Naturally Occurence Of Wolbachia Spp. Infection In Populations Of Aedes Albopictus In The City of Valencia, Spain

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Keywords: Aedes albopictus, Wolbachia, Infection, 16S and wsp genes, Phylogenetic analysis

Posted Date: July 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-41237/v1

Abstract

Background: The presence of *Aedes albopictus* was first reported in Valencia (Eastern Spain) in 2015, having a high sanitary and social impact. Innovative tools for its control include the use of the endosymbiotic bacterium *Wolbachia pipientis*, which suppress *Ae. albopictus* populations. The release of mosquito males infected with the bacteria has proven effective as a control strategy in similar urban areas in Rome, Italy. Before this strategy could be implemented in Valencia, it is crucial to know whether natural local populations of *Ae. albopictus* are infected with *Wolbachia pipientis* and if so, identifying the bacteria strains/supergroups.

Methods: Eggs were collected from water basins from the 19 districts of the city of Valencia between May and October 2019. A total of 50 *Ae. albopictus* reared mosquito adults were processed and analyzed for the presence *Wolbachia* by selected markers for *16S* rDNA and surface protein (*wsp*) genes, under optimized PCR conditions and sequenced.

Results: Our study reveal that 94,7% of the samples carried the endosymbiont, with no differences among sexes. In relation to the *Wolbachia* strains, both wAlbA and wAlbB strains were identified in the mosquitoes analyzed, with some samples carrying mixed infections.

Conclusions: These data provide the first characterization of endosymbionts present in natural populations of *Ae. albopictus* in the Mediterranean area of Spain, and offers relevant information to evaluate the potential use of *Wolbachia* strains in order to achieve mosquito populations suppression through massive releases of males artificially infected.

Introduction

The invasion of *Aedes* (Stegomyia) *albopictus* (Diptera: Culicidae), known as Asian tiger mosquito, has been reported worldwide (1, 2). Its recognized clinical importance lies on its potential to transmit several diseases like dirofilariosis, and different exotic anthroponotic arbovirosis including dengue (DENV), chikungunya (CHIKV), or Zika (ZIKAV) (3). In this context, Mediterranean countries like Italy, France, Croatia and Spain have declared cases of autochthonous DENV, CHIKV and ZIKAV transmission in recent years (2010 and 2018) (4–7), and very recent studies have pointed to the reasons that increase the risk of transmission (8).

Ae. albopictus was first reported in Spain in 2004 (9), and in 2015 was recorded for first time in the city of Valencia (10). In these few years, its control and elimination has become a major goal for municipal public health services of Valencia city. Major control measures include treatment with insecticides of breeding places, community participation, as well as biological strategies. These biological strategies overcome some of the drawbacks of the use of insecticides, namely non specificity against insects, toxicity to humans and environment, as well as the possibility of generate resistances (8, 11).

Among biological strategies, different options have been used, including the release of sterilized males (Sterile Insect Technique, SIT), insect transgenesis techniques, or the use of endosymbionts like *Wolbachia* species (12, 13). In this case, the replacing of natural *Ae. albopictus* populations with a laboratory population carrying the endosymbiont is known as the incompatible insect technique (IIT).

Wolbachia species (Alphaproteobacteria: Rickettsiales) are maternally transmitted endosymbionts, detected in almost 65% of known insect species (14). They cause different reproductive alterations in its host to increase their transmission to the next generation, with cytoplasmic incompatibility (CI) as the most frequent manipulation produced in the insect (15).

Wolbachia has been used for pest and disease vector control since it can not only alter host reproduction, but also blocks virus replication and transmission (16). These endosymbiont-infected populations are being released into the field for two main purposes: population replacement and population suppression. Both approaches rely on the novel *Wolbachia* infection types inducing cytoplasmic incompatibility with the resident mosquito population. Thus, the presence of natural *Wolbachia* infections in mosquitoes may interfere with disease control programs, making population replacement or suppression challenging or even impossible.

The presence of *Wolbachia* in natural populations of *Ae. albopictus* has been widely described (17, 18), were individuals are found frequently infected with two *Wolbachia* strains, wAlbA and wAlbB, corresponding to the A and B supergroups, respectively (19).

Pioneering studies carried out in Italy, described the replacement of those *Wolbachia* strains by the one obtained from *Culex pipiens* (*w*Pip) (20), and very recently, the same group has reported the first field experiment in Europe to assess the capacity of those *Ae. albopictus* populations (in which the natural endosymbiont *Wolbachia* has been replaced with the *Wolbachia* strain from the mosquito *Culex pipiens*), to sterilize wild females (21). These studies provide further support to the IIT as suitable to reduce *Ae. albopictus* populations in the Mediterranean area.

In the present study, as a first step in the evaluation of IIT as suitable in Valencia, Spain, we have investigated the presence and identification of endosymbionts in natural populations of *Ae. albopictus* in the city. Our data reveal that 94,7% of the analyzed samples carried the endosymbiont naturally, with wAlbA and wAlbB strains and some samples with mixed infections.

Materials And Methods

Sampling

Captures of eggs were done through the employment of standard ovitraps (4). Black plastic bowls (0.4 L volume) filled with water (2/3 of capacity), and supplemented with a wooden stick as an oviposition support, were used. Samplings were carried out between May and October, 2019, in 19 districts of the city of Valencia, Spain (Fig. 1; Table 1).

According to published methodological information in similar studies (22), eggs collected in the field were reared in laboratory conditions until adult emergence, and once adult populations reached to 7–10 days of life both males and females were frozen at -20ºC. Species identification and sex separation was definitively confirmed under binocular microscope. Finally, all 50 adult specimens (25 females and 25 males) were maintained individually in a -80ºC freezer until the *Wolbachia* genotyping analyses.

Replicates of all samples are available upon request.

Wolbachia genotyping: DNA extraction, PCR, sequencing and DNA analyses

Individual adult mosquitoes were lysed and homogenized in 200 µL of phosphate buffered saline (PBS) with the help of sterile toothpicks. After centrifugation at low speed (800xg) for 20 min, total genomic DNA of each mosquito was extracted using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Hilden, Germany) following the modified protocol, and using a final elution volume of 100 µL.

Presence of endosymbiotic bacteria of the genus *Wolbachia* was assessed using specific single polymerase chain reaction (PCR) and two molecular markers as previously described (19, 23). Briefly, the presence of the endosymbiont was tested by amplification of a fragment of the *WSP* (*Wolbachia* outer Surface Protein) gene delimited by primers *wsp* 81F and *wsp* 691R as described previously (19, 23, 24). We also performed a PCR to amplify a fragment of the *16S* rDNA (*Wolbachia*-specific primers) with primers *WolbF* and *Wspecr* as recently described by Carvajal et al. (2019)(23).

For the *wsp* gene amplification, we followed the standard *wsp* protocol (24), with some modifications; the number of cycles was increased to 45. In the end, a 25 µL final reaction volume was used consisting of 1X KAPA2G Robust HotStart ReadyMix PCR Kit® (Kapabiosystems, Boston, USA), 0.5 µM of forward and reverse primers, 2 µL of template DNA and PCR-grade water up to final volume. The final thermal profile was as follows: initial denaturation at 95 °C for 3 min; 45 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; final extension at 72 °C for 3 min.

For the *16S* rDNA gene amplification, we used a 25 µL final reaction volume was used consisting of 1X KAPA2G Robust HotStart ReadyMix PCR Kit® (Kapabiosystems, Boston, USA), 0.5 µM of forward and reverse primers, 2 µL of template DNA and PCR-grade water up to final volume. Thermal profiles followed the protocol of Simões et al. 2011 (25), but annealing temperature was reduced to 55 ºC, and the number of cycles was increased to 45 cycles. The final thermal profile, in accordance with the one used for *wsp*, was as follows: initial denaturation at 95 °C for 3 min; 45 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min; final extension at 72 °C for 3 min.

All PCR amplification experiments included positive and negative controls. As a positive control we used a *Wolbachia*-infected *Ae. albopictus*, as well as two *Wolbachia* DNAs (A + B strains) kindly provided by Nuria Busquets and Sandra Talavera, CReSA, IRTA, Universidad Autónoma de Barcelona, Spain. Water was used as negative controls. The products size of each fragment was checked by 2% agarose gel electrophoresis set at 60V for 30 min. The *wsp* primers amplify a DNA fragment ranging from 590–632 bp, depending on the individual *Wolbachia* strain, while the *16S* rDNA fragment is approximately 900 bp. The PCR amplification process underwent two replicates to validate the results. Positive samples of each marker were subjected to sequencing, carried out at Servicio Central de Soporte a la Investigación Experimental (SCSIE)-Universitat de València.

Identity of Wolbachia strains and their positions in phylogroups

All sequences were subjected to the Nucleotide Basic Local Alignment Search Tool (BLAST) (26), and compared to deposited *Wolbachia* sequences in GenBank. Selected sequences of *Wolbachia* defined subgroups were used (sequences M84691.1 and KX155506.1 for subgroups A and B in *16S* gene, respectively; and sequences MF999264.1 and KJ140127.1, for subgroups A and B in the *wsp* gene, respectively) (23). Sequences underwent multiple alignment using MUSCLE in MEGA X (27).

After editing, final lengths used for phylogenetic inference analyses were 512 and 897 bp for *wsp* and 16S rDNA, respectively.

Maximum likelihood (ML) methods in MEGA X were used to analyze phylogenetic relationships using pre-selected parameters, which included Tamura 3-parameter model + Gamma distributed (27). The ML trees were constructed with 1000 bootstrap replicates.

All newly generated sequences were submitted to the GenBank database with accession numbers MT510204-MT510238 for *16S* sequences, and MT569490-MT569515 for *wsp* sequences.

Results

Prevalence of Wolbachia infections

A total of 50 adults (25 females and 25 males) reared in the laboratory were investigated for the presence of *Wolbachia* infections. Of these, 38 samples (20 females and 18 males) were finally analyzed, and 94.7% (36/38) were PCR positives for *Wolbachia* using 16S primers (23) (Table 1). From the 36 samples, 35 gave good quality sequences to be analyzed.

When using a PCR for a fragment of the *wsp* gene (19), 28/38 (73.7%) of samples showed *Wolbachia* infection, and good quality sequences were obtained from 25 samples, with another sample from district 12 (named H2) (Table 1).

Phylogeny of Wolbachia **through** 16S **rDNA sequences**

All sequenced DNA fragments showed high degree of similarity (> 98%) with *16S* rDNA *Wolbachia* sequences in GenBank. Control DNA sequences for markers of wAlbA and B strains were used. As shown in Fig. 2, most of the *16S* rDNA sample sequences (*n* = 25) belonged to supergroup B, with 6 samples belonging to supergroup A, with the remaining 4 samples localized as intermediate forms.

Phylogeny of Wolbachia **through** wsp **sequences**

All sequenced fragments showed high homology to *wsp* genes sequences in GenBank (> 94%). We used control markers of wAlbA and B strains, and the tree obtained with the ML method rendered 18 samples in supergroup B, 7 samples in supergroup A, and the remaining sequence as intermediate forms (Fig. 3). Interestingly, samples ALB12W1H and ALB12W1H2 were located in different clades, being located the former with supergroup A, and the latter in supergroup B.

Comparison of 16S **rDNA and** wsp **for** Wolbachia **detection**

A total of 28 individual samples (73.7%) yielded positive amplification in both markers (Table 1).

In the *16S* rDNA positive samples (*n* = 36), there were 28 samples with successful *wsp* amplification, while 8 had no successful *wsp* amplification. From those, only good quality sequences were obtained in 25 samples (three samples presented mixed sequences, unable to read: ALB2WOL1M, ALB4WOL1M and ALB7WOL1H) (Table 1).

Interestingly, six samples were confirmed as belonging to the wAlbA strain with both markers (Figs. 2 and 3; Table 1). From the four sequences considered intermediate with the *16S* gene, only one (ALB9WOL1H) was also considered as an intermediate form by the *wsp* gene sequencing. Two *16S* sequences considered to belong to the supergroup B, showed to belong to supergroup A when analyzed for the *wsp* marker (ALB10WOL1H and ALB13WOL1H), indicating a mixed infection in those samples, as it could be also the case for samples ALB4WOL1M (with no clean sequence in *wsp* marker, in the only sample analyzed from a male mosquito), and sample ALB12WOL1H (mixed strains in the *16S* sequence, and belonging to the A supergroup by the *wsp* marker) (Table 1). Two other mixed infections detected by the *16S* marker (ALB2WOL1M and ALB8WOL1H) rendered either no sequence or corresponded to supergroup A by the *wsp* marker, respectively (Table 1).

In relation to sex, most of the mosquito females carried wAlbB strains (50%), with also A strains (27.7%) and mixed A + B infections (22.3%). In the case of mosquito males, 83.3% of the samples carried only wAlbB strains, with two samples carrying A + B mixed infections (11%), and one sample carrying only the wAlbA strain.

Overall, our data confirm the presence of both *Wolbachia* strains even in the same mosquitoes isolated in Valencia, Spain.

Discussion

Aedes albopictus is considered a competent vector for at least 23 arboviruses (28). Among them, the situation of DENV is particularly worrying since this arbovirus has emerged as the most important viral mosquito-borne disease globally in recent years. In Southern Europe autochthonous DENV transmission episodes have occurred on several occasions (29), with a recent detection of *Ae. albopictus* mosquitoes infected with DENV in Spain in 2015 (the first detection in Europe) (30). Arbovirus surveillance is extremely important to identify possible foci of transmission, and this relies on multidisciplinary activities, including community engagement to early detect the mosquito and control its population, especially in urban areas (30).

There is a great consensus among the scientific community about the need to search for new, innovative and complementary mosquito control methods in urban environments. Traditional methods employed to reduce *Aedes* populations include source reduction, public education and insecticide application routinely implemented by hundreds of municipalities across Europe (31). However, in some cases success can be limited due to low levels of communities' participation, lack of coordination between different administrations and bad practices in insecticides applications. Moreover, insecticide resistance cannot be discarded as an additional problem to achieve optimal results on *Ae. albopictus* control in Europe (32).

Population suppression of *Aedes* mosquitoes through the implementation of *Wolbachia* strategy is considered a method of high impact on resistance management (33). Some researches support the use of *Wolbachia pipientis* (wPip) infected *Ae. albopictus* strain (ARwP) males for autocidal suppression strategies against the Asian tiger mosquito (34).

Before those initiatives could be implemented, the precise identification of the *Wolbachia* strains present in wild *Ae. albopictus* populations in those areas is required to prevent unexpected effects like inefficient loss of compatibility (35). With this aim, we collected and analyzed mosquito samples from the 19 districts of the city of Valencia, Spain.

To our knowledge, this is the first report and characterization of natural *Wolbachia* infections in wild populations of *Ae. albopictus* in Spain, and one of the first studies in the Mediterranean area, including Italy (36). Since the first report of *Ae. albopictus* in Spain in 2004 (9), it has been spread from Catalonia to the rest of the Mediterranean area including the Balearic islands (37), as well as other inland and northern regions in Spain such as Extremadura and Basque Country (38).

Our data reveal a high rate of *Wolbachia* infection in the wild populations of *Ae. albopictus* (94.7%), which i.s similar to the prevalence recently found in Asian countries like China (93.3%) (39), Thailand (100%) (40), or Korea (99%) (41), as well as in the US (95%) (42), and Brazil (99,3%) (43), and far from the low

prevalence found in Mexico (a median of 38%) (44). Very few reports have been published in the Mediterranean countries, with great differences found in different locations in France (metropolitan and Corsica) (35, 45), and Greece (35).

Based on our phylogenetic studies, only wAlbA and wAlbB supergroups were found in our analysis, with wAlbB being predominant (25/35, 71.4%), with only few individual samples of wAlbA, confirming previous reports (39). Interestingly, we detected both strains in a single mosquito, confirming previous findings (19, 40). Our phylogenetic analysis also detected "intermediate" (mixed) *Wolbachia* infections, some of them confirmed when sequences from both markers were analyzed (i.e. samples ALB10WOL1H and ALB13WOL1H belonged to different supergroups depending on the marker used). The presence of different strains in the same geographical location also supports the presence of mixed infections, and suggest possible crossing between mosquitoes carrying different *Wolbachia* strains.

Regarding to sex, most of the mono-infections were detected in males (88.8%), mostly with wAlbB strain (only one mosquito male showed wAlbA infection). Mosquito females presented more mixed infections than males (22.3% vs 11%), and half of the single infected samples carried wAlbB strains. These results are in agreement with previous reports either in field or laboratory studies, suggesting a recent invasion and spread of *Wolbachia* in mosquito species (21, 35, 36, 46).

The recent studies by Caputo et al. (2020) (21) using ARwP infected males and released in urban areas of Rome (Italy), have confirmed the feasibility of IIT as way of controlling *Ae. albopictus* populations (21). They show promising results characterized by only 30% of females collected in the release spots, showing 100% sterility, and 20% showing strongly reduced fertility compared with control sites (21). Regarding to ARwP males longevity, they survived up to 2 weeks after release, which is considered adequate for the preservation of reproductive fitness in males, and very similar to wild Asian tiger mosquito males longevity in normal situations in the field (47). These results are in the same line that those from Calvitti et al. (2009) (48), since they found no differences between uninfected and infected males (ARwP) of *Ae. albopictus* with respect to longevity, mating rate, sperm capacity and mating competitiveness in laboratory condition and greenhouses.

Finally, it is important to remark which is the current *Wolbachia* situation according to Biocides regulation in Europe. Any products which is used to control unwanted organisms, including mosquitoes, that are harmful to human or animal health or to the environment, or simply can cause damage to human activities, should be registered in Europe as a biocide according to European Directive of biocides (Directive 98/8/EC) (49). After collecting information about the use of *Wolbachia* as a potential biocide in Europe, the European Commission has recently approved its use stating as follows: The bacteria of the genus *Wolbachia*, or any preparation containing those bacteria, used for the purpose of inoculating those bacteria into mosquitoes, with the objective of creating non-naturally infected mosquitoes for vector control purposes, shall be considered a biocidal product; while non-naturally infected mosquitoes, irrespectively of the infection technique used, shall be considered neither a biocidal product nor a treated article" (49). Considering these regulations, now there is a clear route for the introduction process of *Wolbachia* into the vector control programs of Europe according to European laws. In this context, the City Council of València is supporting an initiative regarding the implementation of *Wolbachia* strains for the control of *Ae. albopictus* populations which will be pioneering in Spain.

Declarations

No **Ethics approval** and consent to participate or consent for publication was required.

The authors declare that they have **no competing interests**.

Funding was obtained from the City Council of València (Ref. E-02401-2019-001377-00), and the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions: AM, FQL and RB designed the study; RB provided the samples; MT and AM analyzed the samples. AM and RB wrote the manuscript.

All authors read and approved the final manuscript

Authors want to thank to technicians of the Universitat de València and Lokímica SL, for their collaboration in the analysis and field collection of samples, respectively.

Dr. Nuria Busquets and Dr. Sandra Talavera, from Animal Health Research Center IRTA-CReSA (Barcelona, Spain) are thanked for provide guidance and positive control samples.

This work was carried out within the framework of AIM-COST Action CA17108.

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Tables

Table 1: Demographic profile (sex, site code, location), detection status (16S rDNA and wsp) of all individual adult *Aedes albopictus* mosquitoes used in the st

Sample	Sex	Site Code	Coordinates		16S rDNA Amplification			Sequence	16S rDNA Supergroup		*wsp* Amplificatio	
			Lattitude	Longitude	1	2	Basis			GenBank Acc. No.	1	2
ALB1W0L1H	F	D1	39.4727	-0.3695	positive		positive	ok	A	MT510204	positive	
ALB1W0L1M	M	D1	39.4727	-0.3695	positive		positive	ok	B	MT510205	negative	positi
ALB2W0L1H	F	D2	39.4589	-0.3759	positive		positive	ok	A	MT510206	positive	
ALB2W0L1M	M	D2	39.4589	-0.3759	positive		positive	ok	A+B	MT510207	negative	positi
ALB3W0L1H	F	D3	39.4769	-0.3867	positive		positive	ok	A	MT510208	positive	
ALB3W0L1M	M	D3	39.4769	-0.3867	negative	negative	negative				negative	
ALB4W0L1H	F	D4	39.4916	-0.4036	positive		positive	ok	B	MT510209	positive	
ALB4W0L1M	M	D4	39.4916	-0.4036	positive		positive	ok	A	MT510210	positive	
ALB5W0L1H	F	D5	39.4869	-0.382	positive		positive	ok	B	MT510211	negative	negat
ALB5W0L1M	M	D5	39.4869	-0.382	positive		positive	ok	B	MT510212	positive	
ALB6W0L1H	F	D6	39.4776	-0.3641	positive		positive	ok	B	MT510213	negative	
ALB6W0L1M	M	D6	39.4776	-0.3641	positive		positive	ok	B	MT510214	positive	
ALB7W0L1H	F	D7	39.4667	-0.3958	positive		positive	ok	B	MT510215	positive	
ALB7W0L1M	M	D7	39.4667	-0.3958	positive		positive	ok	B	MT510216	negative	positi
ALB8W0L1H	F	D8	39.4531	-0.4043	positive		positive	ok	A+B	MT510217	negative	negat
ALB8W0L1M	M	D8	39.4531	-0.4043	positive		positive	ok	B	MT510218	negative	negat
ALB9W0L1H	F	D9	39.4446	-0.3944	positive		positive	ok	A+B	MT510219	positive	
ALB9W0L1M	M	D9	39.4446	-0.3944	positive		positive	ok	B	MT510220	positive	
ALB10W0L1H	F	D10	39.4444	-0.3661	positive		positive	ok	A	MT510221	positive	
ALB10W0L1M	M	D10	39.4444	-0.3661	positive		positive	ok	B	MT510222	positive	
ALB11W0L1H	F	D11	39.4666	-0.3267	positive		positive	ok	A	MT510223	positive	
ALB11W0L1M	M	D11	39.4666	-0.3267	positive		positive	ok	B	MT510224	negative	negat
ALB12W0L1H	F	D12	39.4673	-0.34	positive		positive	ok	A+B	MT510225	positive	
ALB12W0L1M	M	D12	39.4673	-0.34	positive		positive	ok	B	MT510226	negative	negat
ALB12W0L1H2	F	D12	39.4673	-0.34	ND						positive	
ALB13W0L1H	F	D13	39.4733	-0.3425	positive		positive	ok	B	MT510227	positive	
ALB13W0L1M	M	D13	39.4733	-0.3425	positive		positive	ok	B	MT510228	positive	
ALB14W0L1H	F	D14	39.4816	-0.358	positive		positive	ok	B	MT510229	positive	
ALB14W0L1M	M	D14	39.4816	-0.358	positive		positive	ok	B	MT510230	positive	
ALB15W0L1H	F	D15	39.493	-0.3815	positive		positive	ok	B	MT510231	positive	
ALB15W0L1M	M	D15	39.493	-0.3815	positive		positive	ok	B	MT510232	positive	
ALB16W0L1H	F	D16	39.4984	-0.3962	positive		positive	ok	B	MT510233	negative	positi
ALB16W0L1M	M	D16	39.4984	-0.3962	positive		positive	ok	B	MT510234	positive	
ALB17W0L1H	F	D17	39.5229	-0.3784	positive		positive	ok	B	MT510235	negative	negat
ALB17W0L1M	M	D17	39.5229	-0.3784	positive		positive	ok	B	MT510236	negative	negat
ALB18W0L1H	F	D18	39.5018	-0.4241	positive		positive	ok	B	MT510237	positive	
ALB18W0L1M	M	D18	39.5018	-0.4241	positive		positive	ok	B	MT510238	negative	negat
ALB19W0L1H	F	D19	39.4222	-0.3396	negative	negative	negative				negative	negat
ALB19W0L1M	M	D19	39.4222	-0.3396	positive		positive				positive	

Figures

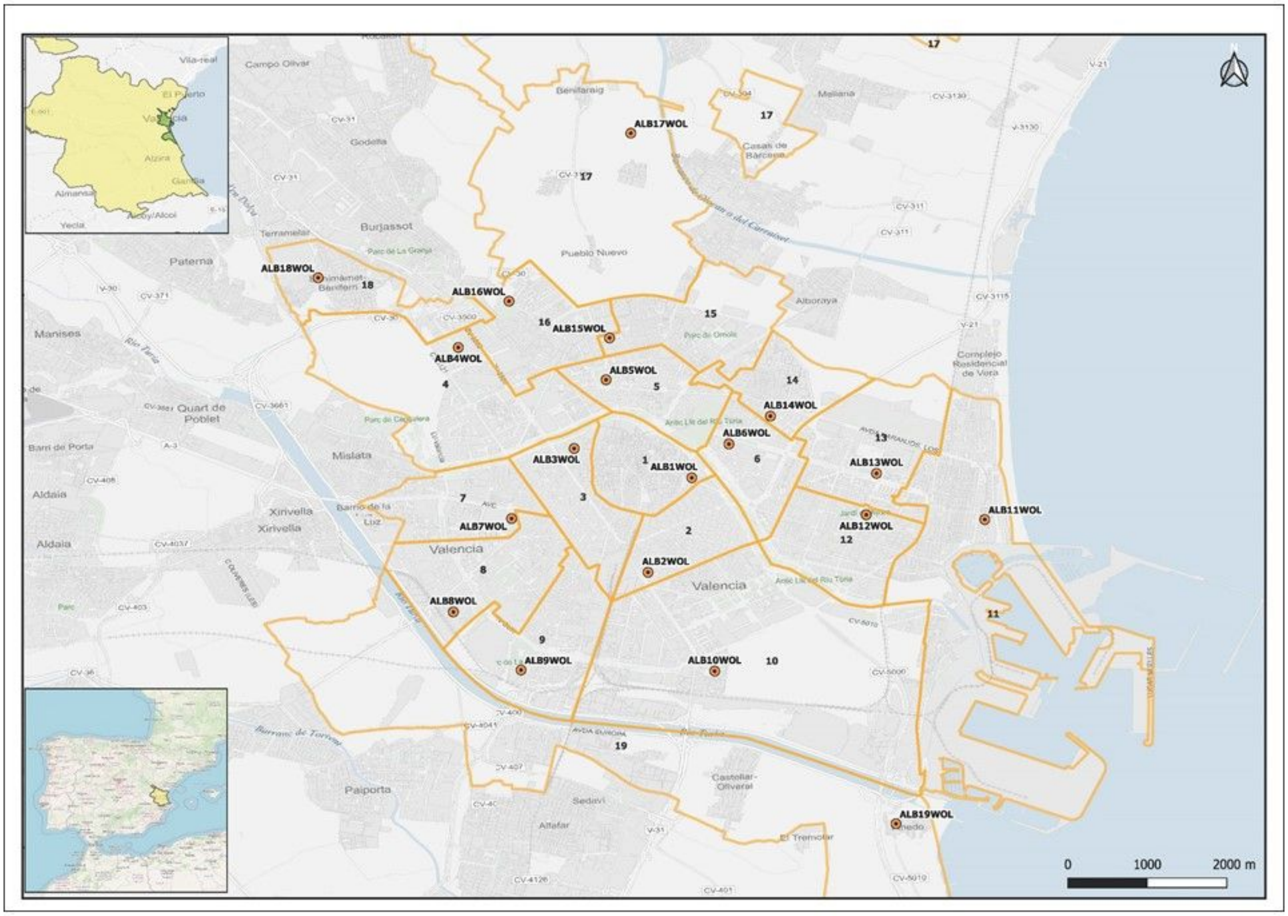

Figure 1

Spatial distribution of the sampling sites (n = 50) for collecting Ae. albopictus eggs in the 19 districts of the city of Valencia, Spain.

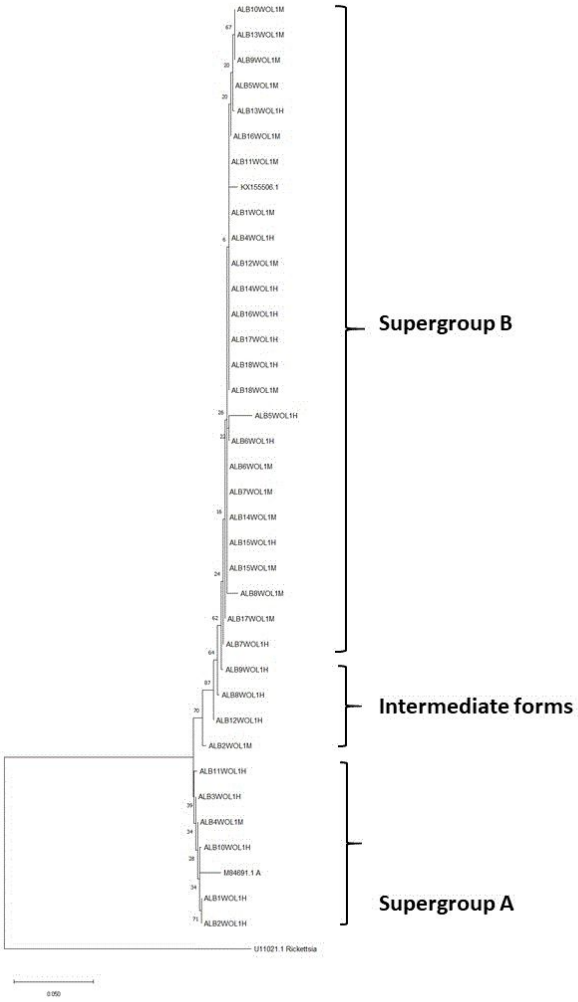

Figure 2

Phylogenic analysis based on 16S rDNA. The alignment was analyzed in using maximum likelihood (ML) methods in MEGA X (27), using Wolbachia pipientis M84691.1 (A supergroup) and KX155506.1 (B supergroup) sequences, as well as Rickettsia rickettsii 16S rRNA sequence (U11021.1) as outgroup.

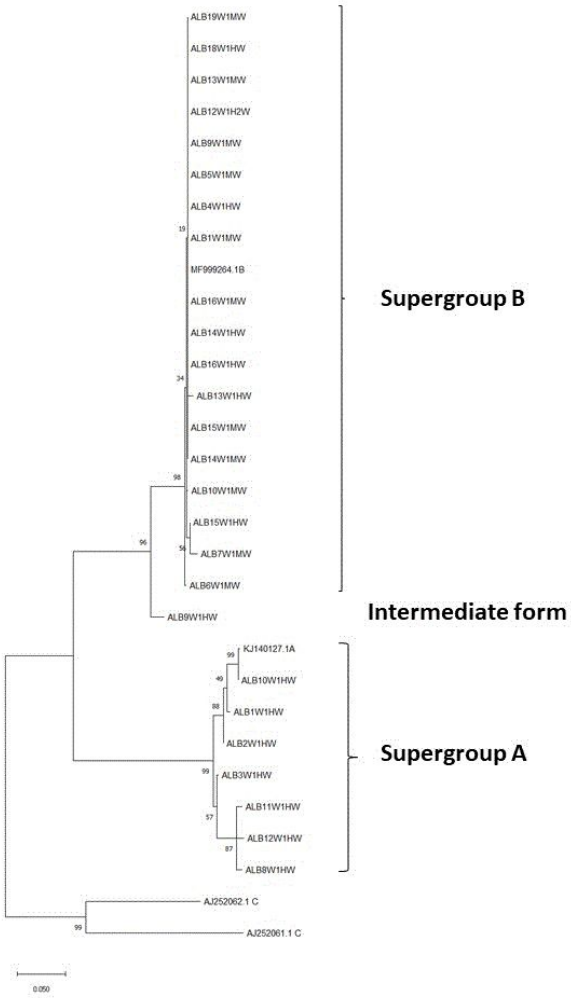

Figure 3

Phylogenic analysis based on wsp gene. The alignment was analyzed in using maximum likelihood (ML) methods in MEGA X (27), using Wolbachia pipientis KJ140127.1 (A supergroup) and M999264.1 (B supergroup) sequences, as well as Wolbachia endosymbiont of Brugia malayi wsp gene (AJ252061.1 and AJ252062.1) as outgroup sequences.