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Molecular Detection, Characterization, and Temperature-Dependence of Wolbachia Infections in Field Populations of *Aedes albopictus* (Diptera: Culicidae) Mosquitoes in Greece

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Abstract: We investigated the prevalence and genetic diversity of *Wolbachia pipientis* strains in *Aedes albopictus* populations in Greece. Using a combination of PCR and Sanger sequencing techniques, we genotyped Wolbachia strains in 105 mosquitoes collected across eight different prefectures in 2021. We found a high prevalence of *Wolbachia* in both male (90%) and female (97%) mosquitoes. Among the infected samples, 84% had double infections with both *w*AlbA and *w*AlbB strains, while 16% had infections with only *w*AlbB. Our comparison of the Multi-Locus Sequence Typing (MLST) profile, employing *gatB-coxA-hcpA-ftsZ-fbpA* genotyping, revealed a single MLST profile for each *w*AlbA and *w*AlbB strain in Greek populations. The same MLST profiles were also reported in populations from China, Russia, and Argentina, suggesting low levels of global diversity in *w*AlbA and *w*AlbB strains. Furthermore, our results indicated a significant association between temperature and the prevalence of single infections (P = 6.498E-07), with higher temperatures correlating with an increased likelihood of single infections. Although male-bias showed a tendency towards single infections, the effect was marginally non-significant (P = 0.053). These results were confirmed using a bootstrap-with-replacement analysis approach. Overall, our findings offer novel insights into the distribution and species diversity of *Wolbachia* strains in Greek *Ae. albopictus* populations, emphasizing the importance of understanding the short-term plastic and adaptive responses of these organisms to environmental stressors and rapid climate change.

Keywords: Asian tiger mosquito; insect-borne disease; microbial ecology; microbiome dynamics; pathogen vector; MLST typing; *Wolbachia* supergroups

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Introduction

The Asian tiger mosquito, *Aedes albopictus,* is a highly invasive mosquito endemic to Southeast Asia, and its adaptation to urban environments has led to its global spread [1]. The first detection of this mosquito in Greece occurred in Corfu and Thesprotia in 2003/4, and since then, it has been found in nearly every prefecture [2,3]. Due to its ability to transmit many important human pathogens, such as Zika, Chinkungunya (CHIKV), Dengue (DENV- 1, 2, 3, and 4), and *West Nile (WNV) viruses*, the Asian tiger mosquito has attracted substantial research interest *[4,5]*. With traditional use of chemical insecticides raising serious concern about toxic effects on organisms and ecosystems, as well as, about the increasing mosquito resistance, alternative environment-friendly methods for the control of mosquito populations have arisen [6]. The Sterile Insect Technique (SIT), a genetic control method that has been used against agricultural pests since decades, is reconsidered as a promising approach against disease transmitting mosquito species, including *Ae. albopictus,* exhibiting considerable progress towards this goal in recent years [7]. Furthermore, symbiont-based approaches are gaining more interest as promising tools for vector control [8].

Wolbachia pipientis is a Gram-negative, intracellular, endosymbiotic bacterium found in several arthropod species and filarial nematodes. It is classified into 17 supergroups A-R (except G) based on genomic diversity [9]. Supergroups A and B are the most prevalent found in arthropod species [10]. Initial reports proposed that 20% of arthropod species are infected by *Wolbachia* [11,12], but a later meta-analysis suggested that the number is closer to 60% [13]. The empirical evidence regarding the intraspecific prevalence of *Wolbachia* infection seems to follow an "all or nothing" pattern, with either very low (<10%) or very high (>90) infection rates in each species [13]. The spread of *Wolbachia* in arthropod species is closely linked to the manipulations and phenotypes that the bacterium induces in its hosts. The most frequently observed reproductive phenotype is cytoplasmic incompatibility (CI). The maternally inherited *Wolbachia* strain induces a modification during spermatogenesis in males, which can be reversed if a compatible strain from the mated female is present in the embryo. If the strains are not compatible, the eggs will not hatch. It is important to note that a non-infected male is compatible with any infected female. This phenotype explains the ability of *Wolbachia* to establish infections in natural populations with a high prevalence, close to 100% [10,14,15]. In mosquitoes, another important Wolbachia-associated observed phenotype is pathogen interference (PI). Certain *Wolbachia* strains can reduce the replication and transmission of specific viruses and other pathogens. This phenotype has been observed in natural infections and has been most successfully demonstrated through transfections [10,16,17]. [10,16,17] In recent years, *Wolbachia* has emerged as a promising tool for reducing mosquito-transmitted diseases, through the introduction of appropriately infected mosquitoes that can exert CI or PI effects in natural populations [8]. It is widely accepted that careful consideration and thorough research on the effects of a particular *Wolbachia* strain in a specific mosquito species must precede the release of mosquitoes in nature to prevent unintended consequences. Furthermore, extensive screening of natural populations for *Wolbachia* prevalence and the strain composition is an equally important step before any *Wolbachia*-based control effort can be considered [8].

In the natural populations of *Ae. albopictus* tested, mainly in Asia and south America, *Wolbachia* infection is established with a high prevalence (>95%) [18–21]. Two commonly found *Wolbachia* strains in *Ae. albopictus* are *w*AlbA and *w*AlbB, belonging to supergroups A and B, respectively [22]. These strains often co-infect the same individual, although single infections with *w*AlbB, more commonly observed in males, have also been reported [23]. Enviromental factors have been shown to affect the density of the *Wolbachia* strains in *A. albopictus*, with higher temperature and lower nutrition resulting to lower *Wolbachia* density [24]. Genetic diversity of the *w*AlbA and *w*AlbB strains has been studied by the MLST (MultiLocus Sequence Typing) system [25], a typing system based on the polymorphism of five conserved genes (*gatB, coxA, hcpA, ftsZ and fbpA*), in natural populations [25]. Both strains were found stable with *w*AlbA presenting a unique profile and *w*AlbB two very similar ones [18,25–27]. In addition to the naturally occurring *Wolbachia* strains in *Ae. albopictus*, *w*AlbA and *w*AlbB, several *Wolbachia* transfections have been explored in the species, including: (a) stable transfection with the wMel strain from *Drosophila melanogaster*, which showed positive results in blocking DENV and CHIKV transmission [28,29], (b) the *w*Au strain from *Drosophila simulans*, which displayed resistance to Zika and DENV with a moderate fitness cost to the mosquitoes [30], and (c) the *w*Pip strain from *Culex pipiens*, which led to a reduction in field populations when triple-infected (*w*AlbA, *w*AlbB and *w*Pip) (line HC) or single infected (*w*Pip)(line ARwP) male mosquitoes were released in large field trials [31–34].

To date, the prevalence and genetic diversity of *Wolbachia* strains in *Ae. albopictus* populations in Europe remain poorly understood. To our knowledge only one study reports a prevalence of 79% in field population of the Iberian Peninsula, with no information provided about the type of stains (*w*AlbA or *w*AlbB) or genotypic diversity [35]. Apart from a field trial of the IIT method with the ARwP line in Italy [34], no *Wolbachia*-based management practices are currently underway in Europe. Notably, most cases of viruses transmitted by *Ae. albopictus* are not autochthonous [36,37]. However,

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sporadic autochthonous cases of DENV-1 in Italy, Croatia, and southern France [38–41], as well as CHIKV outbreaks in Italy and France [40–44], and WNV cases in Italy, Germany, the Netherlands, France [45–51], have been reported. The frequency of *Ae. albopictus*-transmitted diseases is expected to increase in Europe in the coming years, driven by climate change [52–54], and thus increased efforts should be made to study and control the mosquito populations, including the use of *Wolbachia*-based methods.

In this study, our aim was to elucidate the unknown prevalence of *Wolbachia* in *Ae. albopictus* mosquitoes in Greece. As mentioned, *Ae. albopictus* was first reported in Greece relatively recently (around 2003), and in the subsequent years, its populations have expanded throughout the country [3]. We also aimed to determine the detected strains, *w*AlbA or *w*AlbB, and the occurrence of double and single infections. Additionally, we sought to shed light on the genetic diversity of *w*AlbA or *w*AlbB strains in northeastern Europe by comparing our MLST genotypes with those in other studies. Furthermore, we recorded the sex of the mosquitoes and the temperature environment of the studied populations to provide empirical evidence regarding the association between these factors and the propensity for double or single infections. Our experimental design involved a field sampling campaign across eight different prefectures in Greece between July and September 2021, during which we collected a total of 114 adult *Ae. albopictus* mosquitoes. The sex of each mosquito was recorded, and we employed PCR and Sanger sequencing techniques to detect and genotype the *Wolbachia* strains. To assess the impact of environmental temperature, we collected temperature recordings four times a day for 30 days prior to the sampling date. We then analyzed this data in relation to the observed patterns of occurrence of double/single infections. Overall, our findings provide valuable insights into the distribution and genetic diversity of *Wolbachia* in Greek *Ae. albopictus* populations, Furthermore, they highlight the importance of understanding the organisms' short-term plastic and adaptive responses to environmental stressors and rapid climate change. We anticipate these results to contribute to the development of effective *Wolbachia*-based approaches for controlling mosquito-borne diseases in *Ae. albopictus* in Greece but also in other regions.

Materials and methods

Mosquito sampling

Adult mosquitoes were collected between July and September of 2021, in the frame of standard entomological surveillance of mosquito control projects run in four regions of Greece (Central Macedonia, Western Macedonia, Western Greece and Thessaly). For the entomological surveillance, CDC-style traps were used which were supplied with CO_2 and UV light to attract mosquitoes with a constant CO_2 -release rate of 0.5 l/min. The traps are used for wide-area mosquito control projects all over the country since 2010 and were designed, fabricated, and operated by Ecodevelopment S.A. (https://ecodev.gr/en/ecodev-english/). Random individuals were selected from the hundreds of mosquitoes captured in the traps. These individuals were morphologically identified to species level by scientific personnel of the entomological laboratory of Ecodevelopment using dichotomous identification keys [55–57]. Overall, samples from 8 Greek prefectures were obtained: 10 from Achaea, 15 from Aetolia-Acarnania, 9 from Imathia, 8 from Thessaloniki, 27 from Karditsa, 19 from Kozani, 13 from Pieria and 4 from Serres (Table 1). A total of 105 adult *Ae. albopictus* were analyzed, comprising 74 females (70.5%), 31 males (29.5%) (Supplementary Table 1).

Table 1. Information about the studied *Ae. albopictus* sites, the coordinates of the traps, the sampling dates, and the studied sample sizes. More detailed information is provided in the Supplementary Table 1.

Prefecture (abbreviation).	Trap coordinates	Sampling dates	Collected mosquitoes
Achaea (ACH)	38.314695/ 21.814237	30/08	10
Aetolia-Acarnania (AET)	38.61610755/ 21.3825769; 38.39032018/ 21.85072874	12/07; 11/08	15
Imathia (IMA)	40.536217/ 22.20227	26/08	9
Karditsa (KAR)	39.397834/ 22.070087; 39.37078032/ 21.93258031	27/7; 7/9; 21/9	27
Kozani (KOZ)	40.312092/ 21.822304	16/8; 27/9	19
Pieria (PIE)	40.237544/ 22.582148	30/8	13
Serres (SER)	41.205796/ 23.074921	13/7	4
Thessaloniki (THE)	40.64858/ 22.954067	16/9	8

DNA extraction and Wolbachia detection

DNA was extracted from single *Ae. albopictus* mosquitoes using a modified DNA extraction CTAB protocol [58]. To detect *Wolbachia* infection, PCR was performed using the *wspecF/wspecR* primer pair (5'-YATACCTATTCGAAGGGATAG-3'/5'-AGCTTCGAGTGAAACCAATTC-3') which amplifies a 438 bp fragment of the *16S rRNA* gene [11]. The PCR cycling protocol was as follows: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 5 min [11,59]. The DNA from a *Wolbachia*-infected *Drosophila suzukii* laboratory strain was used as a positive control in each reaction. For DNA quality control, the *12SC* primer pair (*12SCFR*: 5'-GAGAGTGACGGGCGATATGT-3'/*12SCRR:* 5'-AAACCAGGATTAGATACC-CTATTAT-3'), which amplifies a 377 bp fragment of the mosquito *12S rRNA* gene was used. The PCR cycling protocol for this control reaction was: 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min, followed by 72°C for 5 min [60–62]. Both PCR reactions were carried out in a final volume of 15 μl, containing 1X reaction buffer, 2.5 mM $MgCl_2$, 1 Unit of Taq DNA polymerase (BIOTAQ, Bioline), 0.2 mM dNTPs (Jena Bioscience), 0.5 mM of each primer and 1μl of the template DNA. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel using the 100bp DNA ladder PLUS BLUE (GeneOn). Samples were characterized as negative for *Wolbachia* only when they repeatedly failed to give an amplification signal for *wspec* (at least two amplification tests) although producing a strong positive signal for *12S rRNA.*

Strain characterization and MLST genotyping

To characterize the Wolbachia subgroup identity in the samples that were positive of Wolbachia presence, two primer pairs were used: *wAlbA_328F/wsp691R* (5'-CCAGCAGATACTATTGCG-3'/5'-AAAAATTAAACGCTACTC-CA-3') for detection of the *w*AlbA strain, and *wAlbB_183F/wsp691R* (5'-AAGGAACCGAAGTTCATG-3'/5'-AAAAATTAAACGCTACTCCA-3') for detection of the *w*AlbB strain. The primer pairs produce amplicons of 341 bp and 463 bp, respectively. A multiplex PCR was conducted with the following cycling profile: 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by 72°C for 3 min [22]. The PCR reaction was performed in a final volume of 15μl containing 1X reaction buffer, 2.5 mM $MgCl_2$, 1 Unit of Taq DNA polymerase (BIOTAQ, Bioline), 0.2 mM dNTPs (Jena Bioscience), 0.5 mM of each primer and 1μl of the template DNA. The results were analyzed using a 1.5% agarose gel and insects were classified as "double-infected" if they exhibited both *w*AlbA (341bp) and *w*AlbB (463bp) specific bands, or as "single-infected" if they showed only a single band.

MLST genotyping was conducted on 11 selected samples: four (two females, two males) double-infected from THE, three (two females, one male) double-infected and one (male) single-infected from KAR, and one (male) double-infected and two (one female, one male) single-infected from AET. For each individual, five loci of the MLST system, as developed for *Wolbachia* by Baldo et al. (2006) were

PCR-amplified and Sanger-sequenced. For four out of the five loci (*gatB*, *hcpA*, *ftsZ*, *fbpA*) *w*AlbA and *w*AlbB strain-specific primers were used to amplify the respective alleles (Baldo et al 2006). For locus *coxA* the *w*AlbA-specific primers were used to produce the A allele, while universal primers in *w*AlbB single-infected individuals were used to amplify the B allele, since the *w*AlbB-specific primers failed to produce the expected amplicon. The PCR cycling profile was: 94 °C for 2 min, followed by 37 cycles of 94 °C for 30 sec, 55-62 °C for 45 sec and 72 °C for 1.5 min, followed by 72 °C for 10 min (https://pubmlst.org/organisms/*Wolbachia*-spp). The PCR reactions were performed in a final volume of 20μl, consisting of 1X reaction buffer, 1.5mM $MgCl_2$, 1 Unit of Taq DNA polymerase (BIOTAQ, Bioline), 0.2mM dNTPs (Jena Bioscience), 1mM of each primer and 1μl of the extracted DNA (Gerth et al., 2013). The success of each amplification was evaluated using 1.5% agarose gels. The PCR products were cleaned using the ExoSAP-IT™ (New England Biolabs) protocol and Sanger-sequenced in both directions by GENEWIZ (Takeley, UK). The chromatograms were inspected using the Geneious Prime software version 2023.0.4 (www.geneious.com). The allele number for each locus was determined according to the *Wolbachia* MLST database (https://pubmlst.org/organisms/*Wolbachia*-spp). All sequences have been submitted to GenBank (Accession Numbers: OQ985406-OQ985492).

Temperature data collection and statistical analysis

For each sampling location, we calculated the mean temperature from the 30 days prior to the date of sampling. This approach captures the temperature exposure of the mosquito populations over a longer period than the temperature of the day of sampling. Using the latitude and longitude of each trap, we recovered for each day the temperature readings at four time points: 6 o'clock in the morning and in the evening, midday, and midnight. We thus averaged 120 temperature readings for each trap for each sampling event. We thus also accounted for the variability in daily temperature, Temperature data were collected from the OpenWeather platform (www.openweather.org) via its API using the Python package 'requests'. OpenWeather is a widely used weather data provider used by a variety of industries, including agriculture, transportation, and energy.

To explore, per sampling date, the correlation between the mean temperature, the ratio of single to double *Wolbachia* infections, and the sex (female, male) of the infected mosquitoes, we employed the non-parametric Kruskal-Wallis test [63] given the limited number of independent observations (n=11). To further examine the effects of different groups, we performed a post-hoc Dunn test [64]. Additionally, to validate our findings and assess their robustness, we utilized a bootstrap-with-replacement approach. We generated 10,000 bootstrap resamples by randomly selecting observations from the original dataset, allowing for duplicates (i.e., with replacement) in each resampled dataset. Kruskal-Wallis and Dunn tests were then performed in each bootstrap resample to assess the effects of temperature and sex ratio. By employing this approach, we were able to estimate the confidence intervals of the observed correlations and strengthen the reliability of our findings.

Results

Wolbachia prevalence

Out of the 105 *Ae. albopictus* individuals examined, 100 (95%) were found to carry a *Wolbachia* infection. The prevalence of infection in males was 90% (28/31) and in females it was 97% (72/74). The infection rates in each district prefecture were as follows: 90% in ACH (9/10), 87% in AET (13/15), 93% in KAR (25/27) and 100% in IMA (9/9), THE (8/8), KOZ (19/19), PIE (13/13) and SER (4/4) (Table 2; Supplementary Table 1).

Table 2. Results of the sex, the *Wolbachia* infection and the type of infection (single or double) in the studied mosquitoes. More detailed information, such as the date of the samplings and the average temperature, is provided in the Supplementary Table 1.

Prefecture (abbreviation)	No of samples/Sex	*Wolbachia* prevalence	Double infection (*w*AlbA+*w*AlbB)	Single infection (*w*AlbB)
Achaea (ACH)	5♂,5♀	90% (9/10)	89% (4♂,4♀)	11% (1♂)
Aetolia-Acarnania (AET)	9♂,6♀	87% (13/15)	54% (5♂,2♀)	46% (3♂,3♀)
Imathia (IMA)	9♀	100% (9/9)	89% (8♀)	11% (1♀)
Karditsa (KAR)	14♂,13♀	93% (25/27)	68% (4♂,13♀)	32% (8♂)
Kozani (KOZ)	19♀	100% (19/19)	100% (19♀)	0%
Pieria (PIE)	13♀	100% (13/13)	100% (13♀)	0%
Serres (SER)	4♀	100% (4/4)	100% (4♀)	0%
Thessaloniki (THE)	3♂,5♀	100% (8/8)	100% (3♂,5♀)	0%

Wolbachia strain characterization

The 100 *Wolbachia*-infected mosquitoes were screened for *w*AlbA and *w*AlbB. The PCR essay detected a double infection (both *w*AlbA and *w*AlbB) in 84%, while a single infection with the *w*AlbB strain was found in 16% of the infected samples. No mosquitoes infected solely with the *w*AlbA strain were found (Supplementary Table 1). Most female mosquitoes were double infected (68/74 female mosquitoes or 92%), whereas double infection was present in 52% of males (16/31 male mosquitoes). When inspecting the results by prefecture, single infected individuals were only found in four regions: ACH (11%), AET (46%), KAR (32%) and IMA (11%) (Table 2; Supplementary Table 1).

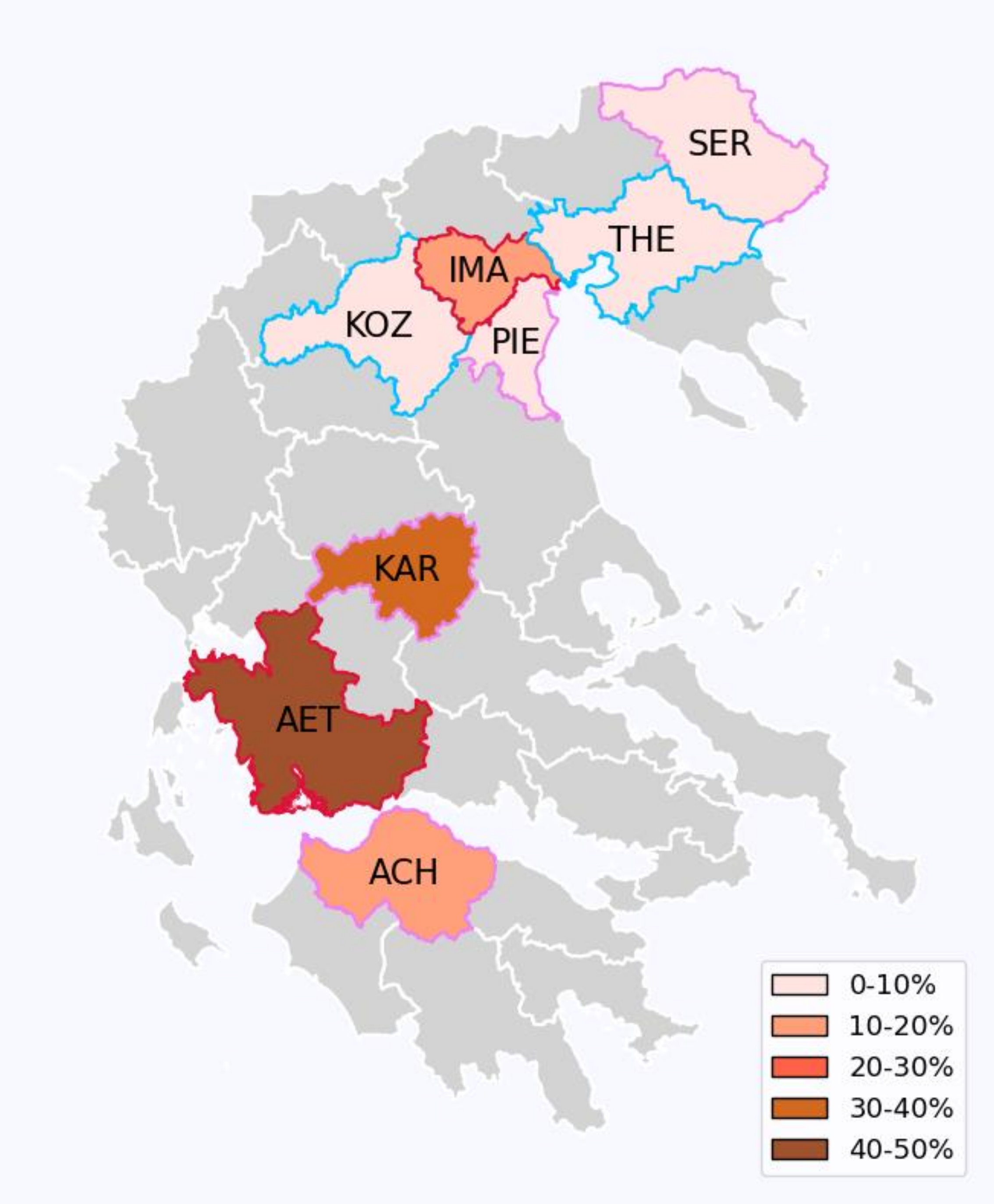

Figure 1. The distribution of Wolbachia proportion of single (wAlbB) to double infection in the natural populations of Ae. albopictus at each of the studied Greek prefectures (colored areas). Prefecture abbreviations are as in Tables 1 and 2. Prefecture colors depict the different single infection proportion levels as shown in the legend. Prefecture boundaries' color indicates average temperature as cyan (23.067 – 25.341°C), pink (25.341 – 27.615°C), or red (27.615 – 29.888°C). These ranges represent equidistant bins across the observed minimum-maximum temperature range. When samples from multiple time points were taken from a prefecture, the overall temperature average was used for this visualization. Additional information is provided in Supplementary Table 1.

MLST genotyping

To gain a deeper understanding of the diversity of *Wolbachia* strains present in *Ae. albopictus* populations in Greece, 11 samples were genotyped by MLST. We genotyped single and double infected samples from both sexes from three different prefectures, attempting to capture possible

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genetic variation and different MLST profiles (if any). The MLST profile obtained for the *w*AlbA strain was identical across all samples. According to the numbering of the *Wolbachia* MLST database the identified alleles were 3, 229, 2, 10, and 3 for the loci *gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*, respectively, presenting four matches with ST code 2. Similarly, the *w*AlbB strain exhibited identical profiles across all samples (alleles 242,229, 166, 210, 27) matching ST code 464. It should be noted that for double-infected individuals the allele for the coxA of the *w*AlbB strain has been assumed to be the same with the one in the *w*AlbB single-infected individuals, given the monomorphic outcome observed for all loci in all samples.

Temperature and sex ratio effect on single Wolbachia infection

The Kruskal-Wallis test revealed a highly significant effect of mean temperature and sex bias on the ratio of single to double *Wolbachia* infection in the studied mosquito populations (P = 1.09E-06; statistic = 27.46). Further analysis using a post-hoc Dunn test distinguished between a highly significant effect of mean temperature (P = 6.50E-07; Figure 2A) and a marginally non-significant effect of sex ratio (P = 0.053). Although the latter may be influenced by the limited number of observations (n = 11), the observed trend appears to be strong (Figure 2B), and the removal of a single outlier value resulted in a significant outcome (P = 0.040). To this end, the bootstrap-with-replacement approach confirmed these observations over 10,000 bootstrap resamples. The mean of P-values for the effect of temperature was P = 2.27E-06, with a high 95% confidence limit of P = 1.69E-05. The mean of P-values for the effect of sex ratio was P = 0.097, with 52.45% of the resamples at P < 0.05 (Figure 3). Overall, these findings suggest a positive relationship between higher environmental temperatures and single *Wolbachia* infections in *Ae. albopictus* mosquitoes, with a negative influence from female sex bias (Figure 2).

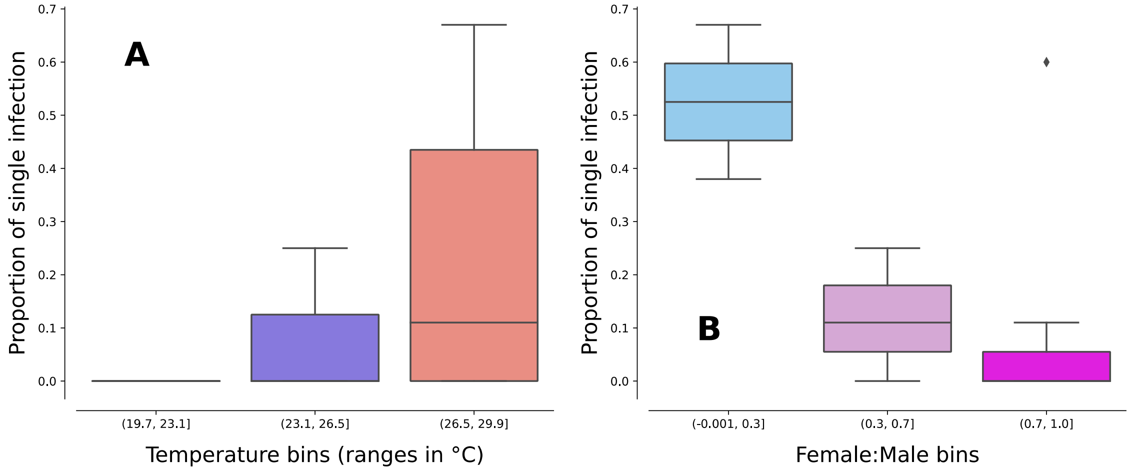

Figure 2. Boxplots showing the distribution of the proportion of mosquitoes infected with single Wolbachia strains across (A) three temperature bins and (B) six female:male sex ratio bins in the studied populations of mosquitoes. The three bins in each cases represent equal splits across the observed min-max range (N = 1, 3, and 8 in temperature bins from lower to higher °C and N = 7, 3, and 2 in sex ratio bins from lower to higher female bias). The lower and upper hinges of the boxes represent the first and third quartiles, while the line inside the box represents the median. Whiskers extend to the lowest and highest values within 1.5 times the interquartile range, and points beyond this range are shown as individual data points. The plot (A) shows that the proportion of single infected mosquitoes increases as temperature increases and the plot (B) shows that the same proportion decreases towards more female-biased sex ratios.

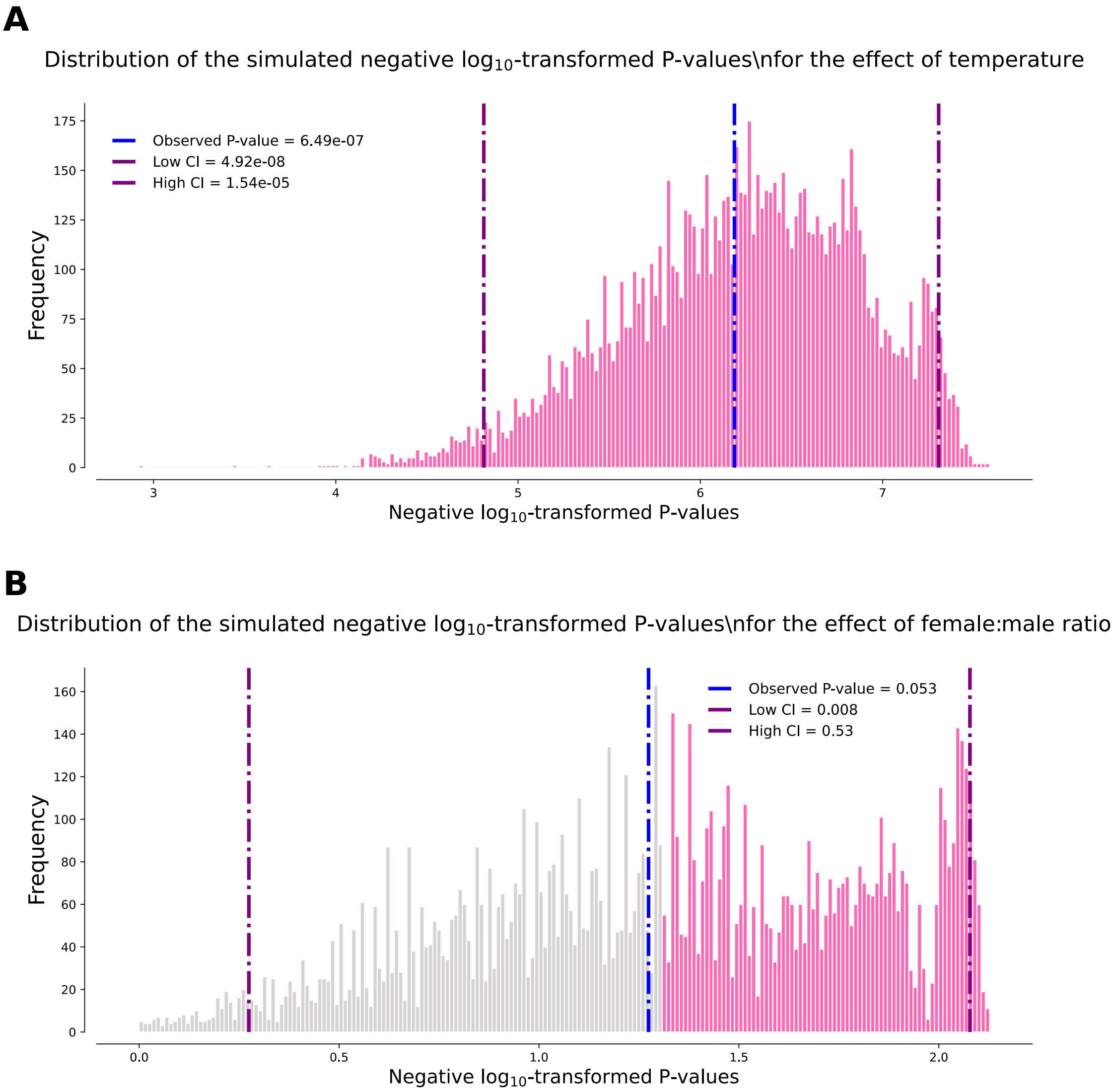

Figure 3. Histograms illustrating the distribution of negative $\log_{10}$-transformed P-values from the simulation analysis (10,000 bootstrap resamples) for the effects of (A) temperature and (B) female:male ratio. Vertical lines indicate the observed significance (in blue), while the 95% confidence intervals of the significance from the simulations are represented in purple. Bars colored in pink correspond to simulations falling below the 5% significance threshold [$-\log_{10}(0.05) = 1.3$].

Discussion

High Wolbachia infection rate in *Ae. albopictus* population in Greece. The high infection rate (95%) in the natural population of *Ae. albopictus* in Greece supports previous studies from other regions and confirms that *Wolbachia* infection has been fixed in *Ae. albopictus* worldwide [13,21,65]. However, we observed slight differences in the infection rates among different prefectures. All the examined prefectures of Northern Greece exhibited an infection rate of 100% unlike the prefectures in central and southern Greece (88% mean rate). Variance in infection rates due to different geographical regions has been previously reported [18]. Understanding the infection dynamics of *Wolbachia* in *Ae. albopictus* holds the promise of establishing biologically based management practices, as the density of the endosymbiont *Wolbachia* plays an important role in crossing sterility (e.g. Werren et al. 1995 [12]).

Low MLST profile diversity for Wolbachia strains in Greek populations and worldwide | The MLST profiles for the *w*AlbA and *w*AlbB strains were monomorphic in the tested Greek populations, which is consistent with the low diversity observed in the MLST genes in the *Ae. albopictus Wolbachia* strains worldwide [18,25–27]. Specifically, the sole identified profile for the *w*AlbB strain (242, 229, 166, 210, and 27 as per the numbering of the MLST database) was also the most common profile identified in almost all populations studied from China, Russia, and Argentina [18,25–27]. There is only one other profile reported for the *w*AlbB strain in China, which differs only in the *gat*B allele [18]. The sole identified *w*AlbA profile (3, 229, 2, 10, and 3) shares identical alleles at four out of the five loci with samples from China and Thailand [18,66]. It only differs in the *coxA* locus, where we detected the same allele (229) as in the *w*AlbB strain. Since it has been proposed that the 229 allele has moved from supergroup-A to supergroup-B through recombination [26], it may be that the supergroup-A-specific primers mis-amplified the 229 allele of the *w*AlbB strain as it is expected to be more abundant in the double infected individuals [67]. Notably, as mentioned in Badieritakis et al. (2018) [3], the Greek populations of *Ae. albopictus* have been found to have some genetic affinity with those of Thailand [68]. As such, our results seem to align with those earlier findings. The Greek populations of *Ae. albopictus* have also been established relatively recently. For example, the first reports in Serres, Aetolia-Acarnania, and Thessaloniki were made between 2007 and 2009 [3]. This recent occurrence of populations may have a role in the observed low MLST diversity due to founder effects and limited time for divergence.

Differential strain distribution of Wolbachia by sex | By examining the double and single infection patterns in relation to the sex of the individual mosquitoes, we noted that almost all female mosquitoes were double infected (92%), whereas only about half of males carried both strains (52%). We observed that in males, the single infection with the *w*AlbB strain is more common in marginally non-significant level (Figure 2B), which agrees with the scientific literature [19–21,23,69]. There are different hypotheses for the detection of both strains in females and only the *w*AlbB strain in males. It is believed that male mosquitoes lose their *w*AlbA infection as they get older as an evolutionary selection of nuclear countermeasures to *Wolbachia* manipulation [23]. Female *Ae. albopictus* carry a fixed infection with both strains, with rare instances of single infected females [19–21,23,69]. The mechanism of CI suggests that females need both strains, to reverse the sperm modification each *Wolbachia* strain induces in males to maximize mating compatibility [10,70]. However, males with a single or no infection remain compatible with double infected females [10]. Male embryos carry both infections since their mothers are almost always double infected, but they tend to lose the *w*AlbA infection as they age. The same pattern is not observed in the *w*AlbB strain. The first detected *Wolbachia* infected *Ae. albopictus* (before 1970) carried only the *w*AlbA strain, indicating that the infection with the *w*AlbB strain is more recent [65].

Differential strain distribution of Wolbachia by environmental temperature | The prefecture-specific infection rates highlighted that non-infected individuals were only found in the three southernmost prefectures examined (ACH, AET, KAR). Furthermore, single-infected individuals were also mainly found in these three prefectures (with one single-infected individual found in Imathia as well) (Table 1). This observation aligns with previous results from China showing that *Wolbachia* prevalence in *Ae albopictus* natural populations is affected by geographical location and climate type [18]. It also agrees with hypotheses that higher temperatures lead to decreased *Wolbachia* density in laboratory populations, affecting both infections but particularly *w*AlbA [24,71]. Our statistical analysis strongly supported this hypothesis, as the effect of the mean temperature from 30 days prior to the sampling date had a highly significant impact on the ratio of single infections in the mosquito populations (P = 6.50E-07; Figure 2A). The mechanistic underpinnings of this observation are not fully understood. Some insight could be provided by studies concluding that maternal transmission under high temperatures and larval density is more often unsuccessful for the *w*AlbA than for the *w*AlbB strain [67,71].Even so, when scoring single- to double-infected individuals it should not be disregarded that the density of the *w*AlbA strain is smaller than that of *w*AlbB in double-infected individuals [18,67] and, thus, it is possible that what appears like absence of the *w*AlbA infection could in fact reflect a reduction beyond PCR detection limits [15,71]. Nevertheless, the above findings raise important points regarding the molecular ecology of the *Wolbachia* infections, which may also be of applied interest.

Conclusions : Altogether: this study provides important insights into the distribution and diversity of *Wolbachia strains* in *Ae. albopictus* populations in Greece and the broader northeastern European region. We report the first MLST genotype information of *Wolbachia* strains in *Ae. albopictus* in Europe and identify a high infection rate in the natural population in Greece, confirming that *Wolbachia* infection has become fixed in populations worldwide. We found a positive correlation between higher environmental temperatures and single *Wolbachia* infections in *Ae. albopictus* mosquitoes, demonstrating that ecological and evolutionary factors play a role in *Wolbachia* dynamics. Overall, this study contributes to our understanding of *Wolbachia* dynamics in natural populations of *Ae. albopictus*. We anticipate that future research with even denser sampling will evaluate our findings, and it will have profound implications for the potential use of *Wolbachia*-based approaches in controlling mosquito-borne diseases in the area.

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