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Wolbachia Infection in European Populations of *Aedes albopictus*

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DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM CIÊNCIAS BIOMÉDICAS

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Sumário

Microrganismos surgiram no planeta Terra há cerca de 3.5 mil milhões de anos e espalharam-se pela Terra, estando presentes no solo, na água e em organismos vivos. Aos microrganismos presentes em organismos vivos dá-se o nome de microbiota. A microbiota dos artrópodes é diversa e uma das bactérias mais abundantes em artrópodes chama-se *Wolbachia*. Esta bactéria é um endosimbionte e infeta naturalmente vários insetos, nomeadamente, o mosquito *Aedes albopictus*. Este mosquito é um vetor de agentes patogénicos e tem-se expandido rapidamente pelo planeta, o que o torna uma ameaça à saúde pública. Assim, é necessário existirem programas de controlo deste vetor. Atualmente, a forma mais comum de combate à disseminação de vetores é o uso de inseticidas químicos, porém, a resistência a estes compostos está a surgir e a disseminar-se nas populações de mosquitos. Deste modo, os cientistas têm-se concentrado na procura de novas formas de controlo como o controlo biológico. Neste contexto, vários investigadores estão a testar *Wolbachia* como ferramenta de controlo, uma vez que *Wolbachia* possui a vantagem de ser um agente causador de incompatibilidade citoplasmática (CI). Assim, é importante compreender o padrão de infeção por *Wolbachia* em populações naturais de *Aedes albopictus*, que grupos e estirpes infetam estes insetos e como é que estas bactérias interagem com o seu hospedeiro. Atendendo a estes factos e à aparente inexistência de um amplo estudo sobre a distribuição da infeção por *Wolbachia* em populações de *Aedes albopictus* na Europa, os objetivos principais deste estudo são avaliar o tipo e o nível de infeção por *Wolbachia* em populações europeias de *Aedes albopictus* e descrever a sua estrutura populacional e distribuição na Europa.

Para atingir os objetivos, foi extraído DNA para se proceder ao PCR Multiplex com o objetivo de detetar se os mosquitos estavam infetados e com que grupo de *Wolbachia*. Em seguida, uma árvore filogenética foi construída e análises bioinformáticas foram realizadas com o objetivo de confirmar a circulação das estirpes circulantes de *Wolbachia* nas populações de *Aedes albopictus*.

Este estudo detetou que 99.5% dos mosquitos estavam infetados com *Wolbachia*. A maioria dos mosquitos estava duplamente infetada com *w*AlbA e *w*AlbB. Apenas 8.8% dos mosquitos estavam infetados somente com *w*AlbA ou com *w*AlbB. Outro aspeto detetado nesta investigação foi que, comparando com machos e larvas, as fêmeas apresentam uma taxa significativamente maior de infeções duplas (K= 28.5; $p < 0{,}0001$). Por outro lado, não houve diferenças significativas entre países ou regiões europeias, mostrando que existe uma distribuição uniforme deste tipo de infeção por *Wolbachia* na Europa.

Este estudo pode ser importante para o desenvolvimento e implementação de novas estratégias de controlo de vetores pelas autoridades de saúde nacionais e europeias. No entanto, novos estudos são necessários para aprofundar o conhecimento sobre a interação da *Wolbachia* com o hospedeiro e compreender melhor como ela evolui e interfere na dinâmica populacional dos mosquitos.

Palavras-chave: *Aedes albopictus*, incompatibilidade citoplasmática, infeção dupla, *Wolbachia*, *w*AlbA, *w*AlbB

Abstract

Microorganisms surged on planet Earth around 3.5 billion years ago and spread around Earth, being present in the soil, water, and in living beings. To the microorganisms present in living beings, it is called microbiota. The arthropods' microbiota is diverse and one of the most abundant bacteria in arthropods is called *Wolbachia*. This bacterium is an endosymbiont and infects naturally several insects, namely, the mosquito *Aedes albopictus*. This mosquito is a vector of pathogens, and it has spread rapidly around the world, becoming a public health threat. Therefore, vector control programmes must exist. The most common form of fighting the spread of mosquito vectors is the use of chemical insecticides. However, resistance to these chemicals is starting to emerge and disseminate among mosquito populations. Therefore, scientists are focused on finding new ways of vector control. One of these is biological control, namely, researchers are testing the endosymbiont bacterium *Wolbachia* as a control tool since *Wolbachia* has the advantage of inducing cytoplasmic incompatibility (CI). Therefore, it is important to understand the pattern of *Wolbachia* infection in *Aedes albopictus* populations, what groups and strains infect this mosquito and how these bacteria interact with its host. Attending on these facts and the apparent inexistence of a continent-wide study in Europe on the distribution of *Wolbachia* infection in *Aedes albopictus* populations, the main goals of this study were to assess the type and level of *Wolbachia* infection within European populations of *Aedes albopictus* and to describe its populational structure and distribution in Europe.

To achieve the goals, DNA was extracted to carry out a Multiplex PCR aimed at detecting whether mosquitoes were infected, and which *Wolbachia* group was infecting mosquitoes. A phylogenetic tree was constructed and bioinformatic analyses were performed to confirm the circulation of the circulating *Wolbachia* strains in *Aedes albopictus* populations.

This study detected that 99.5% of the mosquitoes were infected with *Wolbachia*. Most of the mosquitoes were double infected, with both *w*AlbA and *w*AlbB. Only 8.8% of the mosquitoes were singly infected with either *w*AlbA or *w*AlbB. Females have a significantly higher rate of double infections (K=28.5; p<0,0001) when compared with males or larvae. No significant differences between European countries or regions were observed, showing the uniform distribution of *Wolbachia* infection in Europe.

This study may be important for developing and implementing new strategies of vector control by European and national health authorities. Nevertheless, further studies are needed to deepen the knowledge on the interaction of *Wolbachia* with the host and to better understand how it evolves and interferes with the population dynamics of the mosquitoes.

Keywords: *Aedes albopictus*, cytoplasmatic incompatibility, double infection, *Wolbachia*, *w*AlbA, *w*AlbB

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List of Abbreviations and Acronyms

AICc – Akaike Information Criterion corrected

CTAB – Cetyltrimethylammonium Bromide

CHIKV – Chikungunya Virus

coxA – cytochrome c oxidase

CI – Cytoplasmatic Incompatibility

DENV – Dengue Virus

DNA – Deoxyribonucleic acid

fbpA – Diacylglycerol acyltransferase

FtsZ – Filamenting temperature-sensitive mutant Z

gatB – Glutamyl-tRNA amidotransferase subunit B

hcpA – beta-lactamase coding gene

HVR – Hypervariable Region

IIT – Incompatibility Insect Technique

IS – Insertion Sequence

kdr – Knockdown resistance

LINE – Long Interspersed Nuclear Elements

LTR – Long Terminal Repeat

MLST – Multilocus Strain Typing

RIDL – Release of Insects carrying a Dominant Lethal gene system

rDNA – Ribosomal DNA

SNP – Single Nucleotide Polymorphism

SIT – Sterile Insect Technique

*w*Alb – *Wolbachia* endosymbiont of *Ae. albopictus*

*w*AlbA – *Wolbachia* endosymbiont of *Ae. albopictus* A

*w*AlbB – *Wolbachia* endosymbiont of *Ae. albopictus* B

*w*Mel - *Wolbachia* endosymbiont of *D. melanogaster*

WSP – *Wolbachia* Surface Protein

WGS – Whole Genome Sequence

INTRODUCTION

Microbes are organisms that surged around 3.5 billion years on Earth (1) and spread around the planet. Microbes are present in the soil, in the water, and also in other living beings namely plants and animals such as insects (2). To the microorganisms that live inside other living organisms, it is called microbiota or microbiome. The arthropods' microbiota is diverse and its composition varies depending on various factors such as the environmental characteristics or the breeding site and the developmental stages (2–4). According to some studies, the predominant phylum present in insects is *Proteobacteria* (5) and one of the most abundant genera in arthropods is *Wolbachia* (3,4). But what is this bacterium?

WOLBACHIA

General characterization of *Wolbachia*

Wolbachia is a Gram-negative bacterium that belongs to Class *Alphaproteobacteria* and Order *Rickettsiales* (6). It was discovered by Hertig and Wolbach in 1924 in the ovaries of a mosquito called *Culex pipiens* (7). This obligate intracellular endosymbiont is widespread among the major Arthropoda orders Hemiptera, Coleoptera, Diptera, Hymenoptera, Lepidoptera, Orthoptera, Odonata, Isopoda, and in mites (Subclass Acari) (8). An estimated 1,690,000 to 5,070,000 arthropods might be infected with these parasitic bacteria (8). But how did it spread to so many animals, especially, arthropods?

Researchers think that *Wolbachia's* success might be related to an efficient transmission through female germline and manipulation of the reproductive system of its hosts by conferring different phenotypes to the host, such as parthenogenesis, feminization, differential male mortality, distorting sex ratio, or cytoplasmatic incompatibility (CI) (7,9,10). This increases the frequency of infected females (11). Besides the vertical transmission, it is thought that *Wolbachia* can also be horizontally transmitted which may influence *Wolbachia's* success too (7,12). Some experimental and theoretical studies suggest *Wolbachia* might improve host fitness (7). But how is its genome and how has it evolved?

Genetics and Evolution of *Wolbachia*

Wolbachia has a circular genome that may range from 0.9 to 1.8 Megabases and includes transposable elements like Insertion Sequence (IS) elements (6) The presence of repetitive sequences and mobile elements is common among *Wolbachia* strains infecting arthropods (6). It also has prophages in its genome. However, there is a possible degeneration of this genetic element (6). *Wolbachia* is an endosymbiont and as an endosymbiont *Wolbachia* is found in many mosquito species, particularly, in their gonads. Furthermore, it is present in a wide range of hosts which influenced its evolution. Therefore, it is necessary to understand how it evolved. One of the ways to answer this question is through phylogenetic analysis and characterization of *Wolbachia* strains.

The most common genes used in phylogenetic analyses and strain characterization include the filamenting temperature-sensitive mutant Z *(ftsZ)* gene (a gene responsible for coding a cell division protein (FtsZ) homologous to eukaryotic tubulin) or *16S rDNA* (ribosomal DNA). However, the *Wolbachia* surface protein *(wsp)* gene has become the gene of choice (13). This gene codes for the *Wolbachia* Surface Protein (WSP), which is an abundant major protein located on the outer membrane of the bacterium. This protein is composed of, approximately, 30 amino acids (14). Besides, the WSP encoding gene has homology with other outer membrane proteins encoding genes of other rickettsia bacteria that are closely related to *Wolbachia* (14). This gene is used to genotype *Wolbachia* and to establish phylogenetic relationships. It has a higher mutation rate than *ftsZ,* which facilitates these studies (12,13). Nonetheless, there are other ways to distinguish the *Wolbachia* strains and groups, namely, using Multilocus Strain Typing (MLST) (15). MLST is a technique used to genotype microorganisms that analyze several genes through polymerase chain reaction (PCR). In the case of *Wolbachia*, the genes used are cytochrome c oxidase (*coxA*), glutamyl tRNA amidotransferase subunit B (*gatB*), *ftsZ,* a β-lactamase coding gene (*hcpA*), and diacylglycerol acyltransferase (*fbpA*) (15). This system is also used to infer phylogenetic trees and is a reliable method of strain typing (15).

Through these methods, scientists found that *Wolbachia* is subdivided into 16 supergroups: A – Q (16,17) (Figure 1). In Figure 1 it is possible to observe that there is no Supergroup G. This happens because Supergroup G was decommissioned. After all, it is the result of a recombination between supergroups A and B (17). Supergroups A and

B are more commonly present in arthropods like flies, fleas, mosquitoes, and other insects. Supergroups C and D are present in nematodes (12). As for the other supergroups, these are present mainly in arthropods but also in other animals like nematodes, crustaceans, mammals, and other vertebrates.

There is recombination within the B-*Wolbachia* supergroup genomes and there are also gene exchanges between different supergroups of *Wolbachia* which is proven by the evidence that horizontal transmission between *Wolbachia* species infecting arthropods occurs (12). Moreover, the A-*Wolbachia* supergroup forms a monophyletic group, and the B – *Wolbachia* supergroup forms another monophyletic group (18).

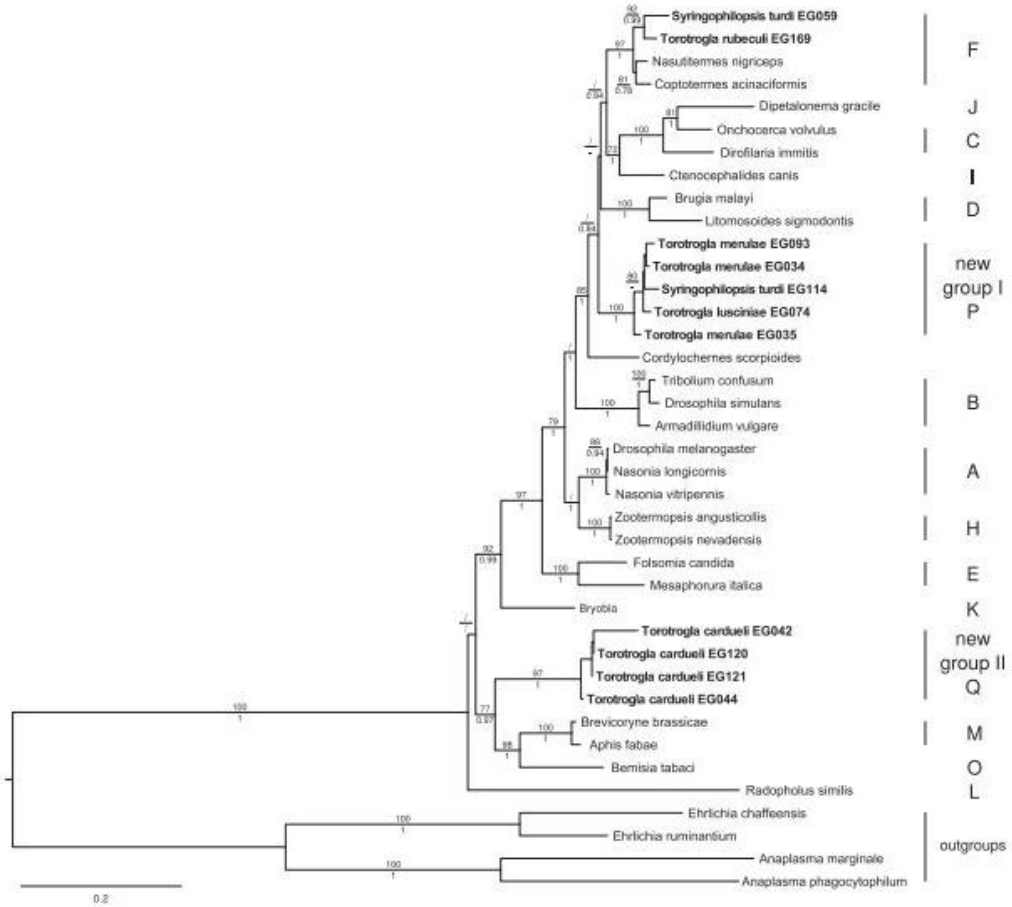

Figure 1 – *Wolbachia* supergroups phylogenetic tree adapted from: Glowska *et al* (16)

It is thought that supergroup A and B diverged from each other 58.3 million years ago – 66.6 million years ago (12).

Wolbachia as endosymbiont of Aedes albopictus

These two supergroups can be found in a lot of arthropods, namely, in a particular mosquito species called *Aedes albopictus*. Most *Ae. albopictus* populations so far analyzed are found naturally infected with *Wolbachia* (19–23). These mosquitoes may harbor three types of infection: double infection with both *Wolbachia* endosymbiont of *Ae. albopictus* A (*w*AlbA) and *Wolbachia* endosymbiont of *Ae. albopictus* B (*w*AlbB) strains, single infection with *w*AlbA or single infection with *w*AlbB. The double infection allows genetic exchanges and recombination between strains leading to the increase of bacterial diversity (24).

Moreover, a particularity of the *w*AlbB strain is that it is not closely related to the other *Wolbachia* strains infecting Culicidae as showed by Chuchuy *et al*. (25). Furthermore, in recent years it was found that there are some differences in the genetic structure of *w*AlbB and other Supergroup B Wolbachia strains, namely, in some metabolic pathways (6) helping explain the evolutionary process of *Wolbachia.*

But why is it important to study this mosquito, the distribution of this bacteria in its population, and its interaction with *Wolbachia*?

AEDES ALBOPICTUS

Taxonomy

Aedes albopictus (Skuse, 1895) (26) belongs to Domain Eukarya, Kingdom Animalia, Phyla Arthropoda, Class Insecta, Order Diptera, and Family Culicidae (26).

General characteristics

Aedes albopictus, commonly known as the Asian tiger mosquito, is a highly invasive mosquito. It has many features, namely, it is aggressive and daylight biter and has its peak of activity in the early morning and late afternoon (27,28). Furthermore, this species is exophagic and exophilic (29). Concerning the feeding habits of *Ae. Albopictus*, this mosquito is considered an opportunistic feeder (27). However, recent studies show that, when given the choice of the two blood meals, *Ae. albopictus* prefers human blood meal (30). Consequently, *Ae. albopictus* may act as a bridge-vector of emerging viruses

between animals and humans, because of its opportunistic behavior (27).

Another characteristic of this species is the involvement in the transmission of arboviruses such as dengue virus (DENV) and chikungunya virus (CHIKV). There is one mosquito species, *Aedes aegypti*, that is usually acknowledged as the primary vector of these pathogens as *Ae. albopictus* is a less competent vector (27,31). However, in recent outbreaks in Europe and some islands of the Indian Ocean, *Ae. albopictus* was the main vector responsible for the transmission of CHIKV and DENV (27,31,32). Therefore, this mosquito is becoming a public health concern, particularly, because of its rapid global spread and ecological plasticity.

Biological and ecological characteristics

Besides the feeding habits referred to above, other reasons contribute to the rapid spread of *Ae. albopictus*. This mosquito is considered a rural vector (27,31) because it usually breeds in forests, tree holes, and other natural habitats. However, *Ae. albopictus* adapted very well to suburban and urban habitats, exploring peridomestic larval habitats of anthropogenic nature, such as used tires and artificial containers (e.g. tires, water containers, cemetery urns), especially surrounded by vegetation or with plant debris (31,33). Kamgang *et al*. (33) showed that despite its adaptation to urban environments with high building densities, *Ae. albopictus* probably still prefers wooded areas or areas with more vegetation and a lower building density.

Among the main reasons for the global spread and establishment of this species are the global trade of used tires (34,35) and the capacity of this mosquito to live in adverse environments with low rainfall and high-temperature ranges (33–35). This characteristic is due to its capacity to adapt to seasonal variations, namely, through photoperiodic diapause (31,36) and successful overwintering of diapause eggs, namely, in central European countries like Germany (37). These features can confer a competitive advantage for *Ae. albopictus* over other mosquitoes, namely, *Aedes japonicus* (another invasive mosquito present in Europe) (38) or *Ae. aegypti* (27). But how did it spread and arrived in Europe?

Biogeographical characteristics

Aedes albopictus is a native mosquito from South-Eastern Asia (39). A study suggests that the expansion of *Ae. albopictus* Asian populations started around 70,000 years ago. However, the expansion to mid-latitudes occurred after the last glacial age (around 12,000 years ago), probably due to the amelioration of the climatic conditions and environmental changes caused by human activities (40). In the last few decades, this mosquito has been spreading all over the globe and it is now present in all continents except Antarctica.

Focusing on Europe, *Ae. albopictus* was first detected in Albania in 1979 (27,31,34). Since then, it has rapidly disseminated around the continent, which is supported by genetic studies that found there is low genetic variation in *Ae. albopictus* European populations(41). *Aedes albopictus* initially established populations mainly in Southern Europe, particularly around the Mediterranean basin, in countries like Italy, Spain, Greece, Croatia, and Bosnia and Herzegovina (42,43). In Portugal, Russia, and other countries, the species was only introduced a few years ago (44,45). Whether these recently introduced populations are already established is not yet clear. However, some studies appear to show that *Ae. albopictus* populations are already locally established in

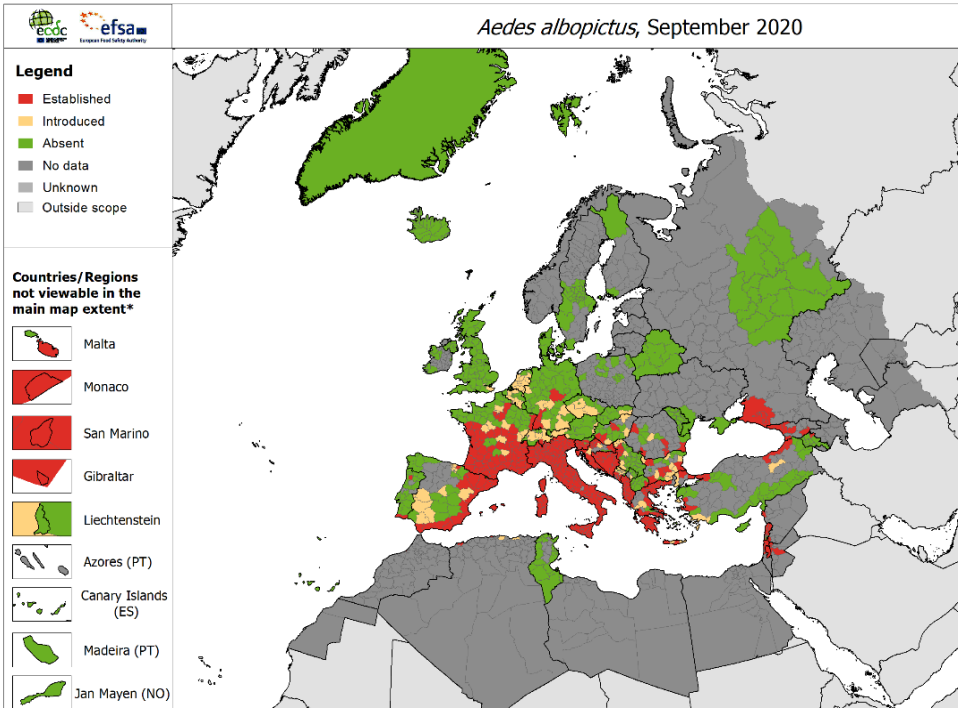

Figure 2 – Map of the distribution of *Ae. albopictus* in Europe adapted from European Centre of Diseases Control. (47)

some regions of these countries (44,46,47) (Figure 2). Furthermore, it is expected that this mosquito will spread to other areas of Europe (35,38,48).

As the invasion happened quite rapidly, it is essential to know the reasons for the proliferation and dispersion of the mosquito populations. Unlike the expansion of the native populations, studies report that the dispersion and invasion of Europe and America were human-driven mainly due to transportation between long distances and multiple introduction events (49,50) associated with the global trade of tires (34). There is evidence for at least three independent introduction events in Europe: Albania, North Italy, and Central Italy (49).

Population Genetics

Due to the rapid spread and great adaptability to new environments, some researchers tried to investigate the population genetics and phylogeography of this species. Studies using genetic markers as microsatellites or Single Nucleotide Polymorphisms (SNPs) found that there is genetic differentiation within native range populations and invasive range populations and there is genetic differentiation between native range populations and invasive range populations (49,50). In Asia, two genetic clusters can be divided into Japanese populations and continental Asia populations (49,50). Furthermore, it was also observed that the continental Asia populations are also divided into Northeast (China) and Southeast Asian (Malaysia and Thailand) populations (50). In this region, most *Ae. albopictus* populations are infected with *Wolbachia* (19–21,51–53) and the most prevalent type of infection is double infection (19–21,51,53). Moreover, in some regions (India and Malaysia), there were not found mosquitoes singly infected with *w*AlbB strain (20,51).

In Europe, the studies to analyze the distribution of *Wolbachia* among *Ae. albopictus* populations are rarer, although, some researchers did it in some countries namely, Spain, Italy, and Russia (22,23). They verified that the pattern of distribution is like the one in the Asian region. However, there is a slight difference. While in some Asian regions, there were not found single infections with *w*AlbB, in these countries, no single infections with *w*AlbA strain were found (22,23). In the case of the study of population genetics concerning Ae. albopictus in Europe, it is possible to observe through

the literature that there is a high admixture of populations derived from the fact that multiple introductions occurred. However, it is still possible to establish some population structure among European populations that are divided into south Balkans, north Italy, central Italy, and Turkey (50). In the case of Western European populations, there is a mixture between north and central Italy (50).

Another question that has been posed throughout recent years is the origin and genetic relatedness to the native populations of the invasive populations if the native populations have a higher genetic diversity than the invasive and if the older established to have a higher genetic diversity than the recently introduced ones. According to various studies, Albanian and South Balkans populations are related to China populations (39,50), instead of north Italian populations that probably came from the United States of America (49,50) that, in turn, are related to Japanese populations (49,50). In the case of central Italy, there is a mix between North Italy and China populations which means that there was probably more than one introduction event (50). Concerning Western Europe, populations are genetically related to both north and central Italy populations. Greek populations have a genetic influence mainly from Albania but also from central and north Italy populations (50). Some studies demonstrate that Albanian populations have lower genetic diversity than Italian ones while the native range harbor a similar level of genetic diversity to Albanian populations (41,50). This might happen due to the political and commercial isolation of Albania and restricted exchanges to China. Therefore, few individuals were introduced leading to a low founding diversity, which possibly led to a low genetic diversity (50,54).

In the last decades, it has been observed a spread of mosquitoes towards new areas in Europe, Africa, and America (55). One of these species of mosquitoes is *Aedes albopictus*. Moreover, mosquitoes such as *Ae. albopictus*, are vectors of emerging or re-emerging arboviruses such as dengue and chikungunya(27,55). This is very problematic and is becoming a very serious public health issue. Therefore, it is important to study the biology of the mosquito, its ecology, its behavior, and new ways of fighting these vectors.

VECTOR CONTROL

Mosquitoes, like other insects, are vectors of pathogens and therefore, are an

important agent of transmission and dissemination of diseases. To fight them, governments and health organizations started to monitor insect populations and tried to eradicate them, or, at least, reduce them to try to eliminate the propagation of diseases.

Chemical control

One of the most common techniques of vector control is the use of chemical insecticides. The major strategies used to fight mosquitoes are the deployment of insecticide-treated nets (in the case of vectors of malaria) and the community-wide spraying of insecticides inside domiciles (in the case of vectors of malaria and *Aedes* mosquitoes) (56). The four major categories of insecticides used are organochlorines, organophosphates, carbamates, and pyrethroids (57). Besides the difficulties to implement some of these techniques, in recent years, researchers identified a growing development of insecticide resistance. This phenomenon in *Aedes* mosquitoes occurs mainly due to target-site mutations, namely knockdown resistance mutations (*kdr*), and increased detoxification (58). Insecticide resistance may be a threat to vector control. Furthermore, concerns with the effects of insecticides in the environment made scientists investigate more sustainable approaches of vector control, such as biological and genetic control (59,60).

Genetic control

Genetic control strategies have been used in recent times with, mainly, two different purposes: suppress the number of competent vectors in a target area, or replace a population with a modified vector with reduced vectorial capacity (59,60). To achieve these goals, it is possible to use several systems, such as sterile insect techniques (SIT), incompatibility insect techniques (IIT), or gene drive systems (59,60).

Sterile insect techniques consist mainly in the release of sterile males to mate with wild females to diminish the reproductive potential, leading to a reduction or collapse of the target population (60,61). The classical SIT normally used DNA damaging agents to cause lethal modifications (60). However, new techniques involving the production of modified germ lines that, for example, express a nuclease that generates chromosome breaks have been developed. The release of insects carrying a dominant lethal genetic

system (RIDL) is an example of a type of these new approaches (60).

Sterility can also be induced through artificial infections with various strains of *Wolbachia*. Male insects are useless for the spreading of *Wolbachia*, as these bacteria are maternally transmitted. Nevertheless, infected males produce modified sperm that originate viable zygotes only with eggs of infected females, a phenomenon named cytoplasmatic incompatibility (CI) (60). CI is the basis of IIT and has already been used in the field with success. However, if an infected male mates with an infected female, the zygotes will be viable. Therefore, if an infected female is released, it spreads *Wolbachia* reducing or eliminating the effect of sterility (60,61). As illustrated in Figure 3, there are two "types" of CI: i) unidirectional CI which originates a non-viable zygote after the mating of an infected male with an uninfected female, and ii) bidirectional CI which also originates a non-viable zygote after the mating of two infected mosquitoes with different strains (60).

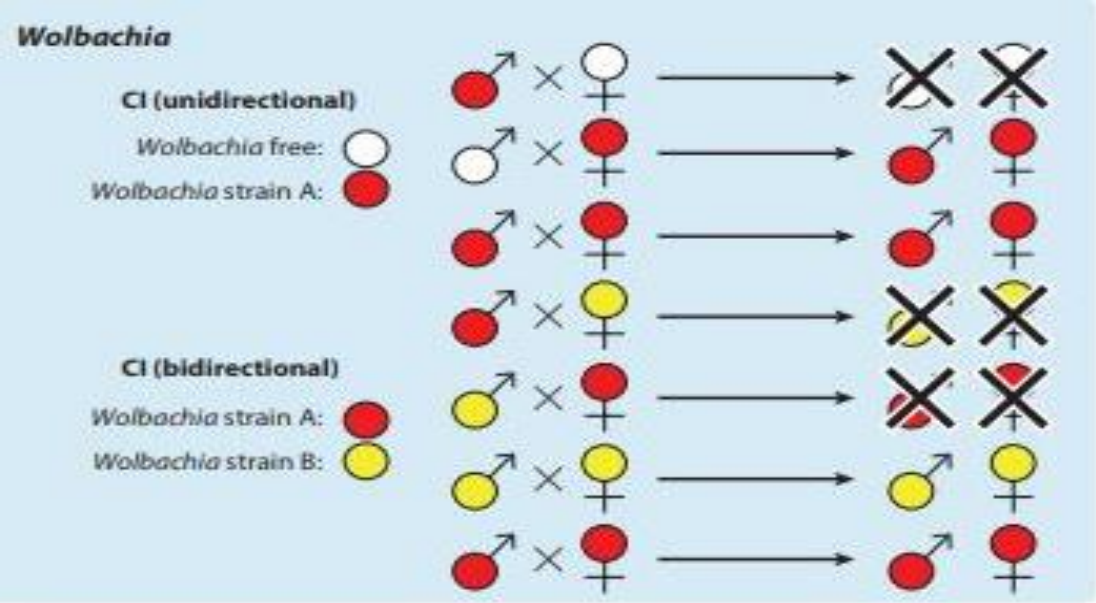

Figure 3 – CI schematic representation. Cytoplasmatic incompatibility is caused by *Wolbachia* and affects the reproductive potential of *Ae. albopictus*. adapted from Alphey *et al* (60)

A clear explanation for this phenomenon is still not available. However, some biochemical models have been suggested. One of them suggests the production by

Wolbachia of a product that disrupts sperm processing in the egg, while, the other model suggests that a still unknown biochemical process caused by the bacteria in the male, binds away a product necessary for the normal processing of the sperm in the egg (62).

Host chromatin-binding proteins have been found to bind to *Wolbachia* within host cells (62). All these processes need further studying but it has been found that CI is associated with early mitotic defects in the fertilized egg, which might make the paternal chromosomes fail to undergo segregation and later, their loss. In diploid species, this may cause embryonic death. It is also common to find CI-induced aneuploidy or aberrations and abnormalities provoked by CI (62). Possibly, CI affects and disrupts one or several processing steps of the paternal pronucleus after fertilization. Another important fact to retain is the role and influence of bacterial density, host genotype, or bacterial strains on CI strength and direction (16,63). Besides CI, *Wolbachia* can also provoke other distortions in the host's reproductive cycle such as feminization and parthenogenesis (60).

As *Wolbachia* infects germ and somatic cells, therefore, spreading within the insects' population through CI, it may also be useful, as a gene drive system, for driving nuclear transgenes, despite transformation in *Wolbachia* is not yet possible (59,60).

***Wolbachia* effect on pathogens transmission**

Wolbachia has also the capacity of reducing the capacity of virus transmission by the mosquito. *Wolbachia* can reduce the ability of DENV and CHIKV from establishing productive infections (64), causing the block or diminishing the transmission of these viruses. However, only some strains can have this effect (65). One of these strains is *Wolbachia* endosymbiont of *D. melanogaster* (*w*Mel). According to a study by Ahmad *et al*. (19), the capacity of *Ae. albopictus* to transmit CHIKV is not significantly affected by *Wolbachia* endosymbiont of *Ae. albopictus* (*w*Alb) because this strain does not impact the dissemination to salivary glands and does not interfere with the replication of the virus in the midgut and salivary glands. Another reason for this to happen is that *w*Alb may have a very restricted tissue tropism in its host (64). The rate of replication of CHIKV and viral load increases during a few days after infecting a *w*Alb-infected individual mosquito, then, keeping the same viral load throughout the rest of the mosquito life but decreasing the density of *w*Alb, contrarily to mosquitoes free of *w*Alb, in which the viral

load and the rate of replication is highly variable (66). However, in the case of DENV, *w*Alb can interfere with the replication in the midgut and salivary glands and it can also affect its transmission suggesting that this symbiont can naturally influence DENV transmission (67). Furthermore, *w*Mel and *w*Alb do not affect only arboviruses, but can also probably affect the transmission of other pathogens like *Plasmodium* (64).

Other *Wolbachia* effects on the host

The *w*Mel strain reduces the longevity and fecundity of *D. melanogaster*, *Ae. albopictus* and halves the lifespan *of Ae. aegypti* (60,68,69), possibly because it induces up-regulation of immune effector genes in the mosquito (64,68). In the case of *Ae. albopictus*, it may also be because *w*Mel is maladaptive to this mosquito and acts as a pathogen, causing a weak CI effect and a high fitness cost which also affects maternal transmission in the mosquitoes, making it unlikely to spread in an uninfected population (69). *Wolbachia* has other effects on the host. It might affect its evolutionary dynamics and so, it may cause rapid speciation (60) and a low DNA mitochondrial variability (70).

Therefore, in the last years, researchers have focused on the study of *Wolbachia* as a possible vector control tool. To understand if there is the possibility to use these bacteria to control vectors, namely, mosquitoes such as *Ae. albopictus* and *Ae. aegypti*, it is necessary to investigate the distribution of *Wolbachia* among these species and to study the characteristics of *Wolbachia* populations that infect these insects. Several studies have been done to study *Wolbachia* in *Ae. albopictus* populations, particularly in Asia (19–21,51). Some have been made concerning field-collected European populations (22,23) but none has covered the continent-wide European distribution of this species.

OBJECTIVES

The main objective of this work is to assess the type and level of *Wolbachia* infection in European populations of *Ae. albopictus* and describe its population structure and distribution in Europe. Specifically:

1. Screening for *Wolbachia* infection in European populations of *Ae. albopictus*.
2. Genotyping the circulating *Wolbachia* strains infecting European populations

of *Ae. albopictus* through Multiplex PCR.

3. Finding a geographical pattern of distribution of *Wolbachia* in Europe.

MATERIAL AND METHODS

MOSQUITO SAMPLES

Mosquitoes were collected in 14 countries and regions of Europe, namely Abkhasia, Albania, Bulgaria, Croatia, France, Georgia, Greece, Italy, Malta, Portugal, Russia, Serbia, Slovenia, Spain, and Turkey as showed in Figure 4. The characteristics of the collection sites are described in Table A1 in Annex 1 section.

From June 2017 until October 2019, all mosquitoes have been collected in the various countries referred above except for mosquitoes from Georgia that were collected in September 2015. All mosquitoes were captured during summer (From May/June to September/ October). The methods of collection varied depending on the developmental stage, namely, to collect adults (female and male), it was used baited trap, human trap, and resting catch. To collect eggs or mosquitoes in larval stages, ovitraps and larval dippers were used. In the case of mosquitoes captured in Spain (Badajoz, Monesterio, Almaraz, and Aldea del Cano), Greece, Italy (Bologna), Albania, Serbia (Novi Sad), and Croatia, adults were obtained from larvae from dippers and ovitraps and lab-reared from eggs from ovitraps. After being captured, mosquitoes had to be conserved. For this, it was used ethanol 80%, although, some samples such as Batumi (Georgia) mosquitoes and Bologna (Italy) mosquitoes were dried out first before being stored in ethanol.

Figure 4 – Map of European continent showing the collection sites of the mosquitoes analyzed in this study, identified by blue dots

DNA EXTRACTION

DNA was extracted using a CTAB (Cetyltrimethylammonium bromide) protocol adapted from Weeks *et al*. (71). Individual mosquitoes were removed from the tube and dried on filter paper. The mosquito was placed in a 1,5 ml tube and 150 µL of CTAB buffer (2%) was added. The mosquitoes were macerated with a pestle. 2 µL of proteinase K were added, mixed thoroughly, and incubated for one hour at 56ºC in a water bath. 4 µL of RNase A were added and tubes vortexed and let at room temperature for 2 minutes. 150 µL of chloroform: isoamyl alcohol solution (24:1) was added, mixed thoroughly for 5 minutes, and centrifuged at 18 000 g for 5 minutes. The upper aqueous phase was transferred to a new tube. 200 µL of frozen absolute ethanol and 5 µL of sodium chloride (5M) were added to each tube, vortexed and let to precipitate at 4ºC overnight. The samples were centrifuged at 20 000 g at 4ºC for 20 minutes, washed with 400 µL frozen ethanol 70%, and centrifuged at 20 000 g for 10 minutes at 4ºC. The pellet was dried using a speed vac Concentrator 5301 (Eppendorf, Hamburg, Germany) and resuspended in 200 µL of distilled water or TE (1/100x).

DNA from a subset of samples was extracted with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Two methods of extraction were used due to different works occurring in parallel

that are part of the same project.

WOLBACHIA GENOTYPING

All the individuals were screened for *Wolbachia* infection and genotyped by a multiplex PCR using *Wolbachia* specific primers 328F, 691R for *w*AlbA, and 183F, 691R for *w*AlbB strains (13). The PCR mixture was composed of 1X GoTaq® Flexi buffer (Promega, Madison, USA), 2.5mM $MgCl_2$, 0.3mM dNTPs each, 0.4 µM of each primer, and 1U of GoTaq® polymerase (Promega, Madison, USA). The reaction volume was 25 µL and the DNA volume used was 2 µL. The thermal cycle conditions were the following: 95ºC for 3 min, followed by 35 cycles of 95ºC, 55ºC, 72ºC for 1 minute, and 72ºC for 10 minutes.

To confirm *Wolbachia* negative samples, universal *wsp* primers 81F and 691R were used (13). The PCR mix and cycle conditions were the same as above. Samples that remained negative were further analyzed by PCR using *28S rDNA* specific primers (21). The PCR mixture was composed of 1X GoTaq® Flexi buffer (Promega, Madison, USA), 2,5mM $MgCl_2$, 0,16mM dNTPs each, 0,4 µM of each primer, and 1U of GoTaq® polymerase (Promega, Madison, USA). The cycling conditions were 94ºC for 5 minutes, followed by 37 cycles of 94ºC for 30 seconds, 56ºC for 45 seconds, 72ºC for 1 minute, and a final extension at 72ºC for 10 minutes. All primers used during the experiment are described in Table 1.

Table 1 – Characterization of the primers used for *Wolbachia* genotyping

	Primer Forward	Primer Reverse	Product Size
*w*AlbA	328F:5'-CCAGCAGATACTATTGCG-3' (18 bp)	691R:5'-AAAAATTAAACGCTACTCCA - 3' (20 bp)	389 bp
*w*AlbB	183F:5'-AAGGAACCGAAGTTCATG- 3' (18 bp)	691R:5'-AAAAATTAAACGCTACTCCA-3'(20 bp)	501 bp

wsp	81F:5'-TGGTCCAATAAGTGATGAAGAAAC –3' (24 bp)	691R:5'-AAAAATTAAACGCTACTCCA-3' (20 bp)	615 bp
28S rDNA	28F:5'-TACCGTGAGGGAAAGTTGAAA– 3' (21 bp)	28R:5'–AGACTCCTTGGTCCGTGTTT–3' (20 bp)	

All PCR products were separated by horizontal electrophoresis (110V, 40 minutes) in 1.5% agarose gels stained with $2.5x10^{-5}$ µg/µL of GreenSafe Premium (NZYTech, Lisbon, Portugal) and visualized and photographed under UV light.

SEQUENCING

Partial sequences of the *wsp* gene were obtained using the *w*AlbA and *w*AlbB primer set and the PCR conditions used for multiplex PCR as described above, except for the final reaction volume and DNA template that were increased to 50 µL and 4 µL, respectively. Products were sequenced in both directions and the amplicons were purified using the JETQUICK™ PCR Purification Spin Kit (GenoMed, Löhne, Germany) as described by the manufacturer. Samples were sequenced using the Sanger method at STAB Vida (Lisbon, Portugal).

PHYLOGENETIC ANALYSIS

The obtained *wsp* sequences were edited with BioEdit (72) and aligned using the multiple alignment algorithm ClustalW. Each sequence was run against BLAST in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm that they were from the *Wolbachia* endosymbiont of *Ae. albopictus*. The maximum-likelihood method using the Tamura-Nei parameter model in MEGA6 software was used to construct a phylogenetic tree. The best fit model of nucleotide substitution was chosen using Akaike Information Criterion corrected (AICc) as implemented in MEGA6 (21,73,74). To estimate the robustness of each node, 1000 bootstrap replications under the Nearest-Neighbor Interchange method

were used. As an outgroup, *wsp* sequences from *Wolbachia* endosymbiont of *Dirofilaria immitis* belonging to Supergroup C; *D. melanogaster*, *Drosophila simulans* strain Riverside, *Wolbachia* endosymbiont of *Aedes aegypti*, *w*AlbA from India belonging to supergroup A; and *w*Pip from *C. pipiens* and *C. quinquefasciatus*, *w*AlbB from India, *w*AlbB from Russia and *Wolbachia* endosymbiont of *Ochlerotatus cantans* belonging to supergroup B were used. All sequences used to construct the tree are described in Annex 2 section.

To confirm the circulation of *w*AlbA and *w*AlbB strains in field populations, a WSP typing system (15), that characterizes each strain based on the amino acid motifs of four hypervariable regions (HVRs), was used. A batch query of the sequences was performed as described in https://pubmlst.org/*Wolbachia*/wsp/info/protocol.shtml (accessed on 21st December 2020) to compare the *wsp* sequences obtained with those available at the PubMLST database. This method was also used to detect the allele of each HVR, to determine the WSP profile of the strains present in the analyzed mosquitoes.

The genetic diversity of the *Wolbachia* population for the fragments sequenced was analyzed using the DNAsp software version 5.0 (75) to detect polymorphisms within strains and different haplotypes within and between strains. For this, sequences were aligned and trimmed. Then, using DNAsp, haplotype analysis was made to check the number of polymorphisms and haplotypes of the sequences analyzed. After that, it was proceeded to draw a haplotype network. For that, it was used Network software. To perform this analysis, it was generated through DNAsp a haplotype file. Then, this file was used on Network software to calculate the network. After the calculations were made, the haplotype tree was drawn with the help of the same software.

STATISTICAL ANALYSES

Wolbachia infection and double infection rates were calculated for every country, sex, and developmental stage. Using GraphPad Prism software (version 8), a comparison between *Wolbachia* single and double infections in *Ae. albopictus* from the different European countries was made with non-parametric Kruskal-Wallis ANOVA. A comparison between *Wolbachia* double and single infections in female, male, and larval

stages of *Ae. albopictus* was also made. These analyses were done using the Chi-square test. A p-value < 0.05 was considered statistically significant.

RESULTS

WOLBACHIA GENOTYPING

A total of 782 mosquitoes were analyzed. Of these, 474 were adult females, 239 were adult males and 69 were larvae. Out of the 782 mosquitoes analyzed, 778 (99.5%) were infected with *Wolbachia*. Of these, four (0.5%) were singly infected with *w*AlbA and 65 (8.3%) were singly infected with *w*AlbB. Co-infection with both strains, *w*AlbA, and *w*AlbB, was observed in 90.7% of the infected mosquitoes (Table 2), meaning that two bands, *w*AlbA, and *w*AlbB, with a size of 389 bp and 501 bp were respectively detected by electrophoresis. Single infections with *w*AlbA were observed in adult females only, while single *w*AlbB infections were found in both sexes and all developmental stages. Single infections were more common in adult males and larvae than in adult females (Table 2 and see Table A2 in Annex 1 section). In males, 18.7% were singly infected with *w*AlbB while in larvae the rate was 24.6%. Nevertheless, double infection was the most predominant type of infection found, being significantly higher in females than in males and in larvae (97.1% in females, 81.2% in males, and 73.9% in larvae; *K*: 28.5; $p<0.001$). However, through the Chi-square test, it was observed that all types of infection vary with developmental stage and sex of the mosquito, which means that variables are not independent of each other (*w*AlbA+B: Chi-square value: 309360.260 $p<0.00001$; *w*AlbA: 19.779 $p=0.011$; *w*AlbB: 159.023 $p<0.00001$).

Table 2 - Rates of infection of *Wolbachia* in *Ae. albopictus* by sex and developmental stage

	Infected	***w*AlbA**	***w*AlbB**	***w*AlbAeB**	**Total**
Males	238 (99.6%)	0 (0.0%)	44 (18.4%)	194 (81.2%)	239
Females	472 (99.6%)	4 (0.8%)	8 (1.7%)	460 (97.1%)	474
Larvae	68 (98.6%)	0 (0.0%)	17 (24.6%)	51 (73.9%)	69
Total of infected mosquitoes	778 (99.5%)	4 (0.5%)	65 (8.3%)	709 (90.7%)	782

There was a predominance of double infections in all countries, with rates ranging from 80.0% to 100.0% (Table 3). There was no statistically significant difference between countries or regions (*K*: 17.3; $p= 0.241$). Mosquitoes singly infected with *w*AlbB were detected in most of the European countries analyzed. Russia, Georgia, and Slovenia were

the exceptions where no single infection was detected. Single infections of *w*AlbA were only detected in mosquitoes from Portugal, Greece, Italy, and Abkhasia.

The four mosquitoes that were negative for *wsp* (including for the additional PCR assay with wsp universal primers) were tested for DNA integrity by PCR targeting the mosquito 28S *rDNA*. These samples gave a positive PCR result showing that the DNA was intact and confirming that the samples were negative for *Wolbachia*.

Table 3 - Rate of *Wolbachia*, *w*AlbA and *w*AlbB infection by country

Country	Total	Infected	*w*AlbA+B	*w*AlbB	*w*AlbA
Portugal	30	29 (96.7%)	26 (86.7%)	2 (6.7%)	1 (3.3%)
Spain	98	97 (99.0%)	79 (80.6%)	17 (17.3%)	0 (0.0%)
Turkey	45	45 (100.0%)	43 (95.6%)	2 (4.4%)	0 (0.0%)
Greece	109	109 (100.0%)	104 (95.4%)	4 (3.7%)	1 (0.9%)
Italy	79	78 (98.7%)	71 (91.0%)	6 (7.7%)	1 (1.3%)
Russia	15	15 (100.0%)	15 (100.0%)	0 (0.0%)	0 (0.0%)
Abkhasia	15	15 (100.0%)	12 (80.0%)	2 (13.3%)	1 (6.7%)
France	64	64 (100.0%)	58 (90.6%)	6 (9.4%)	0 (0.0%)
Albania	46	46 (100.0%)	45 (97.8%)	1 (2.2%)	0 (0.0%)
Malta	50	50 (100.0%)	41 (82.0%)	9 (18.0%)	0 (0.0%)
Bulgaria	54	54 (100.0%)	49 (90.7%)	5 (9.3%)	0 (0.0%)
Serbia	100	100 (100.0%)	91 (91.0%)	9 (9.0%)	0 (0.0%)
Slovenia	40	40 (100.0%)	40 (100.0%)	0 (0.0%)	0 (0.0%)
Croatia	22	21 (95.5%)	20 (95.2%)	1 (4.8%)	0 (0.0%)
Georgia	15	15 (100.0%)	15 (100.0%)	0 (0.0%)	0 (0.0%)
Total	782	778 (99.5%)	709 (90.7%)	64 (8.2%)	4 (0.5%)

PHYLOGENETIC ANALYSIS

Estimates of genetic diversity for *w*AlbA and *w*AlbB sequences were calculated using DNAsp software. Therefore, it was verified that within *w*AlbA, there is no nucleotide variability while within *w*AlbB variability is low (0.041). In total, as it is possible to see in Figure 5, four haplotypes were observed. One corresponds to *w*AlbA and the other three to *w*AlbB. However, two of them were observed only in a single

individual while the other was found in all other mosquitoes infected with *w*AlbB. The two single-individual haplotypes have one polymorphic site but no parsimony informative sites. These results show there is a low diversity within *Wolbachia* strains

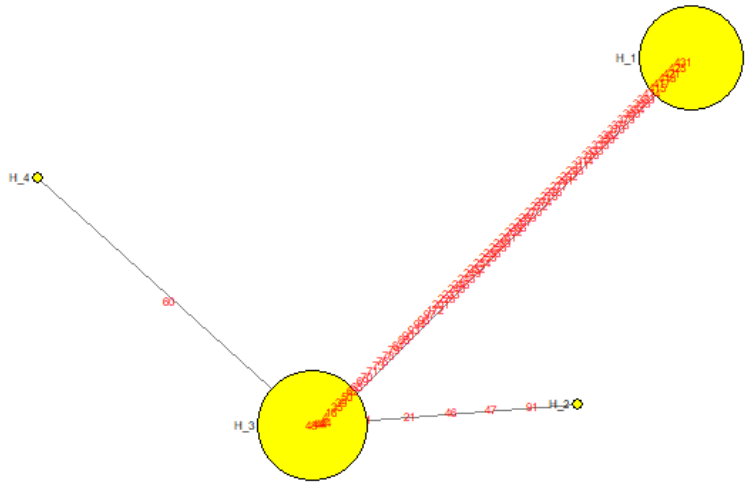

Figure 5 – Haplotype network of *Wolbachia*. H_1 represents haplotype of *w*AlbA strain and H_2, H_3 and H_4 represent haplotypes of *w*AlbB strain.

The phylogenetic tree (Figure 6) shows a clear divergence between the two strains (*w*AlbA and *w*AlbB) that are grouped in two clades with 100% bootstrap confidence. As showed in the literature, the *w*AlbB strain is closely related to *w*Pip strains from *C. pipiens* and *Culex quinquefasciatus*. It is shown that our samples are close to *w*AlbB from other regions, namely, India. It was also observed that there is a clear divergence between our samples and *Wolbachia* endosymbiont of *Ochlerotatus cantans*, even though, both belong to supergroup B. *w*AlbA strain is closely related to *w*AlbA strain from India and *w*AlbA from *Ae. aegypti*. *w*AlbA strain also appears more closely related with non-*Ae. albopictus* strains albeit with lower bootstrap confidence.

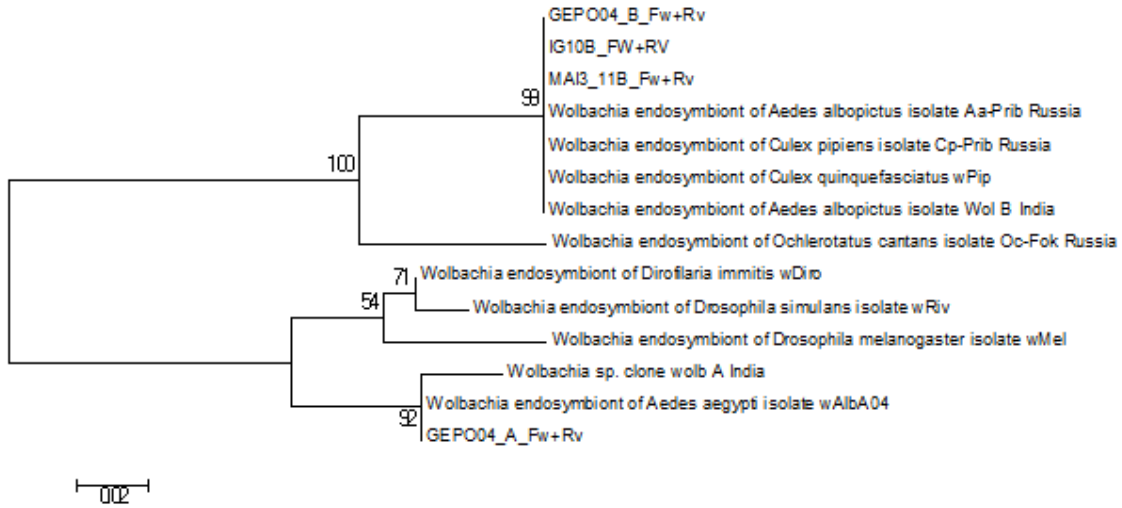

Figure 6 – Phylogenetic analysis of *Wolbachia*. Phylogenetic tree based on *wsp* sequences and constructed by Maximum-Likelihood algorithm. The number on the nodes indicates the bootstrap values of 1000 replicates

The comparison of the obtained WSP sequences with the HVR profiles through the PubMLST database showed that Europe's *w*AlbA strains shared the HVR1:1, HVR2:1, HVR3:1, and HVR4:1 allele whereas *w*AlbB shared the HVR1:10, HVR2:82; HVR3:10, and HVR4:84 alleles. These results confirm the circulation of *w*AlbA and *w*AlbB in *Ae. albopictus* further confirming the phylogenetic results.

DISCUSSION AND CONCLUSIONS

WOLBACHIA GENOTYPING

This work shows that there is a widespread distribution of *Wolbachia* infection in *Ae. albopictus* across the European continent. This was an expected result since *Ae. albopictus* is naturally infected by these bacteria. Other insect species such as *D. melanogaster*, *C. pipiens*, and other organisms such as nematodes like *Dirofilaria* are also naturally infected with *Wolbachia*. Two strains (*w*AlbA and *w*AlbB) (19–23) infect the European populations of *Ae. albopictus*. Furthermore, there are three possible types of infection in *Ae. albopictus*: double infection with both strains, single infection with *w*AlbA, or single infection with *w*AlbB. Globally, double infection is the most prevalent type of infection (9,19–23,51,76–79) which was also observed in this work. This can be explained by various factors, namely, the occurrence of high frequencies of double infection within a species which can lead to a detection bias (6). This observation suggests double infection confers an evolutionary advantage to the mosquitoes (19,20). It may also cause higher rates of speciation or lower rates of extinction (8). Therefore, the loss of one of the strains, particularly, the B strain, may have some evolutionary impact suggesting that the transmission of *w*AlbA+*w*AlbB will, probably, be maintained in the populations (20).

Another fact observed in this work and others (19) is the higher prevalence of double infection in females over males. The reasons why this happens may be related to the way *Wolbachia* is transmitted. Kittayapong *et al* (80) report a high rate of maternal transmission of *Wolbachia* double infection. However, the researchers detected that some of the *Ae. albopictus* offspring was only singly infected, showing that there may be an imperfect transmission which can explain the lower prevalence of double infections in males. Another possible reason might be related to the importance of females for the *Wolbachia* transmission as males do not transmit *Wolbachia* to the offspring. Nevertheless, this phenomenon requires further investigation. Larvae also showed a lower rate of double infection when compared to females, which may be caused by the rise of the density of the strain *w*AlbA during the development of the mosquito that has a lower density during the larval stage (23). However, due to the smaller quantity of larvae collected and because there were adults and larvae collected in only one location, it is

necessary to be careful when concluding this fact. Thus, it would be important to analyze more larvae and compare their rate of infection with adults from the same location to confirm and better conclude whether females have a higher rate of infection than larvae or not.

It was also observed in this work a higher rate of infection of *w*AlbB strain in comparison to *w*AlbA strain corroborating the findings of Shaikevich *et al.* (22) and Tortosa *et al.* (23) made in Europe. In some regions from Asia, namely, Thailand and Orissa region in India, it was found a higher rate of infection of *w*AlbA in comparison to *w*AlbB (20,53). These differences are particularly observed in males. The lower detection of *w*AlbA, particularly in males, might be due to the decrease of *w*AlbA density that is lost after 5 days of emerging in the mosquito which suggests the loss of *w*AlbA or the decrease of its density might be adaptive (22). This can be a possible explanation for *w*AlbA not being detected in males, despite females being infected with both strains (19,23). Another reason to explain the lower detection of *w*AlbA is the lower rate of multiplication of *w*AlbA (63). Therefore, the density of wAlbA may be lower than *w*AlbB, which might make it harder to detect (63) and to transmit (80). Tortosa *et al.* (23) also suggested the hypothesis that *w*AlbA is less efficiently transmitted by females to their progeny, making *w*AlbA infected males suffer a fertility reduction because of CI which increases the selective pressure for a density reduction of *w*AlbA.

As expected, very few uninfected samples were detected, probably, because of the *Wolbachia* leakage related to the environmental factors and the effect of overcrowding during larval developmental stages, which have been associated with reduced transmission of *Wolbachia* (63). The natural *Wolbachia* infection may bring some advantages to the host, for example, females might live longer and have a higher level of fecundity than uninfected ones and if there is a compatible crossing, there is a higher egg hatch rate (81).

Joanne *et al*. (51) described that, in Malaysia, the prevalent type of infection changes according to the region. In the case of this study, the results obtained show a uniform distribution pattern, however, not every country or region of the continent was screened. Expanding the study to Central European populations of *Ae. albopictus* could help to confirm this fact. The observed uniformity is possibly explained by the rapid

spread and fixation of *Wolbachia* infection (20,81,82). Indeed, a study was made with a laboratory population of *Ae. aegypti* showed that *Wolbachia* can spread and fixate throughout an uninfected population within seven generations demonstrating the ability of these bacteria to rapidly invade a mosquito population (82). This rapid spread in Europe and the uniform distribution pattern might have resulted due to multiple introduction events at various countries of the Mediterranean basin, involving the intermixing of the various populations through interpopulation mating, leading to a uniform pattern of *Wolbachia* double infection throughout Europe (39,49,50). As referred above, double infection can be transmitted with efficiency and as *Wolbachia* is transmitted maternally, facilitating the spread of double infection after the multiple introduction events (20,80–82). Another phenomenon that might help explain this fact is CI, since when infected female mate with infected male, they are still compatible, facilitating the spread of *Wolbachia* infection (60).

PHYLOGENETIC ANALYSES

Two strains of *Wolbachia* (*w*AlbA and *w*AlbB) were detected to infect the European populations of *Ae. albopictus*. These results concur with several studies made around the globe (19–23) confirming that, contrarily to *C. pipiens* that harbors a high diversity of strains (18,83), *Wolbachia* infecting *Ae. albopictus* is less diverse. Moreover, according to Ahmad *et al.* (19), Albuquerque *et al.* (78), and Das *et al.* (20), there is a lack of diversity within *Wolbachia* strains infecting *Ae. albopictus* suggesting these strains are stable and highly conserved (78). The lack of detection of diversity within *Wolbachia* strains may be related to the way some studies, like this one, are made using few markers to evaluate the genetic diversity (83). However, two *w*AlbB haplotypes were found when only sequences of this group were analyzed. This was due to the difference in the lengths of the sequences. PCR products from *w*AlbA had a length of 369 bp while *w*AlbB sequences had 501 bp. Consequently, when the sequences were analyzed, the part of the sequence that had the polymorphism within *w*AlbB was not represented in *w*AlbA sequences. This issue might be solved by sequencing a longer fragment of the *wsp* gene, for example, using universal primers 81F and 691R (13) or as in Baldo *et al.* (15) use degenerate primers. These primers allow amplifying a *wsp* fragment of a specific group even if the individual is double infected with both strains, *w*AlbA, and *w*AlbB. However,

sequencing the PCR products will involve the cloning of the products and sequencing with M13 forward and reverse primers (21). Another way of solving this problem is designing new primers so that the primers amplify fragments of *wsp* from *w*AlbA and *w*AlbB strains with the same length. Another solution is to amplify another gene that allows distinguishing between *Wolbachia* strains but that does not require two sets of primers that amplify fragments with very different sizes, like *ftsZ* or *groE* (84). Nevertheless, the solutions referred to above also involve cloning. Another possible solution would be to use MLST or Whole Genome Sequence (WGS) (15,85). Further studies are needed to understand the reason why there is so little *Wolbachia* diversity among *Ae. albopictus* populations.

CONCLUSIONS

The European populations of *Ae. albopictus* have a high prevalence of *Wolbachia* infection. These populations are infected with two strains of *Wolbachia* (*w*AlbA and *w*AlbB); therefore, it is possible to conclude that *wsp* can be used to identify the infecting strains of *Ae. albopictus*. The geographical pattern of distribution of *Wolbachia* infection among the various countries analyzed is homogenous. The type of infection most prevalent in all European regions studied is double infection. Moreover, relative to the sex of the mosquitoes and the developmental stage, the double infection with both strains has also the highest rate. This may lead to concluding that double infection confers some advantage to the host. As referred above, within both strains there is either low or no genetic variability. Attending this fact, it might be possible to draw one of two conclusions: Either *w*Alb strains are highly stable and conserved or *wsp* is not the ideal gene to evaluate variability within *Wolbachia* strains.

Wolbachia infection can influence evolutionarily, genetically, and reproductively the mosquito host determining the number of individuals of each population and the characteristics of the respective population. *Wolbachia* can alter host population genetics through its CI-inducing behavior that causes infected females to produce more offspring than uninfected ones (20). The results obtained also permit us to conclude that probably it is not ideal to use *w*Alb strains as a vector control tool for European populations of *Ae. albopictus* since this mosquito is naturally infected with it and double infection is widespread throughout Europe which might complicate the application of a strategy using *w*Alb strains as a vector control tool, namely, through CI.

However, further investigation is needed to better understand the mechanisms of *Wolbachia* infection, and how *Wolbachia* can induce CI and influence the mosquito lifespan and modulate the infection by an arbovirus. Therefore, it would be interesting to a) continue this investigation by expanding the study to central European populations, namely, from Germany, Netherlands, Czech Republic, among others, to have a broader picture of the *Wolbachia* infection and diversity in Europe. b) to understand why there is little diversity within *Wolbachia* strains infecting *Ae. albopictus* by making phylogenomic analyses and evolutionary studies to investigate these characteristics of *w*Alb. c) study the *Wolbachia* interaction with the host and comprehend if there is a difference in this interaction between double infection and single infection since there is a higher prevalence of double infection. This knowledge is of crucial importance because it can help the health authorities to understand the spread of *Wolbachia* infection and therefore, provide knowledge to help the implementation of new vector control strategies and help to predict the results and effects of the implementation of these novel strategies.

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ANNEX 1

Table A1 – Characteristics of the sampling sites

Country	Municipality	GEO Lat	GEO Long	no larvae	no male	no female	Type of trap
Portugal	Penafiel	41,185548	-8,329371	0	0	15	Human bait
Portugal	Loulé	37,090838	-8,092465	0	0	15	Human bait
Spain	Barcelona	41,353508	2,096833	14	0	0	Larval dipping
Spain	Catarroja	39,402944	-0,395514	15	0	0	Larval dipping
Spain	Magaluf	39,506791	2,530729	15	0	0	Ovitrap
Spain	Es Capdella	39,581278	2,473413	15	0	0	Ovitrap
Spain	Badajoz	38,866222	-6,974194	0	5	6	Ovitrap
Spain	Monesterio	38,028444	-6,219306	0	6	9	Ovitrap
Spain	Almaraz	39,791167	-5,695806	0	3	3	Ovitrap
Spain	Aldea del Cano	39,2899722	-6,329	0	3	4	Ovitrap
Turkey	Igneada	41,877100	27,983500	0	0	15	
Turkey	Aliaga	38,763900	26,944800	0	0	15	
Turkey	Hopa	41,387600	41,437800	0	0	15	
Greece	Chania	35,51816	24,13771	0	15	10	Ovitrap
Greece	Chania	35,53367	24,11715	0	0	5	Ovitrap
Greece	Chania	35,54581	24,14614	0	0	4	Ovitrap
Greece	Chania	35,54036	24,14214	0	0	10	Ovitrap
Greece	Athens(AIA)	37,923603	23,933567	0	6	5	Ovitrap
Greece	Athens(AIA)	37,920436	23,936244	0	0	14	Ovitrap
Greece	Athens(AIA)	37,935464	23,945256	0	1	0	Ovitrap
Greece	Athens(AIA)	37,945808	23,957339	0	0	17	Ovitrap
Greece	Athens(AIA)	37,937186	23,946883	0	7	0	Ovitrap
Greece	Kavala	40,935944	24,395528	0	0	5	Ovitrap
Greece	Kavala	40,925139	24,384111	0	0	5	Ovitrap
Greece	Kavala	40,940056	24,395778	0	0	5	Ovitrap
Italy	Bologna	44,511476	11,386411	0	10	25	Ovitrap
Italy	Bologna	44,484783	11,366584	0	10	19	Ovitrap
Italy	Gemona del Friuli	46,28289	13,138467	5	0	0	Larval dipping
Italy	Gemona del Friuli	46,28327	13,139216	0	0	10	Human bait
Russia	Sochi	43,600471	39,744568	0	0	5	Human bait

Russia	Plastounka	43,676788	39,769154	0	0	5	Human bait
Russia	Maikop	44,618569	40,119033	0	0	5	Larval dipping
Abkhasia	Souckhumi	43,005203	41,024323	0	0	5	Human bait
Abkhasia	Gagra	43,388714	40,044422	0	0	5	Human bait
Abkhasia	Gagra	43,178757	40,294086	0	0	5	Human bait
France	Bischeim	48,611236	7,754512	0	0	5	Resting catch
France	Strasbourg	48,598708	7,735877	0	0	5	Resting catch
France	Hoenheim	48,620438	7,753989	0	0	5	Resting catch
Albania	Durres	41,297042	19,503734	0	21	20	Ovitrap
Albania	Saranda	39,767527	20,002252	0	0	5	Ovitrap
Malta	Luqa	35,860528	14,487028	0	25	25	Baited trap
Bulgaria	Lom	43,80489	23,23634	0	35	19	Ovitrap
France	Saint Martin d'Hères	45,1823	5,7749	0	19	30	Human bait
Serbia	Novi Sad	45,258871	19,818778	0	15	18	Ovitrap
Serbia	Novi Sad	45,251234	19,845873	0	10	7	Ovitrap
Serbia	Loznica	44,531991	19,202970	0	25	25	Baited trap
Slovenia	Ajdovščina	45,887149	13,770997	0	16	24	Human bait
Croatia	Dubrovnik	42,606543	18,226612	0	7	15	Larval dipping
Georgia	Poti	42,147938	41,681530	5	0	0	Larval dipping
Georgia	Batumi	41,604156	41,597897	0	0	10	Resting catch

Table A2 – Rate of *Wolbachia* infections by municipality

Municipality	Total	Infected	wAlbA+B	wAlbB	wAlbA
Penafiel	15	15 (100.0%)	14 (93.3%)	1 (6.7%)	0 (0.0%)
Loulé	15	14 (93.3%)	12 (80.0%)	1 (6.7%)	1 (6.7%)
Barcelona	14	14 (100.0%)	10 (71.4%)	4 (28.6%)	0 (0.0%)
Catarroja	15	15 (100.0%)	8 (53.3%)	7 (46.7%)	0 (0.0%)
Magaluf	15	14 (93.3%)	13 (86.7%)	0 (0.0%)	0 (0.0%)
Es Capdella	15	15 (100.0%)	11 (73.3%)	4 (26.7%)	0 (0.0%)
Badajoz	11	11 (100.0%)	10 (90.9%)	1 (9.1%)	0 (0.0%)
Monesterio	15	15 (100.0%)	14 (93.3%)	1 (6.7%)	0 (0.0%)
Almaraz	6	6 (100.0%)	6 (100.0%)	0 (0.0%)	0 (0.0%)
Aldea del Cano	7	7 (100.0%)	7 (100.0%)	0 (0.0%)	0 (0.0%)
Aliaga	15	15 (100.0%)	14 (93.3%)	1 (6.7%)	0 (0.0%)
Igneada	15	15 (100.0%)	14 (93.3%)	1 (6.7%)	0 (0.0%)
Hopa	15	15 (100.0%)	15 (100.0%)	0 (0.0%)	0 (0.0%)
Chania	44	44 (100.0%)	42 (95.5%)	2 (4.5%)	0 (0.0%)
Atenas	50	50 (100.0%)	48 (96.0%)	2 (4.0%)	0 (0.0%)
Kavala	15	15 (100.0%)	14 (93.3%)	0 (0.0%)	1 (6.7%)
Bologna	64	63 (98.4%)	57 (90.5%)	5 (7.9%)	1 (1.6%)
Gemona di Friuli	15	15 (100.0%)	14 (93.3%)	1 (6.7%)	0 (0.0%)
Sochi	5	5 (100.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)
Maikop	5	5 (100.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)
Plastounka	5	5 (100.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)
Gagra	10	10 (100.0%)	8 (80.0%)	2 (20.0%)	0 (0.0%)
Souckhumi	5	5 (100.0%)	4 (80.0%)	0 (0.0%)	1 (20.0%)
Bischeim	5	5 (100.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)
Hoenheim	5	5 (100.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)
Strasbourg	5	5 (100.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)
Saint Martin d'Hères	49	49 (100.0%)	43 (87.8%)	6 (12.2%)	0 (0.0%)
Durres	41	41 (100.0%)	40 (97.6%)	1 (2.4%)	0 (0.0%)
Saranda	5	5 (100.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)
Luqa	50	50 (100.0%)	41 (82.0%)	9 (18.0%)	0 (0.0%)
Lom	54	54 (100.0%)	49 (90.7%)	5 (9.3%)	0 (0.0%)
Novi Sad	50	50 (100.0%)	42 (84.0%)	8 (16.0%)	0 (0.0%)
Loznica	50	50 (100.0%)	49 (98.0%)	1 (2.0%)	0(0.0%)
Ajdovščina	40	40 (100.0%)	40 (100.0%)	0 (0.0%)	0 (0.0%)
Dubrovnik	22	21 (95.5%)	20 (95.2%)	1 (4.8%)	0 (0.0%)
Poti	5	5 (100.0%)	5 (100.0%)	0 (0.0%)	0 (0.0%)
Batumi	10	10 (100.0%)	10 (100.0%)	0 (0.0%)	0 (0.0%)

ANNEX 2

>*Wolbachia* endosymbiont of *Dirofilaria immitis w*Diro

CAAAAGTTGATGGTATTACCTATACGAAAGACAATAGTGATTACAGTCCAT
TAAAAGCGTCTTTTCTAGCTGGTGGTGGTGCGTTTGGTTACAAAATGGACGA
CATCAGGGTTGATGTTGAAGGAGTTTATTCATACCTAAACAAAAATAATGTT
ACAGATGCAAGATTTATGCCAGATACTATTGCAGACAGTGTAACAGCAATT
TCAGGACTAGTTAACGTTTATTACGATATAGCAATTGAAGATATGCCTATCA
CTCCATATATTGGTGTTGGTGTTGGTGCAGCGTATATTAGCACTCCTTTGAA
AGACGCTGTGAATGATCAAAAAAGTAAATTTGGTTTTGCTGGTCAAGTAAA
AGCTGGTGTTAGTTATGATGTAACTCCGGAAGTCAAACTTTATGCTGGAGCT
CGTTATTTCGGTTCTTTTGGTGCTCATTTTGATAAAGATGCTGCTGCAGGCA
AAGACAAAGGGGAACTCAAAGTTCTTTACAGCACTGTTGGTGCAGAAGC

>*Wolbachia* endosymbiont of *Drosophila simulans* isolate *w*Riv

GATCCTGTTGGTCCAATAAGTGATGAAGAAACTAGCTACTACGTTCGTTTGC
AATACAACGGTGAAATTTTACCTCTTTTCACAAAAATTGAAGGTATTGAATA
TAAAAAGGCCACAGACATTCATAATCCATTAAAAGCATCTTTTATAGCTGGT
GGTGGTGCATTTGGTTACAAAATGGACGACATCAGGGTTGATGTTGAAGGG
CTTTATTCACAGCTAAACAAAAATGATGTTACAGGTGCAGCATTTAACCCAG
ATACTGTTGCAGACAGTTTAACAGCAATTTCAGGGCTAGTTAACGTTTATTA
CGATATAGCAATTGAAGATATGCCTATCACTCCATATGTTGGTGTTGGTGTT
GGTGCAGCGTATATTAGCACTCCTTTGAAAGACGCTGTGAATGATCAAAAA
AGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGTGTTAGTTATGATGTAA
CTCCAGAAGTCAAACTTTATGCTGGAGCTCGTTATTTCGGTTCTTTTGGTGCT
CATTTTGATAAAGATACTGCTGCAGCAAGCAAAGACAAGGGGGAACTCAAA
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>*Wolbachia* endosymbiont of *Drosophila melanogaster* isolate *w*Mel

GATCCTGTTGGTCCAATAAGTGATGAAGAAACTAGCTACTACGTTCGTTTGC
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TAAGAAAGACAAGAGTGATTACAGTCCATTAAAACCATCTTTTATAGCTGG
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GCAAATACTATTGCAGACAGTGTAACAGCAATTTCAGGATTAGTGAACGTG
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GTGCTAATTTTGATGGAAAAAAAACAGATCCTAAAAATTCAACCGGACAGG
CTGCTGATGCAGGCGCATACAAAGTTCTTTACAGCACTGTTGGTGCAGAAG
CTGGAGTAGCGTT

>*Wolbachia* endosymbiont of *Culex quinquefasciatus w*Pip

AAGGAACCGAAGTTCATGATCCTTTAAAAGCATCTTTTATGGCTGGTGGTGC
TGCATTTGGTTATAAAATGGACGATATCAGGGTTGATGTTGAGGGACTTTAC
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GTTGCAAACAGTGTGGCAGCATTTTCAGGATTGGTTAACGTTTATTACGATA
TAGCGATTGAAGATATGCCTATCACTCCATACGTTGGTGTTGGTGTTGGTGC
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TGTTGGTGCAGAAGCTGGAGTAGCGTTTAATTTTT

>*Wolbachia* endosymbiont of *Aedes aegypti* isolate *w*AlbA04

CCAGCAGATACTATTGCGAACAGTTTAACAGCAATTTCAGGACTAGTTAAC
GTTTATTACGATATAGCAATTGAAGATATGCCTATCACTCCATATGTTGGTG
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TCAAAACAGTAAATTTGGTTTTGCTGGTCAAGTAAAAGCTGGTGTCAGCTAT
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TTGGTGCTCACTTTGATAGCGAAACTACTGGTGCAGATAACAAAAAAGTAG
TTACCAAAGATGCATACAAAGTTCTTTACCGC

>*Wolbachia* sp. clone wolb A India

TATTTCGGACGGGTTAACGTTTATTACGATATAGCAATTGAAGATATGCCTA
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AGCTCGTTATTTCGGTTCTTTTGGTGCTCACTTTGATAGCGAAACTACTGGTG
CAGATAACAAAAAAGTATTTAC

>*Wolbachia* endosymbiont of *Aedes albopictus* isolate Wol B India

CTACTATGTTCGTTTGCAATATAATGGTGAAGTTTTACCTTTTAAAACAAGA
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GGGTTGATGTTGAGGGACTTTACTCACAACTAAACAAAAACGACGTTAGTG
GTGCAACATTTACTCCAACAACTGTTGCAAACAGTGTGGCAGCATTTTCAGG
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TACGTTGGTGTTGGTGTTGGTGCAGCATATATCAGCAATCCTTCAGAAGCTA
GTGCAGTTAAAGATCAAAAAGGATTTG

>*Wolbachia* endosymbiont of *Ochlerotatus cantans* isolate Oc-Fok Russia

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GCTCGTTACTTCGGTTCTTATGGTGCTAGTTTTGATAAGGCAACTAAGGATG
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>*Wolbachia* endosymbiont of *Culex pipiens* isolate Cp-Prib Russia

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AAGCTGGTGTTAGTTATGATGTAACCCCAGAAATCAAACTCTTTGCTGGTGC
TCGTTATTTTGGTTCTTATGGTGCTAGTTTTAATAAAGAAGCAGTATCAGCT
ACTAAAGAGATCAATGTC

>*Wolbachia* endosymbiont of *Aedes albopictus* isolate Aa-Prib Russia

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TGCAAACAGTGTGGCAGTATTTTCAGGATTGGTTAACGTTTATTACGATATA
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>IG10B_FW+RV

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TGTTGCAAACAGTGTGGCAGTATTTTCAGGATTGGTTAACGTTTATTACGAT
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TTAATAAAGAAACAGTATCAGCTACTAAAGAGATCAACGTTCTTTACAGCG
CTGTTGGTGCAGAAGCTGGAGTAGCGTTTAATTTTTA