *Podocarpus* trees were identified as *N. podocarpus* and were also included in the analysis.

DNA Extraction and *Wolbachia* Detection

DNA was extracted from a group of four individuals based on the protocol described in Latorre et al. (1986). Presence or absence of *Wolbachia* in aphids was based on PCR amplification of 16S rRNA gene. After many trials and difficulties in amplifying a full-length gene, we opted to use two *Wolbachia*-specific primers (wspecF, the first nucleotide modified from C to Y, and wspecR). Although the size of PCR product was small (about 441 base pairs [bp]), these primers successfully and repeatedly amplified the infected samples, an impossible task when other primer pairs were used. The PCR conditions and the primers used are given in Supp Table S2. Both strands were cycle-sequenced using BigDyeTM Terminators 3.1 Kit (Applied Biosystems) and run on an ABI Prism 3700 sequencer by Stabvida Inc.

Amplification of MLST Gene Fragments

The samples that amplified the 16S rRNA gene fragment were considered *Wolbachia* infected and their sequences were used to characterize the strain. The following MLST genes were amplified: *ftsZ* (cell division protein), *coxA* (cytochrome c oxidase subunit I), *hcpA* (hypothetical conservative protein), and *gatB* (glutamyl-tRNA aminotransferase subunit B). The amplification conditions and primers were as given in Baldo et al. (2006) and Augustinos et al. (2011), with some optimization in annealing temperatures (Supp Table S2).

Cloning and Sequencing

The amplicons were purified using NucleoFast 96 PCR plates (Macherey-Nagel) before being ligated into a T-vector (pGEM-T Easy) and transformed into competent cells according to the manufacturer's instructions. Six transformed clones were used in PCR amplification by the primers T7 and SP6. BigDye Terminators 3.1

Table 1. The infection rate of *Wolbachia* in aphid species and the supergroups of infected samples based on 16S rRNA gene sequences

Aphid subfamily Aphidinae	Aphid species	Number	% positives	Supergroups
	<i>Aphis fabae</i> (Scopoli)	20	0	
	<i>Aphis spiraeicola</i> (Patch)	16	43,7% (7)	M, N
	<i>Toxoptera aurantii</i>	15	0	
	<i>Aphis</i> sp.	10	0	
	<i>Aphis gossypii</i> (Golver)	8	0	
	<i>Aphis hederae</i> (Kaltenbach)	4	25% (1)	M, B
	<i>Aphis craccivora</i> (Koch)	3	0	
	<i>Aphis nerii</i> (Boyer de Fonscolombe)	2	0	
	<i>Aphis ruborum</i> (Börner)	2	0	
	<i>Aphis oenotherae</i> (Oestlund)	1	0	
	<i>Illinoia azalea</i> (Mason)	1	0	
	<i>Illinoia lambersi</i> (MacGillivray)	1	0	
	<i>Macrosiphum euphorbiae</i> (Thomas)	1	0	
	<i>Macrosiphum rosae</i> (Linnaeus)	1	0	
	<i>Melanaphis donacis</i> (Passerini)	1	0	
	<i>Metopolophium dirhodum</i> (Walker)	1	0	
	<i>Uroleucon</i> sp. (Mordvilko)	1	0	
	NI	1	1	
	Total	89	10 (9)	
Drepanosiphinae				
	<i>Neophyllaphis podocarpi</i> (Takahashi)	5	100% (5)	N
	<i>N. podocarpi</i> (Barcelona)	2	100% (2)	M
	Total	7	100%	
Lachninae				
	<i>Cinara</i> sp. (Miller)	20	0	
	<i>Eulachnus rileyi</i> (Williams)	1	0	
	Total	21	0	
	Total number of Azorean samples screened	115	12% (14)	

Number of infected aphids is shown in ().

Kit (Applied Biosystems) and run on an ABI Prism 3700 sequencer by STABVIDA Inc. The obtained sequences were first viewed in BioEdit 7.0.5.3 (Hall 1999), then assembled and edited using the SeqMan program of the DNASTAR Lasergene software package. ClustalW (Thompson et al. 1994) was used to align sequences obtained in this work, together with other related sequences deposited in the GenBank database. The aligned sequences were edited with MacClade (version 4.0) (Maddison and Maddison 2000). The alignment lengths were 441 bp, 369 bp, 414 bp, 402 bp, and 444 bp for 16S rRNA, *gatB*, *ftsZ*, *coxA*, and *hcpA* genes, respectively. All the sequences obtained in this study are deposited in the GenBank database under accession numbers MK053596 - MK053608.

Genetic Distance and Phylogenetic Analysis

The best fit model for each sequence data was determined using Akaike information criteria with the program MEGA version 6 (Tamura et al. 2013). To construct the phylogenetic tree, the following models were used: GTR+G+I substitution model for 16S rRNA data, GTR+G substitution model for *coxA*, *gatB* & *ftsZ* data, and T92+G for *hcpA* data. The trees were constructed using maximum likelihood (ML) method and the confidence level of the inferred topology was estimated by 1,000 bootstrap replicates in MEGA 6. This program was also used to compare evolutionary divergence between the sequences using Kimura 2-parameter (K2P) distance model (Kimura 1980).

Results

Occurrence of *Wolbachia* in Azorean Aphids

The presence of *Wolbachia* was investigated in the samples using 16S rRNA gene-specific primers and results showed that 12%

(14 of 115) of the samples were infected by the bacterium. All the infected samples belonged to only three species: *A. hederae*, *A. spiraeicola*, and *N. podocarpi*, although the infection rates were 25, 43.7, and 100%, respectively (Table 1). The rate of infections also varied among the three islands: 14.8% in Pico (4 of 27) samples, 5.8% in São Jorge (2 of 34) samples, and 14.8% in S. Miguel (8 of 54) samples. Augustinos et al. (2011) reported that only 3.7% (1 of 27) samples from São Miguel were infected while the rate of infection was 15% (6 of 40) in another Azorean Island (Terceira).

Strain Typing and Phylogenetic Analysis

To perform strain typing, the samples identified as infected were amplified for *coxA*, *ftsZ*, *gatB*, and *hcpA* gene fragments. Despite many efforts, we were unable to PCR amplify and sequence all the infected samples for the mentioned gene fragments. Some of the gene fragments could be successfully amplified and sequenced in some of the samples but not in others (Table 2). The difficulties of *Wolbachia* strain typing in aphids have also been reported earlier (Augustinos et al. 2011, Wang et al. 2014).

The phylogeny of 16S rRNA gene sequences shows that all the samples of *N. podocarpi* were infected by *Wolbachia* belonging to two supergroups: M, in the two samples collected in Barcelona and N, in samples collected from different localities of S. Miguel Island, Azores (Fig. 1). The *N. podocarpi* Chinese samples harbored supergroups M and A (Wang et al. 2014). One of the samples of *A. spiraeicola* (collected in a different island) clusters with *N. podocarpi* in supergroup N. Three *A. spiraeicola* samples belonging to supergroup M and one sample of *A. hederae* were infected by M and B supergroups (AZ 136 and AZ 136-1).

Table 2. Aphid samples positives for *Wolbachia* and PCR amplification results for *16S rRNA* and MLST genes

Samples	Plant host	Aphid subfamily	Aphid species	16S rRNA	MLST			
					<i>gatB</i>	<i>ftsZ</i>	<i>coxA</i>	<i>hcpA</i>
AZ 80	<i>Pyracantha angustifolia</i> (Schneid)	Aphidinae	<i>Aphis spiraeicola</i>	+	+	–	–	–
AZ 86	<i>Podocarpus</i> sp.	Aphidinae	<i>Aphis spiraeicola</i>	–	–	+	+	–
AZ 100	NI	Aphidinae	<i>Aphis spiraeicola</i>	+	–	–	–	–
AZ 102	NI	Aphidinae	NI	+	+	–	–	–
AZ 129	<i>Eriobotrya japonica</i> (Lindl)	Aphidinae	<i>Aphis spiraeicola</i>	+	+	–	–	–
AZ 134	<i>Pyracantha coccinea</i> (Roem)	Aphidinae	<i>Aphis spiraeicola</i>	+	+	–	–	–
AZ 135	<i>Ilex perado azorica</i> (Tutin)	Aphidinae	<i>Aphis spiraeicola</i>	+	+	–	–	–
AZ 136	<i>Hedera</i> sp. (Campbell)	Aphidinae	<i>Aphis hederae</i>	+	–	+	+	–
AZ 184	<i>Pyracantha angustifolia</i> (Schneid)	Aphidinae	<i>Aphis spiraeicola</i>	+	–	–	–	–
AZ 81	<i>Podocarpus</i> sp.	Drepanosiphinae	<i>Neophyllaphis podocarpi</i>	+	–	+	+	+
AZ 85	<i>Podocarpus</i> sp.	Drepanosiphinae	<i>Neophyllaphis podocarpi</i>	+	–	+	+	–
AZ 162	<i>Podocarpus</i> sp.	Drepanosiphinae	<i>Neophyllaphis podocarpi</i>	+	+	+	+	–
AZ 172	<i>Podocarpus</i> sp.	Drepanosiphinae	<i>Neophyllaphis podocarpi</i>	–	+	+	–	+
AZ 181	<i>Podocarpus</i> sp.	Drepanosiphinae	<i>Neophyllaphis podocarpi</i>	+	–	–	–	+

NI, not identified; +, amplified; –, not amplified.

Then, we performed phylogenetic analysis for other gene markers (either separately or combined). In *gatB* phylogenetic tree, the samples from supergroup M clustered together and well separated from other supergroups, while those representing supergroup N (namely *N. podocarpi*) although clustered together, the cluster was localized within supergroup B (Supp Fig. S2a). The same typology is observed in *ftsZ* phylogenetic tree (Supp Fig. S2b). More complex phylogenetic relations emerged in *coxA* analysis (Supp Fig. S2c): cluster 1 includes the sequences from supergroups M, N, and B (based on *16S rRNA* gene data); clusters 2 and 3 include the sequences representing exclusively supergroup B, although cluster 2 occupies an intermediate position between clusters 1 and 3. Both in *ftsZ* and *coxA* phylogenetic trees, one sample of *A. spiraeicola* (AZ-86) grouped with samples of *N. podocarpi*. Interestingly, this sample and *N. podocarpi* (AZ-85) were taken from the same host plant (*Podocarpus* sp.). In these phylogenetic trees, *A. hedera* (AZ-136-1) is well positioned in supergroup B. Moreover, the position of *C. cedri* from Spain (specially Salamanca strain) in the three phylogenetic trees should be noted, the *coxA* and *gatB* sequences clustered in supergroupB, while in *ftsZ* tree a separate clade (close to supergroup B) was found. For *hcpA* gene, we could only obtain three sequences, all from *N. podocarpi* samples, which were clustered in supergroup B (Supp Fig. S2d).

We also performed phylogenetic analysis for concatenated data, with available sequences of the same strain for *16S rRNA*, *ftsZ* and *coxA* genes (Table 2). The tree shows (Fig. 2) that the Azorean samples of *N. podocarpi* are well separated from those of supergroup B as well as from *C. cedri* (Salamanca).

Genetic Distances

The pairwise genetic distances between the sequences mainly representing supergroups B, M, and N for each gene fragment and concatenated data are shown in Supp Table S3. In *16S rRNA* gene data, all the distances between the supergroups are above 2%, the value required for separating the supergroups (Breeuer et al. 1992, Stouthamer et al. 1993). The genetic distances are higher between N & M compared with N & B supergroups.

Considering supergroups B-N, the pairwise distances are lower than 2% in three of the protein-coding genes (*ftsZ*, *gatB*, and *hcpA*), with one exception in *ftsZ* data. In *coxA* data, the genetic distances

are between zero to 8.9% depending on the *Wolbachia* strains. Very low values (<1%) within cluster 1 (which includes different supergroups), intermediate values (about 3%) between clusters 1 and 2 and high values (about 8%) between clusters 1 and 3. Thus, depending on the *Wolbachia* strain, the pairwise genetic distances can either support the separation of the strains in different supergroups or not. The pairwise genetic distances between supergroups N-M and M-B are above 20% in *ftsZ* and *gatB* data.

Considering the concatenated data (*16S rRNA*, *ftsZ*, and *coxA* genes), all the distances between supergroups N and B are above 3%, and comparable values are obtained between *C. cedri* (Salamanca) and supergroups B and N. We could not include any other strain from supergroup M due to unavailability of adequate sequences.

Discussion

The Infection Dynamics and Supergroups

The infection rates reported here and those in Augustinos et al. 2011 and Wang et al. 2014 demonstrate a high variability in different geographical areas. Altogether, it seems that the geographical localization of aphids plays a key role in shaping the prevalence of *Wolbachia* in these organisms. In the present work, the level of infections among the species that harbored *Wolbachia* was variable (*N. podocarpi* 100%, *A. spiraeicola* 44%, and *A. hedera* 25%), indicating that *Wolbachia* seem infect either few or most individuals within a species, as previously suggested (Hilgenboecker et al. 2008). Since all the samples and all different DNA extractions of *N. podocarpi* tested were always positive, it is quite possible that all individuals of this species are infected by *Wolbachia*. Furthermore, *N. podocarpi* sampled in Barcelona as well as Chinese samples were all infected by *Wolbachia*. Hence, we can postulate that although the supergroups infecting these samples are different, *Wolbachia* may play an essential role in this species.

Based on phylogenetic analysis, the three supergroups (M, N, and B) detected in our samples can be clearly differentiated by *16S rRNA* gene sequence data, the same picture emerges in the concatenated phylogenetic tree. The data based on two of the gene markers (*gatB* and *ftsZ*) can separate the supergroup M from supergroups N and B. However, the separation of supergroup N from supergroups (B

Fig. 1. *Wolbachia* 16S rRNA phylogenetic tree. The maximum likelihood (ML) phylogenetic tree reconstruction based on 16S rRNA gene fragment (length: 441 bp) under the GTR+G+I substitution model. The bootstrap values above 60 estimated from 1,000 replicates are indicated. The host species names are used to characterize *Wolbachia* strains. The samples of the present study are marked.

and/or M) was not clearly supported by MLST data, and the phylogenetic trees demonstrated incongruent grouping (Supp Fig. S2). The occurrence of conflicting results among different gene markers was also reported by Augustinos et al. (2011). It's worth nothing that in whiteflies, some strains of *Wolbachia* were clustered into a new

supergroup (O) by 16S rRNA gene analysis; however, the same strains were clustered into supergroup B by several protein-coding genes (Bing et al. 2014), once more indicating the limited reliability of the currently available protein-coding gene markers in supergroup identifications. The samples of the two species of aphids (*A. spiraecola*

Fig. 2. *Wolbachia* concatenated phylogenetic tree. The maximum likelihood (ML) phylogenetic tree reconstruction based on concatenated data (16S rRNA, *ftsZ* & *coxA*) gene fragments (total length = 1,080 bp) under the GTR+G+I substitution model. Only the bootstrap values above 60 are shown. The host names characterize *Wolbachia* strain, wPip (*Culex quinquefasciatus*), wHa (*Drosophila simulans*), and wCle (*Cimex lectularius*).

AZ-86 and *N. podocarpi* AZ-85), collected from the same host plant, cluster together in *coxA* and *ftsZ* phylogenetic trees (sequences are not available for the other genes). *Aphis spiraeicola* is a polyphagous species but had not been reported in *Podocarpus* sp. hosts. The high number of *A. spiraeicola* individuals sampled from this host plant as well as its absence in the sampling area rules out any accidental sampling, indicating a new adaptation of the aphid species to *Podocarpus* sp. host plant. *N. podocarpi* is an ancient, oligophagous aphid associated with conifers and is a native of East Asia and Australia that was introduced to North America and was recently recorded in Europe (Pérez Hidalgo et al. 2015). The time and the mode of introduction of this species together with the pant host are unknown but judging by the size two adjacent plants are much older than the others.

The Origin of the New Supergroups (M and N) in Azorean Aphids

Regarding supergroup assignment, the present results reiterate the differential power of the available gene markers in the identification, which is further complicated by the discordance between different genes and strains. Due to lack of information on most of MLST gene sequences in different supergroups, a thorough comparison of our results with other published studies was not possible. Supergroup M is the most abundant supergroup in European and Chinese aphids (Augustinos et al. 2011, Wang et al. 2014) and it was suggested that it was rapidly and recently spreading in all host species in China. Unfortunately, few aphid species have been tested for the presence of *Wolbachia* in North America and Africa (4 and 2 species, respectively) and data are not available for other regions (Niriganaki et al. 2003, Zytynska and Weisser 2016). On the other hand, supergroup N was only reported in samples from Azores (Augustinos et al. 2011 and present study) where it seems to be as abundant as the supergroup M (present study). Our data show that the main aphid species harboring supergroup N is *N. podocarpi*, while *A. spiraeicola* and *A. hederae* are mainly infected by supergroup M and occasionally by supergroup N.

The most likely explanation is that *N. podocarpi* was introduced to Azores together with the host plant (*Podocarpus* sp.). The multiple introduction of plants or propagation of the trees from the first introduction are both plausible. If the latter is correct then *N. podocarpus* harboring *Wolbachia* was entered once and then scattered around the island, which would explain that the plants, aphids and are related. The aphids could have harbored supergroup N at the time of the introduction or it was acquired afterward. The continental species of *N. podocarpi* are infected by supergroup M (Europe) and M or A (China); therefore, it is unlikely that the host plant was already harboring supergroup N at the time of its arrival. However, to validate this hypothesis, more data from various parts of the world are needed. It is possible to conclude that *N. podocarpi* harboring *Wolbachia* supergroup N was not introduced to Azores from China (where supergroup N has not been found). Nevertheless, its introduction from other regions (e.g., Australia) cannot be ruled out. If *N. podocarpi* acquired supergroup N after its introduction, then it should have been through horizontal transmission from other species.

On Horizontal Transmission

In arthropods, *Wolbachia* are recognized as the most successful endosymbionts that manipulate the reproduction of their hosts. Recent studies showed that the bacteria infect about 40% of species (Hilgenboecker et al. 2008, Zug and Hammerstein, 2012). However, infection is expected to be lost over evolutionary time scale as hosts acquire resistance to the manipulator (Rigaud and Juchault 1993, Hornett et al. 2006, Koehncke et al. 2009).

It has been proposed that *Wolbachia* maintenance is an outcome of the rate of horizontal transmission between species as well as the rate of loss within species (Werren et al. 2008, Bailly-Bechet et al. 2017). Evidence for this type of transmission is mainly based on the lack of phylogenetic congruence between *Wolbachia* and the insect hosts (Schilthuizen and Stouthamer 1997, Vavre et al. 1999, Baldo

et al. 2008, Raychoudhury et al. 2009). An epidemiological approach indicated that vertical transmission alone is not sufficient for the prevalence of *Wolbachia* in many hosts and that horizontal transmission has also been occurring between unrelated and phylogenetically distant species (Zug et al. 2012). The horizontal transmission can occur either between closely related hosts (Werren et al. 2008, Zug et al. 2012) or ecologically linked hosts (Sintupachee et al. 2006, Stahlhut et al. 2010). The ecological relatedness and physical contact are considered key factors in strain distribution and horizontal *Wolbachia* transmission (Engelstädter and Hurst 2006, Gerth et al. 2013). The present data shows the existence of supergroup N in two phylogenetically different aphids (Aphidinae and Drepanosiphinae) sharing the same host plant (*Podocarpus* sp.), indicating the possibility of *Wolbachia* horizontal transmission between the aphids. Plants are already known to mediate the Rickettsia and *Wolbachia* transmission in whiteflies (Caspi-Fluger et al. 2012, Li et al. 2017).

Although the direction of transmission is not clear, one scenario is the transmission from *Neophyllaphis* to *Aphis*, based on the abundance and consistence of supergroup N infection in the former species. Another question to address is how this supergroup came to infect sporadically *Aphis* sp., in different islands. One possibility can be through introduction of host plants, together with the aphids and the *Wolbachia* from one island to another. Another less likely explanation is the existence of a source yet not detected (such as another host plant(s), harboring aphids with *Wolbachia* supergroup N, parasitoid wasp) that led to the emergence of this supergroup in Azorean aphids.

Augustinos et al. (2011) reported that most aphid species infected belong to Lachninae subfamily and suggested a potential nutritional function for *Wolbachia* in this group. However, this tendency was detected in neither Chinese populations nor in the present work. Here, we find a close association between *Wolbachia* and *N. podocarpi* from Drepanosiphinae subfamily; we also detected another member (*Drepanosiphum* sp.) of this subfamily to be infected by *Wolbachia*, although its supergroup was not identified. These results indicate that *Wolbachia* have an important function in Drepanosiphinae subfamily similar to that reported in Lachninae. It will be interesting to test more species from the Drepanosiphinae subfamily for the rate and type of *Wolbachia* infection and elucidate their involvement in the host life and their interaction with other coendosymbionts.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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Disclosure

The authors have no conflict of interest to declare.

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