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Wolbachia Screening in *Aedes aegypti* and *Culex pipiens* Mosquitoes from Madeira Island, Portugal

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Simple Summary: Mosquitoes can spread serious diseases like dengue and West Nile virus. On Madeira Island, two mosquito species—*Aedes aegypti* and *Culex pipiens*—are present and may pose a risk to public health. Scientists are exploring new ways to control these mosquitoes using a natural bacterium called *Wolbachia*, which can reduce a mosquito's ability to transmit viruses and even lower mosquito populations. However, for these methods to work, it is important to know first if the mosquitoes in the area already carry this bacterium. In this study, we tested *Ae. aegypti* and *Cx. pipiens* from Madeira for *Wolbachia*. We found that *Ae. aegypti* did not have the bacterium, but *Cx. pipiens* did—all individuals tested were infected. We also found that the *Wolbachia* in *Cx. pipiens* belonged to a group commonly seen in other parts of the world. These results are important because they help us understand which mosquito control strategies might work in Madeira. Specifically, if scientists want to use *Wolbachia* to control *Ae. aegypti* on the island, they would need to introduce it artificially. This information can help improve public health efforts and reduce the risk of mosquito-borne diseases in the region.

Abstract: Mosquito-borne diseases such as dengue and West Nile virus pose serious public health risks. On Madeira Island, the presence of the mosquito species *Aedes aegypti* and *Culex pipiens* raises concerns about local transmission. In this study, we tested 100 *Ae. aegypti* and 40 *Cx. pipiens* mosquitoes to assess the presence and diversity of *Wolbachia*, a naturally occurring bacterium known to reduce mosquito ability to transmit viruses. Molecular identification confirmed that all *Cx. pipiens* specimens belonged to the *molestus* biotype, with 3 individuals identified as hybrids between *molestus* and *pipiens* forms, this is the first evidence of such hybrids in Madeira. *Wolbachia* was not detected in any of the *Ae. aegypti* samples. In contrast, all *Cx. pipiens* mosquitoes were positive, showing a 100% prevalence. Genetic characterization placed these infections within the *w*Pip clade, supergroup B, sequence type 9. These findings provide key baseline data to inform future mosquito control strategies on the island. As *Ae. aegypti* showed no natural *Wolbachia* infection, introducing *Wolbachia*-infected mosquitoes may be necessary to implement such biocontrol approaches in Madeira.

Keywords: *Wolbachia*; *Aedes aegypti*; *Culex pipiens*; Madeira Island; vector control

1. Introduction

Aedes aegypti (Linnaeus, 1762) and *Culex pipiens* (Linnaeus, 1758) are mosquito species of medical and veterinary importance [1,2]. *Ae. aegypti* is recognized as the main vector of several arboviruses – including dengue, Zika and chikungunya [1] – while *Cx. pipiens* is a well-established transmitter of West Nile virus (WNV) [2]. These diseases pose significant public health challenges, with an estimated four billion people at risk of arbovirus infections worldwide [3]. Over the past five decades, the rapid growth of populations, expanding urban areas, increased travel, and the rising resistance to both larvicidal and adulticidal insecticides have collectively driven the spread of mosquito-borne diseases (MBD) worldwide [4]. Recent environmental shifts—such as rising temperatures, urban expansion, and enhanced global mobility—have contributed to the broader distribution and activity of mosquito populations. The number of human cases derived from MBD has risen significantly in Europe, particularly in Central and Mediterranean regions [5]. The spread of *Ae. aegypti* on Madeira Island, Portugal, in 2005 triggered the first dengue outbreak in 2012, underscoring the island's vulnerability to future dengue epidemics [6]. Similarly, the presence of *Cx. pipiens* on the island raises concerns about the potential for local WNV transmission, similar patterns observed elsewhere in Europe [7].

Over the past ten years, *Wolbachia* has emerged as a promising strategy for controlling mosquito-borne diseases [8]. *Wolbachia* is an intracellular endosymbiotic bacterium that lives within arthropods and nematodes and often interferes with host reproduction and/or blocks the transmission of arboviruses such as dengue, Zika, and chikungunya [9]. The most common form of this interference is cytoplasmic incompatibility (CI), in which mating between *Wolbachia*-infected males and uninfected females results in reduced embryo viability. Additionally, in some hosts, *Wolbachia* can induce processes like parthenogenesis (the development of offspring from unfertilized eggs), feminization (transforming genetically male individuals into females), or even the elimination of male embryos [10]. Since *Wolbachia* is maternally transmitted, these reproductive modifications ensure that a greater proportion of females in the population become carriers of the infection. Field trials have explored two main approaches: one aimed at suppressing mosquito populations by releasing infected males, and another aimed at replacing the target population with infected individuals of both sexes [5]. To curb dengue transmission in communities, programs releasing *Wolbachia*-infected mosquitoes are currently underway across multiple countries: United States [11], Brazil [12], Italy [13], Australia [14], Vietnam [15], Indonesia [16], Singapore [17], China [18], and Malaysia [19]. Studies employing this approach have demonstrated significant reductions in dengue incidence, with suppression rates of 40% in Kuala Lumpur [19], over 70% in Yogyakarta [16], and up to 96% in northern Queensland [15]. These findings highlight the potential of *Wolbachia*-based strategies as a sustainable and environmentally friendly alternative for global mosquito-borne disease control.

Despite *Wolbachia*'s proven efficacy in reducing pathogen transmission, there are critical gaps in our understanding of its prevalence and genetic diversity in mosquito populations on Madeira Island. Previous studies have focused on the genetic structure, insecticide resistance, and vector competence of *Ae. aegypti* in Madeira [6,20,21], but no research to date has examined *Wolbachia* prevalence in this population. Given that *Ae. aegypti* populations in Madeira have demonstrated resistance to pyrethroids and organophosphates [21], alternative vector control strategies—such as *Wolbachia*-based approaches—may be necessary to mitigate future arbovirus outbreaks.

This study aims to fill this knowledge gap by screening local populations of *Ae. aegypti* and *Cx. pipiens* for *Wolbachia* infection using molecular techniques, including amplification of the *wsp* gene and MLST to genotype and differentiate *Wolbachia* strains. By determining infection rates and strain diversity, this research will provide essential insights into *Wolbachia* dynamics in Madeira's mosquito populations and inform future vector control programs on the island.

2. Materials and Methods

2.1. Mosquito Collection

Adult *Cx. pipiens* mosquitoes, were collected in August 2023 and August 2024 using BG-Sentinel traps (Biogents, Regensburg, Germany). These traps were strategically placed in urban and suburban areas of Madeira, within the municipal limits of Funchal, where mosquito activity was known to be high. Figure 1 illustrates the geographic location of Madeira Island, with Funchal highlighted as the collection site. Of the collected mosquitoes from each year, 10 males and 10 females from each year were selected, creating a total of 40 specimens. Captured mosquitoes were placed in 0.5 mL Eppendorf tubes that contained RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) and were stored at room temperature for transport. Regarding *Ae. aegypti* mosquitoes, eggs were collected in 2023 using oviposition traps placed beside the BG-Sentinel traps in which the adult mosquitoes were collected. The eggs were transported to the laboratory, where they were hatched under controlled conditions in an insectary, with 28°C temperature and 80% humidity conditions. The emerging adults were kept under the same standard insectary settings. To ensure consistency, we analyzed 50 males and 50 females, all tested within 2-4 days post emergence to minimize any age-related physiological differences.

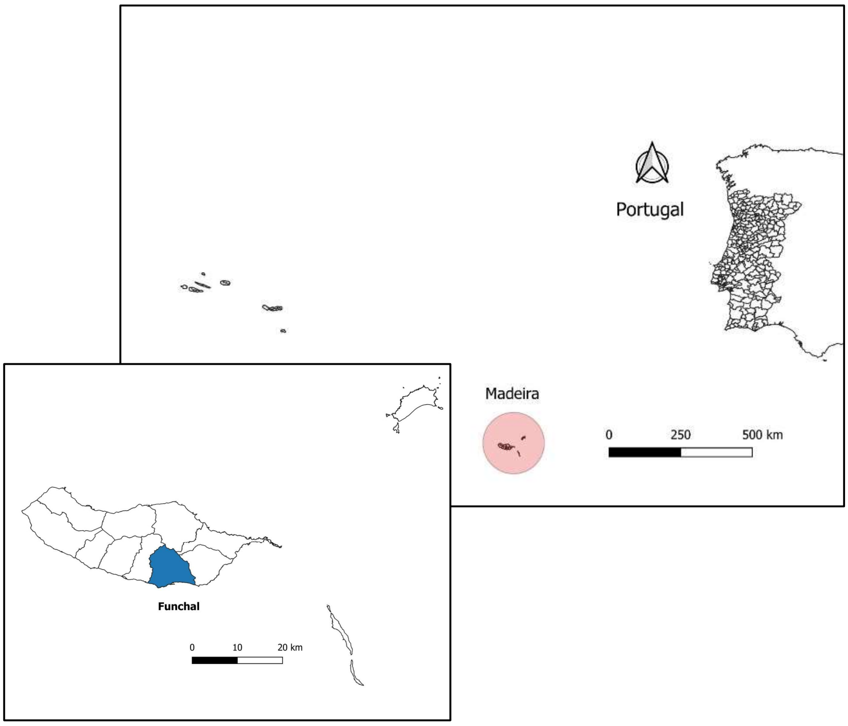

Figure 1. Map showing the geographic location of Madeira Island, Portugal. The highlighted area represents Funchal, where mosquito traps were placed within the municipal limits.

Species identification was conducted under a stereo microscope (SZX7, Stereo Microscope, Olympus LS) using the Ribeiro and Ramos (1999) identification key, allowing precise differentiation of *Cx. pipiens* from other mosquitoes [22].

Whole mosquitoes were used for individual genomic DNA extractions with the NzyTech Tissue gDNA Isolation Kit (NzyTech, Lisbon, Portugal). The extraction process followed the manufacturer's protocol. DNA samples were stored at -20°C until further analysis.

For species identification within the *Cx. pipiens* complex, PCR amplification of the acetylcholinesterase-2 (*ace*-2) gene was performed using the primers ACEpip-F (5′-GGAAACAACGACGTATGTACT-3′), ACEquin-F (5′-CCTTCTTGAATGGCTGTGGCA-3′), and B1246s-R (5′-TGGAGCCTCCTCTTCACGG-3′), following the protocol described by Smith & Fonseca [23]. The PCR reaction mix consisted of 10 µL of NZY Master Mix (NZYTech), 0.8 µM of ACEquin-F and B1246s-R primers, 0.4 µM of ACEpip-F primer, 1 µL of DNA template, and water to a final volume of 20 µL. The amplification protocol included one cycle at 94°C for 5 min, 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 min, and a cycle at 72 °C for 5 min. In this approach, *Cx. pipiens* produces a 610 bp fragment, while *Culex quinquefasciatus* generates a 274 bp fragment, allowing for clear species differentiation [23].

To further distinguish between *Cx. pipiens* biotypes and their hybrids, the CQ11 microsatellite locus was amplified using the forward primer CQ11F2 (5′-GATCCTAGCAAGCGAGAAC-3′) and the reverse primers pipCQ11R (5′-CATGTTGAGCTTCGGTGAA-3′) and molCQ11R (5′-CCCTCCAGTAAGGTATCAAC-3′), following the protocol outlined by Bahnck & Fonseca [24]. The polymerase chain reaction (PCR) mix contained 10 µL of NZY Master Mix (NZYTech, Lisbon, Portugal), 0.5 µM of each primer, 1 µL of DNA template, and water to a final volume of 20 µL. PCR conditions were: one cycle at 94 °C for 3 min, 40 cycles at 94 °C for 30 seconds, 54 °C for 30 seconds, 72 °C for 40 seconds, and one cycle at 72 °C for 5 min.The *Cx. pipiens* has two distinct forms: form *pipiens* and form *molestus*. The different biotypes are represented by the amplification of a 200 bp band for *Cx. pipiens* f. *pipiens*, a 250 bp band for *Cx. pipiens* f. *molestus*. For hybrids of *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus* the two bands specific for each biotype are simultaneously amplified (200 bp and 250 bp).

2.2. Wolbachia Detection

Wolbachia detection in mosquito samples was performed using PCR targeting a 610 bp region of the *wsp* gene using primers 81F and 691R, as described by Zhou *et al.* [25]. The amplification reaction was carried out in a 10 µL total volume, consisting of 5 µL of NZY Master Mix (NZYTech), 0.25 µL of forward primer (10 µM), 0.25 µL of reverse primer (10 µM), 1 µL of DNA template, and 3.5 µL of PCR-grade water. Cycling conditions were optimized as follows: a cycle at 94°C for 2 min, followed by 37 cycles of 94°C for 30 seconds, 54°C for 45 seconds, and 72°C for 90 seconds, and a cycle at 72°C for 10 min.

Amplified PCR products were analyzed via 2% agarose gel electrophoresis, stained with GreenSafe (NZYTech, Lisbon, Portugal), and visualized under UV light to confirm successful amplification.

2.3. Multilocus Sequence Typing

The MLST was performed on *wsp*-positive mosquitoes, targeting five conserved housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*) to characterize *Wolbachia* strains present in the samples [26]. Additionally, the *wsp* hypervariable region (*wsp*HVR) was amplified to provide further strain differentiation. The primer pairs used for each locus, along with the corresponding amplicon sizes, are provided in Appendix A.

PCR reactions were conducted using a Veriti™ Thermal Cycler, (Applied Biosystems, Foster City, CA, USA) following the same reaction mix conditions as for the *wsp* amplification. The thermal cycling conditions were optimized for each gene. For *gatB*, *hcpA*, *ftsZ*, and *coxA*, the cycle consisted of an initial denaturation at 94°C for 30 seconds, followed by 37 cycles of 54°C for 45 seconds and 72°C for 90 seconds. For *fbpA*, the annealing temperature was set at 55°C for 45 seconds, with all reactions including a final extension step at 72°C for 10 min.

All PCR products were analyzed via 2% agarose gel electrophoresis, stained with GreenSafe (NZYTech, Lisbon, Portugal), and visualized under UV light. Successfully amplified DNA fragments were purified using the ExoProStar™ 1-Step PCR Purification Kit (Cytiva, Marlborough, MA, USA) before sequencing.

A total of 20 *Cx. pipiens* specimens (5 males and 5 females from 2023, and 5 males and 5 females from 2024) were selected for sequencing. Bidirectional sequencing was performed for two representative samples from each year to improve sequence accuracy and obtain consensus sequences. Sequencing was carried out using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Raw sequencing reads were quality-checked, and base-calling errors were corrected using BioEdit (version 7.2.5) [27]. Individual loci (*gatB*, *coxA*, *fbpA*, *ftsZ*, *hcpA*, and *wsp*HVR) were aligned and concatenated to generate a complete MLST profile. Consensus sequences were submitted to GenBank, and both individual loci and concatenated sequences were queried in the

Wolbachia MLST database (https://pubmlst.org/wolbachia/) to determine sequence type (ST) assignments.

2.4. Phylogenetic Analysis

Homology searches were performed using the BLASTN algorithm [28] and all partial sequences were aligned with via ClustalW in BioEdit version 7.2.5 [27]. For integration of *Wolbachia* circulating in Madeira Island into the global genetic diversity, the obtained nucleotide concatenated consensus sequences of MLST loci (*coxA*, *gatB*, *ftsZ*, *fbpA*, *hcpA*) and the *w*sp hypervariable region were aligned against multiple sequences available at PubMLST and manually inspected using BioEdit. The obtained nucleotide alignments were further used to build maximum-likelihood phylogenetic trees applying the obtained bestfit model for each alignment using MEGA 11 [29].

3. Results

3.1. Mosquito Morphological Identification

In total, 140 mosquitoes from two genera, *Aedes* and *Culex*, were sampled for testing. Among them, 100 samples were identified as *Ae. aegypti* and were evenly distributed between 50 females and 50 males.

For *Cx. pipiens,* 40 field-collected mosquitoes were morphologically identified and selected for DNA testing. The ace-2 fragment-size analysis provided no evidence of *Cx. quinquefasciatus* in all of the 40 samples, rather all of them had fragments of 610 bp, corresponding to the size of *Cx. pipiens* molecular amplification ID [23,30]. Based on the CQ11 fragment-size analysis, 37 specimens were assigned to *Cx. pipiens* f. *molestus¸* as they all exhibited the 250 bp specific fragment. Additionally, three samples were identified as hybrids[24].

3.2. Wolbachia Screening Through Amplification of wsp Gene

A total of 140 mosquitoes were screened for *Wolbachia* presence by PCR amplification targeting the *wsp* gene. None of the 100 *Ae. aegypti* mosquitoes tested positive for *Wolbachia* presence. In contrast 100% of *Cx. pipiens* samples from both 2023 and 2024 collections tested positive for *Wolbachia* infection.

3.3. Multilocus Sequence Typing (MLST)

All five MLST loci (*gatB*, *coxA*, *ftsZ*, *fbpA*, *hcpA*) and the *wsp*HVR were successfully sequenced for all samples.

Allelic profiles generated from these loci were compared against the *Wolbachia* MLST database (https://pubmlst.org/), confirming that all 20 sequenced *Cx. pipiens* specimens belonged to sequence type (ST) 9, placing them within the *w*Pip clade, associated with *Wolbachia* supergroup B (Table 1). No novel alleles or mixed infections were detected in any of the samples. Phylogenetic analysis using concatenated MLST loci sequences (*coxA*, *gatB*, *ftsZ*, *fbpA*, *hcpA*) and *wsp*HVR supported these findings, reinforcing their classification within the *w*Pip lineage (Figure 2).

Table 1. MLST Allelic Profiles, *wsp*HVR Variants, and corresponding Sequence Types (ST) of *Wolbachia* detected in *Cx. pipiens*.

Sex	Year	*gatB*	*coxA*	*hcpA*	*ftsZ*	*fbpA*	*HVR1*	*HVR2*	*HVR3*	*HVR4*	*ST*
Female	2023	4	3	3	22	4	10	8	10	8	9
	2024	4	3	3	22	4	10	8	10	8	9
Male	2023	4	3	3	22	4	10	8	10	8	9
	2024	4	3	3	22	4	10	8	10	8	9

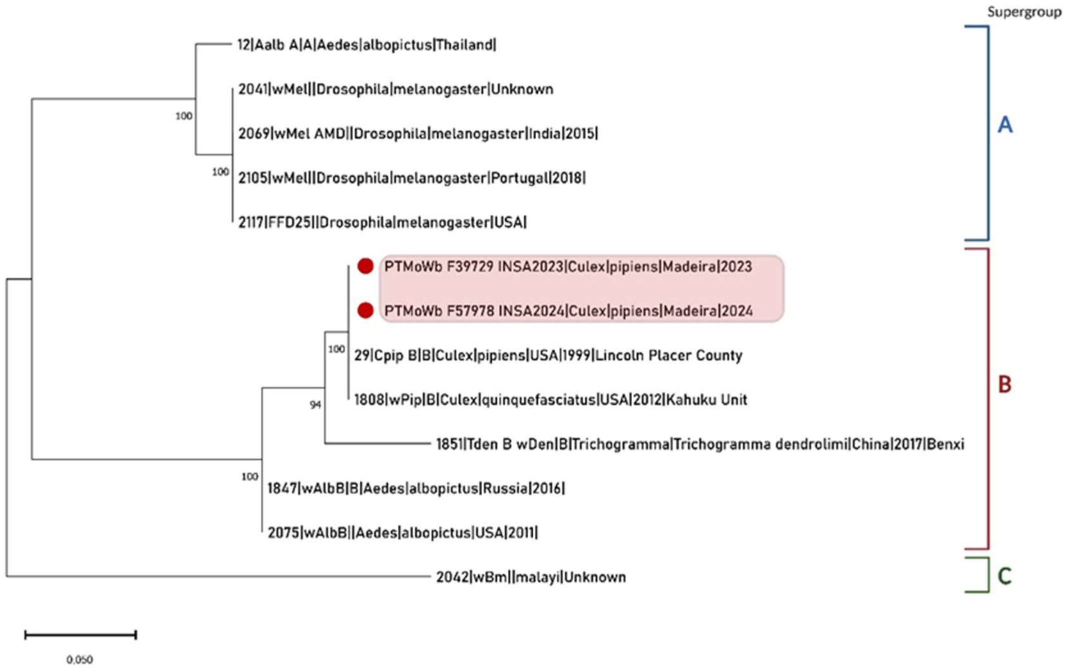

Figure 2. Phylogenetic tree was inferred using Maximum likelihood method and Hasegawa-Kishino-Yano model (with discrete Gamma distribution and some evolutionary invariable sites; HKY+G+I) and 14 (2 novel) COX sequences obtained from mosquitoes circulating in Madeira Island (Table 1) and 12 sequences available in PubMLST. Bootstrap values (1000 replicates) are shown below the branches. *Wolbachia* sequences are identified by PubMLST ID numbers, insect species, country region, country, and year of collection (if available). Colored circles and sequences highlighted in red indicate sequences from Madeira Island. *Wolbachia* supergroups are presented with a different color: blue, supergroup A; red, supergroup B and green, supergroup D. Composite figure was created in https://BioRender.com.

4. Discussion

4.1. Prevalence of *Wolbachia* in *Ae. Aegypti*

The absence of *Wolbachia* in all *Ae. aegypti* specimens analyzed confirms previous studies indicating that this species does not naturally harbor the bacterium [31,32]. While *Wolbachia* has been successfully introduced into *Ae. aegypti* populations for vector control, natural infections are rarely reported [33]. This study provides the first data on *Wolbachia* screening in *Ae. aegypti* populations in Madeira Island and shows relevance for potential *Wolbachia*-based interventions in Madeira, as the feasibility of such strategies depends on the need for artificial transinfection. If *Wolbachia* is to be introduced into Madeira's *Ae. aegypti*, factors such as strain selection, host compatibility, and environmental stability must be considered [34,35].

Although PCR-based detection is highly sensitive, it is still possible that very low-density infections remain undetected [33]. Future studies could complement these findings using quantitative PCR (qPCR) or next-generation sequencing (NGS) to rule out low-level infections [36,37]. Given the confirmed presence of insecticide resistance in Madeira's *Ae. aegypti* populations [20,21], alternative control strategies should continue to be explored.

4.2. High Wolbachia Prevalence in Cx. pipiens

Distinguishing between members of the *Cx. pipiens* complex can be challenging, as the two forms, *pipiens* and *molestus*, are morphologically identical, even in key structures such as the male genitalia. To overcome this limitation, we used molecular tools for accurate identification. The *ace-2* fragment analysis confirmed that none of the tested specimens belonged to *Cx. quinquefasciatus*, as all exhibited the expected 610 bp fragment characteristic of *Cx. pipiens* [23]. Subsequent analysis using the CQ11 microsatellite locus revealed that 37 individuals belonged to *Cx. pipiens* f. *molestus*, while three mosquitoes showed both the 250 bp and 200 bp fragments, clear evidence of hybridization between the *molestus* and *pipiens* forms [24]. To our knowledge, this is the first report of *Cx. pipiens* hybrids in the Madeira Island. The predominance of the *molestus* form suggests that this biotype is well adapted to the island's urban environment, likely aided by traits such as its ability to reproduce without a blood meal (autogeny), to mate in confined spaces (stenogamy), and to remain active year-round without undergoing diapause [24]. These characteristics give this mosquito a clear ecological advantage in human-altered habitats.

This study represents the first molecular screening of *Wolbachia* in *Cx. pipiens* from Madeira Island, revealing a 100% infection rate across both 2023 and 2024 samples. The *Wolbachia* strain detected in all samples belongs to the *w*Pip clade, within *Wolbachia* supergroup B, as confirmed by MLST typing. Similar findings have been reported in other global *Cx. pipiens* populations, supporting the idea that *w*Pip strains form a distinct evolutionary lineage within supergroup B [38–40].

Phylogenetic analysis indicated that *Wolbachia* sequences from Madeira *Cx. pipiens* exhibit high genetic similarity to those found in North America, particularly those associated with ST9. This finding suggests a possible link between Madeira's *Wolbachia* strains and those from geographically distant *Culex* populations. The strong bootstrap support values (94–100%) confirm the robustness of these relationships (Figure 2). Given Madeira's historical role as a maritime trade hub, multiple introduction events of *Wolbachia* in *Cx. pipiens* cannot be ruled out. Future research comparing *Wolbachia* sequences from Europe, North Africa, and the Americas could help clarify whether Madeira's *w*Pip strains originated from a single introduction or multiple independent colonization events.

4.3. Implications for Vector Competence and Control

The detection of *Wolbachia* in Madeira's *Cx. pipiens* has important implications for vector competence and vector control. The presence of hybrids is particularly noteworthy, as interbreeding between forms with different host preferences, the *pipiens* form tending to feed on birds and the *molestus* form on mammals, including humans, may lead to mosquito populations with expanded host ranges and potentially greater capacity to transmit zoonotic pathogens like WNV [24].

Studies have shown that *Wolbachia* can influence pathogen transmission in *Cx. pipiens*, particularly for WNV and filarial nematodes [41,42]. However, the impact of *Wolbachia* on WNV transmission is complex, with some studies reporting reduced viral replication, while others suggest it may enhance pathogen transmission depending on the specific *Wolbachia* strain-host interaction [43]. Understanding how *Wolbachia* affects WNV dynamics in Madeira's *Cx. pipiens* is critical for assessing its role in arbovirus epidemiology.

Given the high prevalence of *Wolbachia* in Madeira's *Cx. pipiens*, the Incompatible Insect Technique (IIT) could be a viable tool for population suppression or modification. IIT relies on *Wolbachia*-induced cytoplasmic incompatibility to reduce mosquito fertility, effectively lowering population densities over time [44]. This technique has already shown promise in *Cx. quinquefasciatus*, where field and semi-field trials demonstrated the potential of *Wolbachia*-based IIT to significantly reduce mosquito populations and interrupt disease transmission cycles, including that of *Wuchereria bancrofti* [45]. Since all tested *Cx. pipiens* mosquitoes in this study were naturally infected with *w*Pip, strategic releases of incompatible males could be considered as part of an integrated vector management approach.

Future research should focus on quantifying *Wolbachia* density using qPCR, evaluating whether infection levels vary under different environmental conditions. Additionally, vector competence studies should be conducted to determine whether *Wolbachia* ST9 influences WNV transmission in Madeira's *Cx. pipiens*.

5. Conclusions

This study represents the first screening of *Wolbachia* in *Ae. aegypti* and *Cx. pipiens* from Madeira Island. The fact that no evidence of *Wolbachia* infection was found in *Ae. aegypti* aligns with previous studies that reported the absence of natural *Wolbachia* infections in *Ae. aegypti* populations. The detection of *Wolbachia* in all tested *Cx. pipiens* f. *molestus* and hybrid forms highlights its widespread prevalence in the local mosquito population, confirming the presence of the *w*Pip clade, supergroup B, ST 9. Given *Wolbachia's* potential to influence vector competence and population control, these findings contribute to the broader understanding of *Wolbachia* diversity in *Cx. pipiens* and its potential role in arbovirus transmission.

Author Contributions: Conceptualization, R.F., T.M., G.S. and H.O.; methodology, R.F., T.M., E.D.; software, R.F. and L.Z.Z.; validation, R.F., G.S. and H.C.O.; formal analysis, R.F.; investigation, R.F. and T.M.; resources, L.Z.Z., G.S., B.R.G. and H.C.O.; data curation, R.F. and L.Z.Z.; writing—original draft preparation, R.F. and T.M.; writing—review and editing, R.F., T.M., L.Z.Z., I.F., M.S., N.C.S., B.R.G., G.S. and H.C.O.; supervision, G.S. and H.C.O.; funding acquisition, L.Z.Z., N.C.S., G.S., B.R.G. and H.C.O. R.F. and T.M. contributed equally to this work and share first authorship. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data supporting the results of this article are included. The nucleotide sequence data reported in this paper have been deposited in the NBCI GenBank database under the accession numbers PV224315 - PV224326.

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Abbreviations

The following abbreviations are used in this manuscript:

ace-2	Acetylcholinesterase-2
IIT	Incompatible Insect Technique
MBDs	Mosquito-borne diseases
MLST	Multilocus Sequence Typing

NGS	Next-generation sequencing
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
ST	Sequence type
WNV	West Nile virus
wsp	*Wolbachia* surface protein gene

Appendix A

Appendix A.1

Table A1. Primers used in this work, for *Wolbachia* detection by PCR and for the amplification of the MLST complex and *wsp*HVR.

Target	Primer Sequences (5′-3′)	Reference
wsp	81F – TGGTCCAATAAGTGATGAAGAAA 691R - AAAAATTAAACGCTACTCCA	[25]
coxA	wsp_F1: GTCCAATARSTGATGARGAAAC wsp_R1: CYGCACCAAYAGYRCTRTAAA	[26]
gatB	gatB_F1: GAKTTAAAYCGYGCAGGBGTT gatB_R1: TGGYAAYTCRGGYAAAGATGA	[26]
ftsZ	ftsZ_F1: ATYATGGARCATATAAARGATAG ftsZ_R1: TCRAGYAATGGATTRGATAT	[26]
hcpA	hcpA_F1: GAAATARCAGTTGCTGCAAA hcpA_R1: GAAAGTYRAGCAAGYTCTG	[26]
fbpA	fbpA_F1: GCTGCTCCRCTTGGYWTGAT fbpA_R1: CCRCCAGARAAAAYYACTATTC	[26]
*wsp*HVR	wsp_F1: GTCCAATARSTGATGARGAAAC wsp_R1: CYGCACCAAYAGYRCTRTAAA	[26]

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