LOW LEVELS OF MITOCHONDRIAL DNA AND SYMBIONT DIVERSITY IN THE WORLDWIDE AGRICULTURAL PEST, THE GREENHOUSE WHITEFLY *TRIALEURODES VAPORARIORUM* (HEMIPTERA: ALEYRODIDAE)

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ABSTRACT

Trialeurodes vaporariorum, the greenhouse whitefly, is a cosmopolitan agricultural pest. Little is known about the genetic diversity of *T. vaporariorum* and the bacterial symbionts associated with this species. Here, we undertook a large phylogeographic study by investigating both the mitochondrial diversity and the infection status of 38 *T. vaporariorum* collections from 18 countries around the world.

Genetic diversity of *T. vaporariorum* was studied by analyzing sequence data from the mitochondrial cytochrome oxidase I (*COI*), cytochrome b (*cytb*) and NADH dehydrogenase subunit 5 (*ND5*) genes. Maximum-likelihood phylogeny reconstruction delineated two clades characterized by limited sequence divergence: one clade comprised samples only from the Northern hemisphere whereas the other comprised samples from a broader geographical range.

The presence of secondary symbionts was determined by PCR using primers specific for *Hamiltonella*, *Rickettsia*, *Arsenophonus*, *Cardinium*, *Wolbachia* and *Fritschea*. Most individuals examined harbored at least one secondary endosymbiont, and *Arsenophonus* was detected in almost all male and female individuals. *Wolbachia* was present at a much lower frequency, and *Cardinium* was detected in only a few individuals from Greece. *Rickettsia*, *Hamiltonella* and *Fritschea* were not found. Additionally, we set out to further analyze *Arsenophonus* diversity by Multilocus Sequence Typing (MLST) analysis, however, the *Arsenophonus* sequences did not exhibit any polymorphism. Our results revealed remarkably low diversity in both mitochondrial DNA and symbionts in this world-wide agricultural pest, contrasting sharply with that of the ecologically similar *Bemisia tabaci*.

Keywords: genetic diversity, mtDNA polymorphism, symbiont communities, *Arsenophonus*

INTRODUCTION

Trialeurodes vaporariorum Westwood (Hemiptera: Aleyrodidae), often called the greenhouse whitefly, is a cosmopolitan and extremely polyphagous species of temperate regions (Inbar 2008). Together with the *Bemisia tabaci* species complex, *T. vaporariorum* is one of the most common and economically important whitefly species, affecting a large number of vegetables and ornamental crops from several families (Solanaceae, Cucurbitaceae, Fabaceae, Brassicaceae, Rosaceae, Euphorbiaceae), cultivated in both open and protected environments (Martin 2000). All life stages, except the egg, cause damage to plants both directly, through feeding on phloem sap, and indirectly, not only by secreting large amounts of honeydew which encourages fungal growth on plant leaves, but also by transmitting Crini- and Clostero- plant viruses (Jones 2003). Most *Trialeurodes* species are found in the New World and the evolutionary origin of *T. vaporariorum* is thought to be in Central or South America, possibly in Brazil, but most likely in Mexico. It should also be noted that Mexico is also considered to be the origin of the first *T. vaporariorum* introductions to Europe (Russell 1948), although the absence of an extensive phylogeographical survey of *T. vaporariorum* has left the origin of this species open to discussion. In order to develop effective pest management strategies, it is important to have some understanding of its intraspecific variation, especially regarding aspects such as dispersal patterns, host plant specialization and gene flow (important, for example, in the spread of insecticide resistance genes). At the same time, it is becoming increasingly apparent that both genetic variation and different adaptive traits of insect pests are affected by their associated symbiotic communities (Feldhaar 2011). For many biogeographic, phylogenetic as well as species delineation and identification studies, mitochondrial DNA provides the markers of choice, due to its useful properties, such as clonality, lack of introns, expected neutrality and high mutation rate. However, using mitochondrial (mt) DNA sequences to study genetic variation may present complications, resulting from the association of mitochondrial evolutionary history with that of vertically transmitted symbionts. When symbionts, like mt DNA, are transmitted maternally, linkage disequilibrium between them could affect mt DNA polymorphism, e.g. in the case of selective sweeps (Ilinsky 2013).

Most research on the population genetics of whiteflies has focused on *Bemisia tabaci* and little is known about *T. vaporariorum*. Previous, small-scale DNA-based studies have revealed low levels of diversity and sequence divergence among neighboring populations of *T. vaporariorum* (Roopa et al. 2012, Prijovic et al. 2014). However, earlier studies on *T. vaporariorum* (Williams 1917) proposed the existence of two races, the "English" and the "American", defined by reproductive differences, the former characterized by thelytokous and the latter by arthenotokous parthenogenesis. This interesting phenomenon may be associated with the presence of symbionts but this has not been confirmed.

The occurrence of symbionts is a common feature among arthropods and particularly insects (Duron et al. 2008, Zug and Hammerstein 2012). Symbiosis can have substantial influence on insect host biology through a wide range of effects (Oliver et al. 2010) thus greatly contributing to their ecological and evolutionary success (Feldhaar and Gross 2009). A diverse array of bacterial species engages in obligatory or facultative symbioses, ranging from parasitism to mutualism (Zchori-Fein and Bourtzis 2011). To date, bacterial symbionts have been reported to have effects on aspects of host biology that may affect genetic diversity, including nutrition, survival or fecundity, host plant utilization, reproduction, insecticide resistance and the ability to cope with environmental factors (Saridaki and Bourtzis 2010, Kikuchi et al. 2012).

Whiteflies are known to host the obligatory primary symbiont Candidatus *Portiera aleyrodidarum*, which has a long co-evolutionary history with all members of the Aleyrodinae subfamily (Thao and Baumann 2004). Portiera is known to supplement hosts' diet with important nutrients like amino acids as well as with carotenoids that present significant antioxidant action (Santos – Garcia et al. 2012). In addition to the primary endosymbiont, whiteflies contain a range of facultative / secondary symbionts, including species of Candidatus *Hamiltonella defensa* and *Arsenophonus* (Enterobacteriaceae), *Cardinium* (Bacteroidetes), *Fritschea bemisiae* (Chlamydiales), *Wolbachia* and *Rickettsia* (Rickettsiales) (Zchori-Fein and Brown 2002, Nirgianaki et al. 2003).

The functions of these secondary symbionts in whitefly biology are largely unknown, but can be expected to be broader than that of *Portiera* (Moran et al. 2008). In *B. tabaci*, bacteria of the genus *Rickettsia*, are known to increase tolerance to heat stress (Brumin et al. 2011) and to enhance host survival, development rate and reproductive potential (Himler et al. 2011). *Hamiltonella defensa* and *Arsenophonus* increase *B. tabaci's* susceptibility as a virus vector (Gottlieb et al. 2010, Rana et al. 2012). *Fritschea*, which has so far only been reported in one whitefly, *B. tabaci* (Thao et al. 2003), as well as the widespread *Cardinium* and *Wolbachia* have unknown effects on whitefly biology.

In other host species, secondary symbionts have variable effects on the host promoting their own persistence. The endosymbiont *Wolbachia*, induces various phenotypes in its hosts, such as male-killing, feminization, parthenogenesis and cytoplasmic incompatibility (Werren et al. 2008, Saridaki and Bourtzis 2010) or affects host physiology including immunity, nutrition and fitness (Bian et al. 2010, Hosokawa et al. 2010). *Cardinium* also manipulates host reproduction in a wide range of arthropod species by inducing cytoplasmic incompatibility, feminization or parthenogenesis (Ros and Breeuwer 2009). *Rickettsia* has been associated with both male-killing (von der Schulenburg et al. 2001), and parthenogenesis (Hagimori et al. 2006). *Arsenophonus* is responsible for a suite of phenotypes in a variety of insects, such as male killing (Gherna et al. 1991) and obligatory mutualism in hematophagous lice (Allen et al. 2007).

Both vertically-transmitted symbionts and mt DNA, are linked to the evolutionary history of the insect host, and thus may be used to shed light on evolutionary processes relating to both sides of a symbiosis. The majority of

recent literature has focused separately on a few *T. vaporariorum COI* sequences from India, China and from Serbia and neighboring countries, as well as on a limited ITS-1 rRNA gene sequences, mostly from India and a single sequence from each of a few other countries (Pakistan, Germany, Australia) (Roopa et al. 2012, Prijovic et al. 2014). These studies provided evidence that in this species, both mitochondrial and nuclear genes display remarkably low levels of genetic variation. Moreover, the *COI* gene sequences from *T. vaporariorum* from Spain (Canary Islands), Netherlands and UK (Malumphy et al. 2007) as well as from the USA (Papayiannis et al. 2009) also indicated very limited variation. Additionally, Calvert *et a*l. (2001) found 99% identity between 16S *rRNA* gene sequences from samples collected in Arizona and Colombia. Regarding the occurrence and diversity of secondary symbionts in populations of *T. vaporariorum,* there are only two studies from Croatia, Montenegro and Bosnia – Herzegovina (Skaljac et al. 2010, Škaljac et al. 2012). The authors report *Arsenophonus* infection near fixation in the populations examined, while *Hamiltonella* occurred at intermediate frequencies and *Wolbachia* and *Cardinium* were only sporadically detected. Recently, in another *Trialeurodes* species (*Trialeurodes* sp. nr. *abutiloneus*), Cass et al. (2014) found that populations from USA, harboured *Arsenophonus* and *Wolbachia* together with *Portiera*, *Pseudomonas* and *Serratia*. The image drawn by the aforementioned studies shows *T. vaporariorum* as a species with very high mitochondrial sequence uniformity, almost universally infected by *Arsenophonus*. Each one of them deals with restricted geographic sampling and is limited to a few *COI* sequences mostly. The population genetics and secondary symbiont associations of this species, however, merit further exploration and the identified patterns remain to be confirmed by additional sampling. Moreover, there is no published information regarding symbiont distribution in *T. vaporariorum* in relation to host genetic structure, in the same populations.

In this context, the aim of this study was to investigate cytoplasmic genetic diversity in a global collection of *T. vaporariorum*, in terms of both mitochondrial DNA polymorphism using three of the mitochondrial genes (*COI*, *cytb*, *ND5*) and endosymbiont prevalence and distribution. The occurrence of symbiotic bacteria that whiteflies are already known to harbor was recorded and we aimed to further analyze the genetic diversity of the prevailing endosymbiont using a Multi-Locus Sequence Typing (MLST) approach (Mouton et al. 2012, Jousselin et al. 2013). Based on the results of the above approaches, we seek to describe the correlation between polymorphisms of the two cytoplasmic components and we discuss how our findings reflect species complexity of *T. vaporariorum* as well as the role of endosymbionts in this species.

METHODS

Trialeurodes vaporariorum collections

Thirty eight *T. vaporariorum* collections from global locations and diverse host plant species were analyzed. Each collection comprised whiteflies sampled from several plants at the same location. Most collections originated mainly from cultivated vegetables (eggplant, zucchini, bean, tomato, cucumber), ornamentals or non-food crops (tobacco), growing either in open environments or in greenhouses. Table 1 lists the population codes, sample characteristics and the number of individuals used for each analysis. At each location, live adult whiteflies were collected and were preserved until use, either at -80 ^{o}C or in 95 % ethanol. Insects were examined under a binocular stereoscope to determine sex based on size and the shape of the abdomen. Genomic DNA for PCR was extracted from single individuals according to the protocol described by Tsagkarakou et al. (2007).

PCR amplification of the mt DNA genes

DNA extracts from single females were used as templates for the amplification of specific fragments of mt DNA: a 769 bp fragment of *COI*, a 549 bp fragment of *cytb* and an 810 bp fragment of *ND5*. Four µl of genomic DNA extract were used in 50 µl reactions containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.0 µM of each primer, 1 unit Kapa HiFi Taq DNA polymerase (KAPABIOSYSTEMS) and 1x enzyme buffer (KAPABIOSYSTEMS). Amplifications were done with an Eppendorf Gradient Mastercycler using the following conditions: initial denaturation at 93 ^{o}C for 3 minutes, followed by 35 cycles of 93 ^{o}C for 40 seconds, 52-58 ^{o}C (depending on primer pairs) for 45 seconds and 72 ^{o}C for 1 minute. The PCR products were purified using the Nucleospin Extract kit (Macherey-Nagel) and sequenced in both directions (Macrogen Europe, Amsterdam, Netherlands). See Supplementary Material online for the conditions and the primers used for amplification and sequencing.

Sequence analysis

Both forward and reverse partial *COI*, *cytb* and *ND5* gene sequences were obtained from a total of 471 females, from at least one specimen per sample (Table 1). Multiple sequence alignment was carried out using the software package ClustalW v.1.7 with default parameters (Higgins et al. 1996) and corrected manually using BioEdit v.7.0 (Hall 1999). A single sequence of each haplotype was deposited in GenBank under the accession numbers KJ475452 to KJ475464 (Table 1).

Diversity and genetic analysis

Identical DNA sequences at a given locus were assigned the same allele number (unique identifier). Each unique allelic combination comprises a haplotype. Genetic diversity was assessed using several functions of the DnaSP package (Rozas et al. 2003) that allowed calculation of the parsimony informative (P) and singleton variable (S) sites, the synonymous (SM) and nonsynonymous (NSM) substitutions, the synonymous (SS) and nonsynonymous (NSS) sites, the number of haplotypes (H), the number of polymorphic sites (PS), haplotype diversity (Hd) and nucleotide diversity (Pi). In addition, the Ka/Ks ratio (number of nonsynonymous substitutions per total nonsynonymous sites / number of synonymous substitutions per total synonymous sites), was calculated.

Phylogenetic analysis

Phylogenetic relationships between specimens were determined with PhyML v.3.0 (Guindon and Gascuel 2003) (available on the Web at http://atgc.lirmm.fr/phyml/) using the maximum-likelihood method (ML) implemented in PAUP, for each locus separately and for the concatenated data set. The most suitable model of DNA substitution was determined with the program jModeltest (Posada 2008). Based on the Akaike Information Criterion (AIC), the HKY85 model was chosen as the statistically appropriate model for the data. Under the selected model, the parameters were optimized and ML analyses were performed. Branch supports were assessed by 1000 bootstrap resamplings, as implemented in PhyML. We carried out an analysis on the concatenated dataset for the three genes (*COI*, *cytb* and *ND5*) in order to improve the reliability of phylogenetic information and the resolution of relationships between the *T. vaporariorum* populations. This analysis was performed only for those specimens of each population for which all three gene sequences were available.

Detection and molecular identification of endosymbionts

To detect the presence of secondary bacterial symbionts, PCR reactions were performed using primers specific for the 16S rRNA genes in *Wolbachia*, *Rickettsia*, *Hamiltonella* and *Cardinium* and the 23S rRNA genes in *Arsenophonus* and *Fritschea* (See Supplementary Material online). The materials and general protocol of the PCR amplification were the same as described above for the mitochondrial genes. The respective annealing temperatures are listed in Supplementary Material online. A total of 640 females (4–20 per population) were tested for the presence of the symbionts. The occurrence of *Arsenophonus* was also examined in 218 males (up to 10 per population). Suitable positive controls from *B. tabaci* infected with the respective bacterium were successfully performed for each endosymbiont in PCR. To verify the identity of the amplicons, PCR products were sequenced (Macrogen Europe, Amsterdam,

Netherlands). The sequences obtained were compared to those in the GenBank database using the BLAST algorithm of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). In order to check the quality of the DNA extraction, the samples that tested negative for all endosymbionts were cross-checked for the obligate endosymbiont *Portiera aleyrodidarum*, using specific primers for the 16S rRNA gene as described by Zchori-Fein and Brown (2002) (See Supplementary Material online).

Characterisation of *Arsenophonus* diversity

One to two *Arsenophonus*-positive individuals were randomly chosen from each sample (representing almost all mt haplotypes and geographic locations) for use in Multi Locus Sequence Typing (MLST). PCR and sequencing of three different genes of *Arsenophonus* (*ftsK*, *yaeT* and *fbaA*) was carried out as reported previously (Mouton et al. 2012) (See Supplementary Material online). Briefly, PCR amplifications were performed in 50 µl reactions containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, each primer at a concentration of 1 µM, *Taq* buffer (KAPABIOSYSTEMS), 5 U Kapa HiFi Taq DNA polymerase (KAPABIOSYSTEMS) and 4 µl DNA. Cycling conditions were: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55-59 °C for 30 s, and 72 °C for 1.5 min and a final elongation step at 72 °C for 10 min (See Supplementary Material online for respective annealing temperatures). PCR products were purified using the Nucleospin Extract kit (Macherey-Nagel) and both strands were sequenced (Macrogen Europe, Amsterdam, Netherlands). Editing of the sequences was performed using BioEdit v.7.0. The sequences were deposited in GenBank under the accession numbers KJ475466, KJ475467 and KJ475465 for *Arsenophonus ftsK*, *yaeT* and *fbaA*, respectively.

RESULTS

Mitochondrial DNA polymorphism

Sequences of mitochondrial *COI*, *cytb* and *ND5* gene fragments were obtained from a total of 471 specimens, sampled from 38 populations of *T. vaporariorum* across a wide geographic range (Figure 1). For each gene, the genetic diversity measures and an alignment showing the polymorphic sites are presented in Table 2 and Supplementary data online, respectively. Analysis of the mitochondrial gene sequences revealed high homogeneity between the *T. vaporariorum* samples.

For the *COI* gene, we obtained a fragment of 769 bp with five different haplotypes, in which four sites were polymorphic and two of them were parsimony informative. For the *ND5* and the *cytb* gene, the alignments of the 810 and 549 bp fragments, respectively, showed three polymorphic sites, one of which was parsimony informative, and four haplotypes. The mt *COI* gene

therefore seems to be the most variable and informative of the three phylogenetic markers used. Tajima's D for all three genes was negative, indicating that the population size may be increasing, or that there is purifying selection. The Ka/Ks ratio was below one for all genes, also providing evidence for purifying selection, as would be expected for housekeeping genes (Table 2).

In most cases, populations were homogenous: all individuals sampled from a single population shared the same haplotype. However, there were some exceptions. Populations from Russia (RU_PU), Ecuador (EC), Australia (AU_VI) and the Netherlands (NL_LI), consisted of individuals carrying different *COI* haplotypes. Likewise, regarding the *cytb* gene, the populations from the Netherlands (NL_LI), Italy (IT_SI) and Turkey (TR), comprised specimens with two different haplotypes. Finally, populations from Russia (RU_PU), Japan (JP_TS), Australia (AU_VI) and Colombia (CO) included specimens with two different *ND5* haplotypes (See Supplementary Material online).

Concatenated sequences of COI, *cytb* and *ND5* (2126 bp) from 67 individuals, for which each of the three mitochondrial gene sequences were available, were analysed and used to construct the phylogenetic tree shown in Figure 2. This analysis revealed seven polymorphic sites, three of which were parsimony informative and four were singleton. A total of nine haplotypes were recorded (Table 2). The tree inferred from the concatenated sequences was nearly identical to the phylogenetic trees obtained when the sequences of each gene were analysed separately (not shown). ML inference resulted in two well-defined clades supported with > 85% bootstrap values. One clade (clade 1) was dominated by samples from the Northern Hemisphere, whereas the other (clade 2) contained sequences sampled from a broader geographic range. Generally, all individuals sampled from a single location fell into the same clade. Interestingly, in the case of France, four (FR_AL, FR_PA, FR_PE, FR_CA) out of five populations sampled belonged to clade 1, while the population from Montferrier (FR_MO) belonged to clade 2 (Figure 2). The genetic diversity of the two clades was compared, and clade 1 showed more variable sequences than clade 2 (Table 3).

Presence of secondary symbionts

Ten to twenty females from each sample were used for screening the six secondary symbionts (*Arsenophonus*, *Hamiltonella*, *Wolbachia*, *Rickettsia*, *Cardinium* and *Fritschea*), and the primary symbiont, *Portiera aleyrodidarum*. In addition, male individuals were examined for *Arsenophonus* in order to explore male killing effects of this symbiont on the whitefly populations. All 38 *T. vaporariorum* collections were tested with species-specific 16S or 23S rRNA gene primers and the results show that all were associated with secondary symbionts. *Portiera aleyrodidarum,* which is essential for the insect's survival, was detected in all tested individuals, confirming that our DNA extracts were of good quality.

Infection status of *T. vaporariorum* varied from 0 % to 97 %. Almost all individuals were associated with *Arsenophonus*. The incidence of *Wolbachia* and *Cardinium* was more sporadic, and there was no evidence for the presence of *Fritschea*, *Hamiltonella* or *Rickettsia* in our samples. The primers specific to the latter symbionts, however, produced DNA fragments of the expected size with positive control DNA templates (*B. tabaci* infected with the respective symbiont).

Arsenophonus showed the highest prevalence among the symbionts and was completely or almost fixed in all populations and in both sexes; found in 97.19 % of females and 91.74% of males. In most cases, it was the only symbiont detected. The males of the Japanese population (JP_TS) were an exception: none (10/10) harbored either *Arsenophonus* or any other secondary endosymbiont, despite the fact that females from this population were all infected by *Arsenophonus*. A few individuals from different populations (GR_FI; GR_SI; GR_NA; FR_AL; FR_MO; FR_CA; CO; AU_Lab) were also infected with *Wolbachia* (2.75 %) besides *Arsenophonus*. And, a minority (0.78 %) harbored *Cardinium*: five of 20 females belonging to a single population (GR_NA). There were no *Wolbachia* / *Cardinium* co-infections, and the few individuals that were not infected by *Arsenophonus* also did not carry any other secondary symbiont (Figure 3).

Wolbachia / *Arsenophonus* co-infection was detected in two Greek populations (GR_FI; GR_SI), three French populations (FR_MO; FR_CA; FR_AL), one population from Colombia (CO) and one from Australia (AU_Lab). In each of these populations, only one individual was doubly infected, except for the population from Colombia, in which half of the individuals tested (10/20) were doubly infected. *Cardinium* / *Arsenophonus* co-infection appeared in only one Greek population (GR_NA), where five out of 20 individuals tested positive for both bacteria. All other symbiont-carrying specimens were mono-infected by *Arsenophonus*.

Overall, *Arsenophonus* infection was widespread across the globe with only 2.81 % females and 8.26% males being uninfected (Figure 3), but no clear geographical distribution trends emerged for *Wolbachia*, which occurred sporadically at very low frequencies in a few widely distributed populations, or for *Cardinium* which was found in only one population. It is also worth noting that the individuals that were co-infected by *Arsenophonus* and *Wolbachia* or *Cardinium*, did not belong exclusively to one of the two clades of the mitochondrial phylogenetic tree shown in Figure 2.

Arsenophonus characterization

Obtained sequences exhibited 100 % identity to *Arsenophonus* sequences from *T. vaporariorum* available in the NCBI database (http://www.ncbi.nlm.nih.gov). In the three bacterial genes used for MLST analysis (*fbaA*, *yaeT* and *ftsK*), no polymorphism was detected in the 38 samples. Given the low diversity of bacterial communities and the low levels of genetic differentiation revealed by

mitochondrial genes, an analysis of association between infection status and genetic groups was not warranted.

DISCUSSION

We present a comprehensive study of *T. vaporariorum* populations from a wide geographic range, undertaken to gain a more thorough understanding of this insect's diversity. We explored the cytoplasmic genetic variation by combining genetic information from mitochondrial gene sequences (*COI*, *cytb*, *ND5*) with a survey of potentially associated reproductive manipulators (*Wolbachia*, *Rickettsia*, *Cardinium* and *Arsenophonus*) and other secondary symbionts (*Hamiltonella* and *Fritschea*).

The three mitochondrial genes showed very low levels of intra-specific variation and the nine resulting haplotypes were separated into only two maternal lineages. One of these lineages comprised specimens from distant locations around the world, whereas the other comprised specimens sampled only in the Northern hemisphere. According to the calibrated molecular clock for the mt *COI* gene of insects, -3.54 % sequence divergence per million years (Papadopoulou 2010)-, the estimated time of divergence between the two lineages should be approximately 42 thousand years ago [0.15 % sequence divergence in *COI* was detected between the two clades of *T. vaporariorum*, (*COI* tree is not shown)].

The low genetic variation found within and between the populations studied here is in accordance with the findings of Roopa and co-workers (2012) who analyzed *COI* gene sequences from Indian and Chinese populations of *T. vaporariorum* and with those of Prijovic and co-workers (2014) who examined sequences of the same gene from Serbia, Montenegro, Republic of Macedonia and Croatia. Thus, all available evidence indicates very low levels of genetic variation in mitochondrial DNA sequence data from *T. vaporariorum* samples from different geographical locations.

Interestingly, the diversity of endosymbionts of *T. vaporariorum* is very low as well. We found that *Arsenophonus* is almost fixed in world-wide natural populations of *T. vaporariorum,* while *Wolbachia* and *Cardinium* are present only at very low frequencies. On the other hand, *Hamiltonella*, *Rickettsia* and *Fritschea* were not detected at all. There are only two studies that have investigated the presence of the same symbionts (except *Fritschea*) in natural populations of *T. vaporariorum* (Skaljac et al. 2010, Škaljac et al. 2012). These studies included individuals from Croatia, Montenegro and Bosnia – Herzegovina and also showed the presence of *Arsenophonus* at near-fixation levels, whereas *Hamiltonella* was detected at intermediate levels (ca, 15-30%), and *Wolbachia* and *Cardinium* were found at relatively low frequencies (Skaljac et al. 2010, Škaljac et al. 2012). The main differences between these studies and ours are the absence of *Hamiltonella* in our screen and the higher frequencies of *Cardinium* detected by Skaljac et al. (2012).

The near fixation of *Arsenophonus* in *T. vaporariorum*, combined with the remarkably low mitochondrial genetic variation, could reflect the effects of a selective sweep, during which a strain of the symbiont could have spread across the host species, carrying along an associated mitochondrial haplotype. Such an effect could be elucidated by examining the polymorphism of nuclear genes, since nuclear genetic diversity would not be reduced by a symbiont-driven selective sweep to the same degree as would mitochondrial diversity. Although there is evidence that rRNA gene sequences from samples from a few countries (India, Pakistan, Germany, Australia) (Roopa et al. 2012) also show very limited polymorphism (i.e a few non parsimonious point mutations), more nuclear sequence information is needed to provide an unambiguous image. Our findings do not exclude the occurrence of genetic polymorphism inferred by other nuclear markers and the presence of genetically structured populations within the species. Very recently, Ovčarenko et al. (submitted) found a high degree of genetic population structure within one clade (Clade 1) of *T. vaporariorum*, even at restricted geographic ranges using microsatellite nuclear markers.

Symbionts engage in complex population dynamics with their hosts and an observed intermediate frequency may be transient or reflect equilibrium. The rate of spread and the equilibrium frequency of symbionts within a host population depends on multiple factors, including environmental and stochastic effects, the intensity of reproductive effects on the host, transmission fidelity, relative metabolic costs, benefits and trade-offs (Oliver et al. 2008, Unckless et al. 2009) and even interactions between symbionts co-infecting a host (Oliver et al. 2006). Furthermore, the density of bacteria within individuals may affect transmission fidelity, leading to variable infection frequencies, as well as our ability to detect symbionts in a reproducible manner. It is possible that low symbiont titers may be hindering reliable detection (Pan et al. 2012), leading to discrepancies in the reported infection frequencies within and between studies, and therefore more sensitive detection techniques may be required.

The very high prevalence of *Arsenophonus* reported here and by Skaljac et al. (2012) is consistent with the extensive survey of a wide range of arthropods conducted by Duron et al. (2008), who found that in almost every species where this symbiont was detected it reached very high infection rates or fixation. Recent studies have shown that *Arsenophonus* is widespread across numerous insect species (Duron et al. 2008, Duron et al. 2010, Taylor et al. 2011) including important pests such as whiteflies, louse flies and triatomine bugs (Dale et al. 2006, Novakova et al. 2009, Gueguen et al. 2010). It has a high diversity of lineages, and due to horizontal transfer, the same host species can harbor more than one lineage (Duron et al. 2010, Mouton et al. 2012).

However, our data points to a lack of diversity within *Arsenophonus* infecting *T. vaporariorum*, despite its prevalence in this species. The sequences we obtained from the 23S *rRNA*, *fbaA*, *ftsK* and *YaeT* genes were identical for all our samples of *Arsenophonus* in *Trialeurodes vaporariorum*. On the contrary,

sequence analysis of *fbaA*, *ftsK* and *YaeT* revealed high genetic diversity within *Arsenophonus* infecting *B. tabaci,* but this diversity was highly correlated with the different genetic groups defined in the *B. tabaci* species complex (Mouton et al. 2012). In the same study, almost no polymorphism was found in the *Arsenophonus* gene sequences from African *T. vaporariorum* samples, which were also identical to the ones we obtained.

Although *Arsenophonus* is a prevalent endosymbiont of many species, how it affects its hosts, including *T. vaporariorum* and *B. tabaci*, has not been elucidated. Male-killing effects of *Arsenophonus* as hinted by the species name (arsên: male; phonos: murder) have been described in the parasitoid wasp *Nasonia vitripennis*, (Ferree et al. 2008, Duron et al. 2010). However, our results indicate that this is not the case for *T. vaporariorum*, as both sexes carry *Arsenophonus*, except for the Japanese population, where all females but no males were infected. It would be interesting to see if this is due to feminization- or male killing-inducing action of *Arsenophonus* in this population. Infection of *Arsenophonus* not leading to male killing has also been reported for other species, e.g. *Triatoma infestans* (Hypša and Dale 1997) and *Pseudolynchia canariensis* (Dale et al. 2006). In psyllids, *Arsenophonus* is a facultative mutualist giving protection to the host against parasitoids (Hansen et al. 2007) and in planthoppers, *A. phytopathogenicus* is a phytopathogenic factor transferred to / from the phloem of plants (Bressan et al. 2008).

Dale et al. (2006) studied hematophagous louse flies and found that *Arsenophonus* is a mutualistic symbiont. They argued that due to the diverse diets (including vertebrate blood and plant sap) of the hosts of *Arsenophonus* it is unlikely that this bacterium plays a nutritional role in these symbioses, but we find this view to be quite unsupported and contradicted by studies on other symbionts. For example, *Wolbachia* appears to play a role related to nutrition and metabolism in insect species with completely different diets, such as the hematophagous *Cimex lectularius* (Hosokawa et al. 2010) and the yeast-feeding *Drosophila melanogaster* (Brownlie et al. 2009). In fact, in some cases, e.g. in aphids infected by the symbiont *Regiella* (Koga et al. 2003), bacterial symbionts have been implicated in expanding the diet range of their hosts. The very high prevalence of *Arsenophonus* in *T. vaporariorum* is likely to be indicative of a close mutualistic association, and it is possible that the bacterium plays a role in supplementing the host's nutrition. This is not uncommon among members of the sap-feeding Sternorryncha that often rely on symbionts for obtaining amino acids not available in their protein-poor diets. The primary endosymbiont *Portiera* plays such a role in whiteflies and particularly in *T. vaporariorum*, as they provide the host with essential (threonine, tryptophan) and nonessential (serine) amino acids and carotenoids. *Arsenophonus* may complement the role of *Portiera* in providing nutrition to *T. vaporariorum*. Since *Portiera* lacks the ability to complete the biosynthesis pathways of several amino acids (phenylalanine, valine, leucine, isoleucine, lysine, histidine, methionine), these pathways could be complemented by the host or by other secondary endosymbionts (Santos-Garcia et al. 2012). The co-existence of *Portiera* and *Arsenophonus* may even

indicate a process in which the former is being replaced by the latter. It has been proposed that the sequestration of secondary endosymbionts may be a mechanism for the replacement of primary endosymbionts (Koga et al. 2003, Gómez-Valero et al. 2004), as these may be subject to long-term genetic effects associated with their small effective population size and lack of recombination, leading to the accumulation of deleterious mutations (Muller's rachet) and detrimental effect on host fitness (Moran 1996, Lambert and Moran 1998).

There may be a range of other effects of *Arsenophonus* on *T. vaporariorum*, besides nutrition. For example, it could be playing a role in the defense of its host against parasites. However, Wulff et al. (2013) failed to demonstrate any protective effects of *Arsenophonus* against parasitoids or fungal pathogens in *Aphis glycines*. On the other hand, the involvement of this symbiont in the ability of its whitefly host to transmit viruses might seem more likely, as such an effect has already been demonstrated in the case of *B. tabaci* and Cotton Leaf Curl Virus (Rana et al. 2012).

Endosymbionts have been associated with the induction of reproductive abnormalities in their hosts, including cytoplasmic incompatibility, parthenogenesis, feminization and male-killing (Stouthamer et al. 1999, Saridaki and Bourtzis 2010). The infection pattern observed here, cannot implicate any symbiont in the clustering of *T. vaporariorum* into the two races. There is no evidence of our collection set clustering into thelytokous or arrhenotokous reproductive races in relation to symbionts, as all collections contained *Arsenophonus* as well as both sexes of whiteflies (data not shown). However, the "English" and the "American" races of *T. vaporariorum* reported first by Williams (1917) were associated with thelytokous and arrhenotokous parthenogenesis respectively. Whether this is associated with the presence of reproductive parasites, including ones that have not been screened for in this study, remains an open question.

The low polymorphism of *Arsenophonus* within *T. vaporariorum*, along with its high prevalence in this species is consistent with a stable, vertically transmitted infection, established before the (recent) separation of the two mitochondrial *T. vaporariorum* clades described in our study. The combined symbiont and mitochondrial diversity supports the conclusion that *T. vaporariorum* is a single species, which contrasts with the well-defined complex of 31 morphologically indistinguishable cryptic species formed by its ecological analogue, *B. tabaci* (Lee et al. 2013). Our study provides a first description of the genetic diversity of *T. vaporariorum* on a global scale, indicating that sequence polymorphism is low within and between the populations sampled and that, unlike the case of *B. tabaci, T. vaporariorum* is a single species which has colonized agricultural ecosystems all over the world. This conclusion is also supported by the genetic uniformity of the highly prevalent *Arsenophonus* symbionts in *T. vaporariorum*. Additional sampling and endosymbiont screening and further, higher resolution analysis of the population genetics of *T. vaporariorum* including nuclear loci is necessary to make more confident interpretations on the population genetics of this species and promise to

provide useful tools for the development of efficient pest management programs.

Funding

This work was supported by SYNERGASIA 09SYN- 22-617, within the National Strategic Reference Framework of the Hellenic Secretariat General for Research and Technology. KEK was supported by the Academy of Finland (project 258365). IO was supported by The Finnish Ministry of Education and Culture, Finnish Ministry of Agriculture and Forestry, BIOINT Doctoral Programme at the University of Jyväskylä and the Oiva Kuusisto Foundation.

Acknowledgements

This study would not have been realized without the help of all the colleagues who sent us *T. vaporariorum* samples and to whom we are grateful: Nathalie Gauthier, Nikos Katis, Andreas Perrakis, James Altman, Taysya Chermenskaya, Gregory Evans, Murad Ghanim, Devid Guastella, Maurice Jansen, Nikos Karatolos, Izumi Ohta, Chronis Remboulakis, Stephan Winter, Bill Wintermantel and Guifen Zhang. The authors would like also to thank an anonymous reviewer for the valuable comments and suggestions that substantially improved the quality of this paper.

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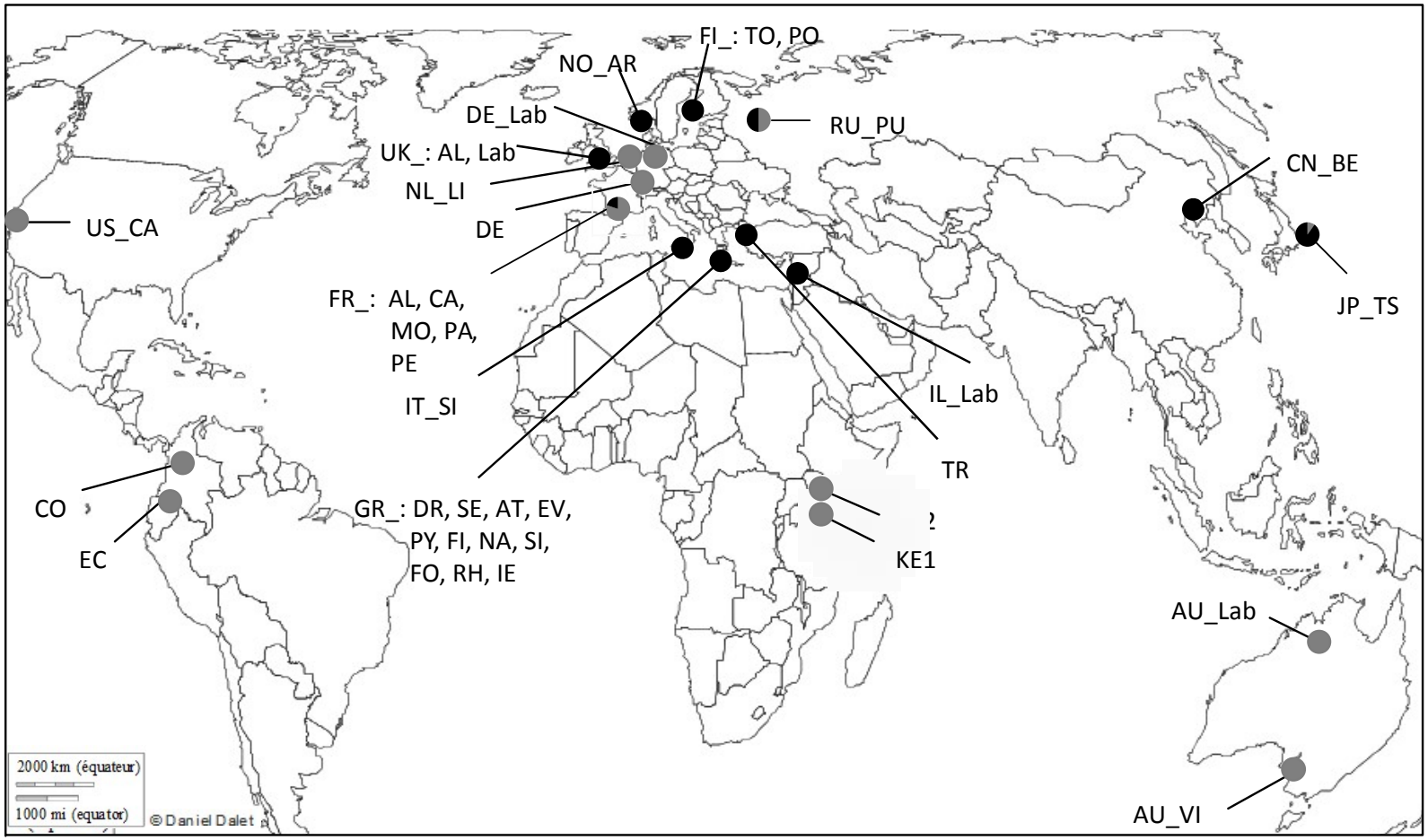

FIGURE 1 *T. vaporariorum* collection sites (See Table 1 for explanation of population codes). Clade 1 is presented in black and Clade 2 in grey.

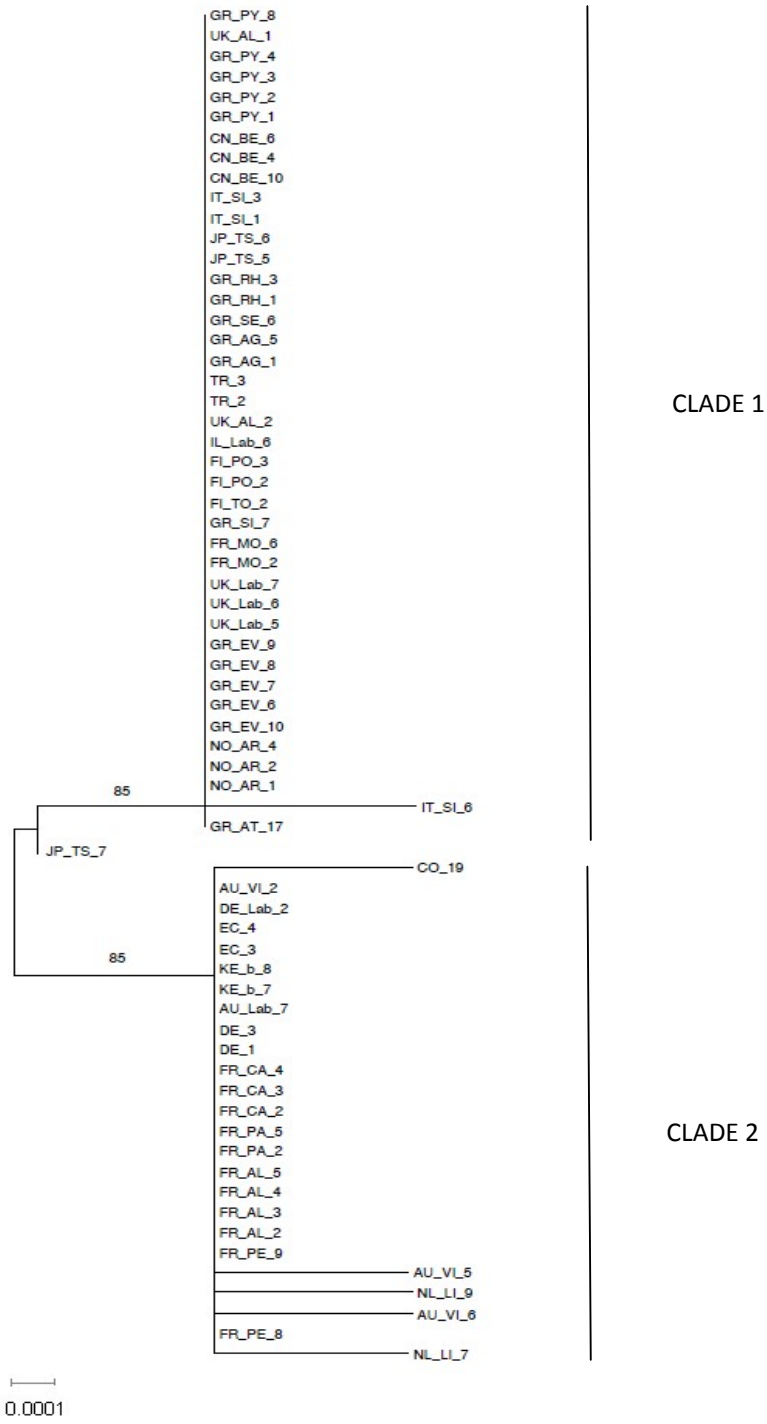

FIGURE 2 Phylogeny inference from concatenated *COI*, *cytb* and *ND5* gene sequences of *T. vaporariorum*, using the maximum likelihood method based on the HKY85 model. Percentage bootstrap supports (1000 replicates) superior to 50% are given at each node. The different populations are denoted by letter codes, suffixed by numbers indicating the respective individual analyzed.

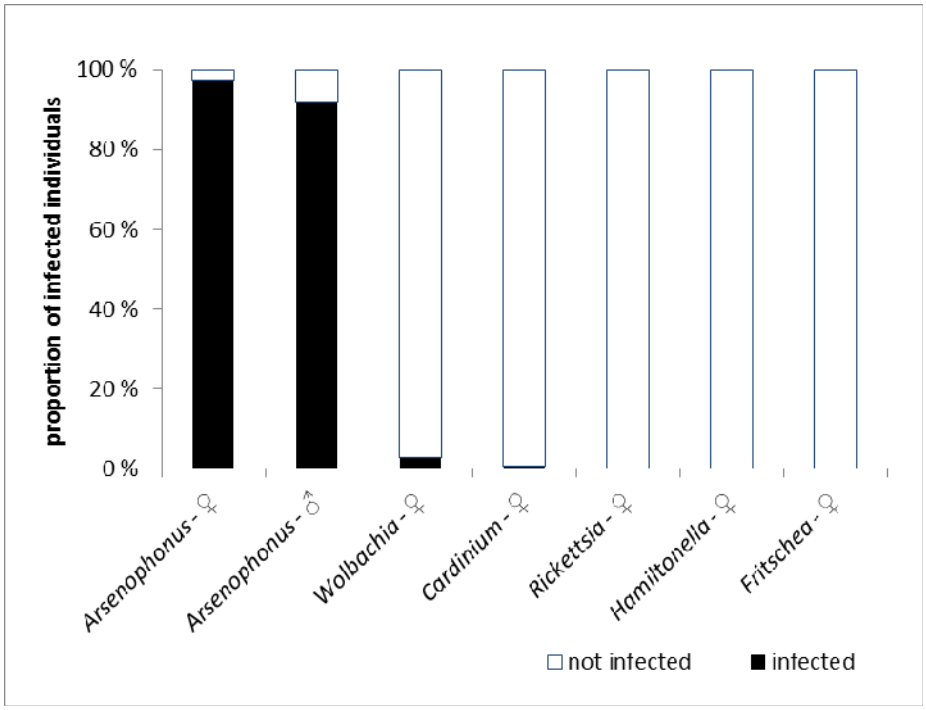

FIGURE 3 Proportion of individuals infected (black bars) or not infected (white bars) by each of the six symbionts tested. Males were tested only for infection by *Arsenophonus*.

TABLE 1 Characteristics of sampled *T. vaporariorum* populations used in the study, including population codes, number of individuals from which the three mitochondrial genes (COI, ND5, cytb) were sequenced from each location and the respective GenBank accession numbers, and number of males / females tested for endosymbiont infection.

Population Codes	Locality	Host plant	Habitat	Collection Year	mt genes						Secondary endosymbionts						
											Arsenophonus		Wolbachia	Cardinium	Rickettsia	Hamiltonella	Fritschea
					COI	Accession number	ND5	Accession number	cytb	Accession number	♀	♂	♀	♀	♀	♀	♀
GR_IE	Greece, Ierapetra	zuccini	gr	2002	3	KJ475452											
GR_AG	Greece, Agrinio	tomato	gr	2011	6	KJ475452	6	KJ475461	2	KJ475457	20	10	20	20	20	20	10
GR_SE	Greece, Serres	tomato	gr	2011	3	KJ475452	3	KJ475461	1	KJ475457	20	10	20	20	20	20	10
GR_DR	Greece, Drama	pepper	gr	2011	1	KJ475452	4	KJ475461	1	KJ475457	20	10	17	20	20	20	10
GR_AT	Greece, Athens	eggplant	gr	2005	3	KJ475452	5	KJ475461	7	KJ475457	20	5	12	20	20	20	10
GR_EV	Greece, Evia	marrow	gr	2008	6	KJ475452	6	KJ475461	5	KJ475457	20	10	20	20	20	20	10
GR_PY	Greece, Pyrgos	cucumber	f	2004	7	KJ475452	6	KJ475461	7	KJ475457	20	10	19	20	20	20	10
GR_FI	Greece, Filiatra	zuccini	f	2004	3	KJ475452	3	KJ475461	2	KJ475457	20	10	17	20	20	20	10
GR_NA	Greece, Nafplio	bean	f	2011	1	KJ475452	3	KJ475461	1	KJ475457	20	10	18	20	20	20	10
GR_SI	Greece, Sissi	rose	gr	2011	4	KJ475452	4	KJ475461	1	KJ475457	20	10	20	20	20	20	10
GR_FO	Greece, Fodele	rose	gr	2010	3	KJ475452	3	KJ475461	1	KJ475457	20	10	20	20	20	20	10
GR_RH	Grrece, Rhodes	tomato	gr	2011	5	KJ475452	6	KJ475461	3	KJ475457							
FR_AL	France, Alenya	tomato	gr	2007	5	KJ475453	4	KJ475462	5	KJ475457	10		9	10	10	10	10
FR_PA	France, Palau de Vivre	zuccini	f	2007	5	KJ475453	5	KJ475462	3	KJ475457	15		15	15	15	15	10
FR_MO	France, Montferrier	tobacco	gr	2007	6	KJ475452	4	KJ475461	3	KJ475457	13	2	13	13	13	13	10
FR_CA	France, Cabrera	zuccini		2007	4	KJ475453	3	KJ475462	3	KJ475457	9		8	9	9	9	8
FR_PE	France, Pernes	eggplant-tomato	gr	2008	4	KJ475453	2	KJ475462	3	KJ475457	20		20	20	20	20	10
IT_SI	Italy, Sicily	*Borrago officinalis*	f	2011	6	KJ475452	6	KJ475461	3	KJ475457, KJ475459	20	10	20	20	20	20	10
TR	Turkey	vegetables		2006	11	KJ475452	8	KJ475461	3	KJ475460	20	10	20	20	20	20	10
IL_Lab	Israel	cucumber	lab	2009	7	KJ475452	7	KJ475461	3	KJ475457	20	10	20	20	20	20	10
KE2	Kenya	rose	f	2011	4	KJ475453	6	KJ475462	2	KJ475457	10		10	10	10	10	10
KE1	Kenya	ornamentals	f	2011	1	KJ475453	6	KJ475462	1	KJ475457	6		6	6	6	6	6
US_CA	USA, California	*Physalis wrightii*-*Malva parriflora*	lab	1960	11	KJ475453	6	KJ475462	3	KJ475457	20	10	20	20	20	20	10
EC	Equador	rose	f	2011	3	KJ475454, KJ475453	7	KJ475462	3	KJ475457	20		20	20	20	20	10
CO	Colombia	*Solidago* sp.	f	2011	4	KJ475453	8	KJ475462,	1	KJ475459	20		20	20	20	20	10

AU_Lab	Australia	tobacco	lab	1992	5	KJ475453	6	KJ475463 KJ475462	1	KJ475457	20	10	20	20	20	20	10
AU_VI	Australia, Virginia	eggplant	gr	2011	5	KJ475455, KJ475453	6	KJ475462, KJ475464	3	KJ475457	20	10	20	20	20	20	10
JP_TS	Japan, Tsu-city	tobacco	lab	2004	6	KJ475452	8	KJ475461, KJ475462	3	KJ475457	20	10	20	20	20	20	10
CN_BE	China, Beijing	tomato	gr	2011	7	KJ475452	8	KJ475461	4	KJ475457	10	6	10	10	10	10	10
RU_PU	Russia, Pushkin	ornamental	gr	2011	7	KJ475452, KJ475453	7	KJ475461, KJ475462	3	KJ475457	20		20	20	20	20	10
FI_TO	Finland, Tojby	cucumber	gr	2011	4	KJ475452	4	KJ475461	3	KJ475457	21		21	21	21	21	10
FI_PO	Finland, Pörtom	tomato	gr	2011	3	KJ475452	3	KJ475461	3	KJ475457	22		20	22	22	22	10
NO_AR	Norway, Arendal	*Datura*	gr	2008	3	KJ475452	3	KJ475461	3	KJ475457	4	5	4	4	4	4	3
UK_AL	UK, Albrighton	rose		2010	4	KJ475452	3	KJ475461	3	KJ475457	20	10	20	20	20	20	10
UK_Lab	UK	bean	lab	1971	5	KJ475452	4	KJ475461	3	KJ475457	20	10	20	20	20	20	10
NL_LI	Netherlands, Lienden	*Pelargonium radens*	f	2011	7	KJ475456, KJ475453	7	KJ475462	2	KJ475457, KJ475458	20		20	20	20	20	10
DE	Germany	ornamentals		2008	3	KJ475453	3	KJ475462	3	KJ475457	20	10	20	20	20	20	10
DE_Lab	Germany	various hosts	lab		5	KJ475453	5	KJ475462	2	KJ475457	20	10	20	20	20	20	10
					180		188		103		640	218	619	640			

gr, greenhouse; f, field; lab, laboratory culture

TABLE 2 Sequence diversity of the mitochondrial datasets: the three mt genes individually and concatenated.

Genes	**N**	**PS**	**P**	**S**	**H**	**Hd**	**Pi**	**Tajima's D**	**Fu's Fs**	**SM**	**NSM**	**SS**	**NSS**
COI	163	4	2	2	5	0.493+0.025	0.00069+0.00005	-0.45081, p>0.10	-0.967	1	3	182.58	582.42
cytb	167	3	1	2	4	0.493+0.018	0.00063+0.00003	-0.06159, p>0.10	-0.075	1	2	127.50	421.50
ND5	99	3	1	2	4	0.079+0.037	0.00015+0.00007	-1.54871, p>0.10	-4.922	2	1	167.33	639.67
Concatenated	67	7	3	4	9	0.561+0.049	0.00053+0.0005	-0.56548, p>0.10	-3.013	4	6		

N, Total number of sequences; **PS**, Number of polymorphic sites; **P**, Parsimony informative sites; **S**, Singleton variable sites; **H**, Number of haplotypes; **Hd**, Haplotype (gene) diversity; **Pi**, Nucleotide diversity (per site); **SM**, Synonymous substitutions; **NSM**, Nonsynonymous substitutions; **SS**, Synonymous sites; **NSS**, Nonsynonymous sites

TABLE 3 Sequence diversity of the two clades, 1 and 2.

Clades	N	PS	Nm	Nd	Pi
Clade 1	24	5	5	0.417	0.00020+0.00007
Clade 2	42	2	2	0.095	0.00004+0.00003

N, Total number of sequences; **PS**, Number of polymorphic sites; **Nm**, Number of mutations;
Nd, Average number of nucleotide difference; **Pi**, Nucleotide diversity (per site)

SUPPLEMENTARY TABLE 1 Nucleotide sequences of primers used in this study.

Targeted taxon	Targeted gene	Primers	Sequences (5' - 3')	Anealing (°C)	Amplicon size (bp)
T. vaporariorum	mtCOI	C1-j-2195	TTGATTTTTTGGTCATCCAGAAGT	52	879
		tRNA1576	TATAAATCTTAAATTTACTGCA		
T. vaporariorum	mtND5	Tv_ND5_F	GATTATTGAGTACGAGGTGC	58	892
		Tv_ND5_R	GAACGTACTATATGAGCCAC		
T. vaporariorum	mtcytb	Tv_cytb_F	CATTATACGAGATGTTAGTGG	58	623
		Tv_cytb_R	CAGATAATACCACTCTGGCTG		
P. aleurodidarum	16SrRNA	28F	TGCAAGTCGAGCGGC	57	1000-1100
		1098R	AAAGTTCCCGCCTTATGCGT		
Arsenophonus	23SrRNA	Ars23S.1	CGTTTGATGAATTCATAGTCAAA	58	600
		Ars23S.2	GGTCCTCCAGTTAGTGTTACCCAAC		
Wolbachia	16SrRNA	Wol16S-f	CGGGGGAAAAATTTATTGCT	55	600
		Wol16S-r	AGCTGTAATACAGAAAGTAAA		
Hamiltonella	16SrRNA	Ham_F	TGAGTAAAGTCTGGAATCTGG	60	700
		Ham_R	AGTTCAAGACCGCAACCTC		
Rickettsia	16SrRNA	RB_F	GCTCAGAACGAACGCTATC	60	900
		RB_R	GAAGGAAAGCATCTCTGC		
Cardinium	16SrRNA	CFB_F	GCGGTGTAAAATGAGCGTG	58	400
		CFB_R	ACCTMTTCTTAACTCAAGCCT		
Fritschea	23SrRNA	U23F	GATGCCTTGGCATTGATAGGCGATGAAGGA	64	600
		23SIGR	TGGCTCATCATGCAAAAGGCA		
Arsenophonus	fbaA	fbaAf	GCYGCYAAAGTTCRTTCTCC	58	700-800
		fbaAr	CCWGAACCDCCRTGGAAAACAAAA		
Arsenophonus	yaeT	YaeTF496	GGCGATGAAAAAGTTGCTCATAGC	55	500
		YaeTR496	TTTTAAGTCAGCACGATTACGCGG		
Arsenophonus	ftsK	ftsKFor1	GCCGATCTCATGATGACCG	59	400
		ftsKRev1	CCATTACCACTCTCACCCTC		

SUPPLEMENTARY TABLE 2 Variable sites in the mt genes COI, cytb and ND5. Nucleotide positions are in relation to the published sequence (Genbank Accession No. AY521265).

Haplotype	Nucleotide position					Populations sharing haplotype
	COI					
	237	**394**	**413**	**513**	**N**	
1	T	G	A	A	102	RU_PU, CN_BE, IT_SI, GR_NA, JP_TS, GR_FI, GR_PY, GR_SE, GR_DR, GR_RH, GR_AG, GR_SI, GR_FO, GR_IE, GR_AT, GR_EV, TR, UK_AL, IL_Lab, FI_PO, FI_TO, FR_MO, UK_Lab, NO_AR,
2	C	.	.	.	56	RU_PU, AU_VI, CO, DE, DE_Lab, NL_LI, EC, KE1, KE2, AU_Lab, US_CA, FR_CA, FR_PA, FR_AL, FR_PE
3	C	T	.	.	1	EC
4	C	.	G	.	1	AU_VI
5	C	.	.	G	3	NL_LI
	cytb					
	322	**375**	**547**		**N**	
1	G	G	A		95	GR_AT, NO_AR, GR_EV, FR_PE, GR_FO, GR_NA, GR_PY, GR_FI, GR_SI, GR_AG, GR_SE, GR_DR, GR_RH, FR_AL, FR_PA, FR_MO, FR_CA, FI_TO, FI_PO, IL_Lab, UK_AL, UK_Lab, TR, JP_TS, US_CA, AU_Lab, KE1, KE2, EC, NL_LI, IT_SI, DE, DE_Lab, CN_BE, AU_VI, RU_PU
2	T	.	.		1	NL_LI
3	.	T	.		2	IT_SI, CO
4	.	.	C		1	TR
	ND5					
	20	**79**	**407**		**N**	
1	T	C	C		100	NO_AR, UK_Lab, FR_MO, FI_TO, FI_PO, IL_Lab, UK_AL, TR, GR_AT, GR_EV, GR_FO, GR_SI, GR_AG, GR_PY, GR_FI, GR_SE, GR_DR, GR_RH, GR_NA, JP_TS, IT_SI, CN_BE, RU_PU
2	C	.	.		65	FR_PE, FR_AL, FR_PA, FR_CA, JP_TS, US_CA, AU_Lab, AU_VI, KE1, KE2, EC, NL_LI, DE, DE_Lab, CO, RU_PU
3	C	T	.		1	4
	C	.	T		1	AU_VI

N, total number of individuals sharing haplotypes.