ORIGINAL ARTICLE

Screening of Natural *Wolbachia* Infection in *Aedes Caspius* and *Culex Pipiens* as Potential Vectors of Arboviral Diseases in Shiraz, South of Iran

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Abstract

Background: Mosquitoes transmit many diseases to humans, including malaria, dengue, yellow fever, chikungunya, and Zika. Controlling mosquitoes with endosymbiont bacterium *Wolbachia* is a new approach in this field. This study aimed to determine the *Wolbachia* infection of two mosquito species, *Aedes caspius* and *Culex pipiens*, in the city of Shiraz, southern Iran.
Methods: Samples of *Ae. caspius* and *Cx. pipiens* were collected from four localities in Shiraz City, Fars Province. The samples were identified using the morphological identification keys. Collected samples were screened for *Wolbachia* infection using a PCR assay targeting the *Wolbachia* surface protein (wsp) gene.
Results: Eight species from four genera were collected in this study; the most caught species was *Cx. Pipiens,* and the lowest abundant species was *An. hyrcanus*. From 110 adult *Cx. pipiens* screened using the wsp primer, 75 (68%) samples were infected with *Wolbachia*. The *Wolbachia* sequences in *Cx. pipiens* were like *Wolbachia* strains belonging to supergroups B. There was no *Wolbachia* infection in 204 *Ae. caspius* investigated samples.
Conclusion: Our study revealed the presence of the supergroup B *Wolbachia* strain in *Cx. pipiens* samples. The present study did not detect any *Wolbachia* infection in *Ae. caspius*; however, it remains plausible to introduce *Wolbachia* populations into *Wolbachia*-free populations of this species. Such an introduction holds promise as a viable tool for vector control and mitigating the transmission of arboviral diseases such as *West Nile virus* and *Chikungunya* through cytoplasmic incompatibility.

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Keywords: *Wolbachia*, Arbovirus, Symbionts, *Aedes caspius, Culex pipiens*

Introduction

The family Culicidae is considered one of the most important insects affecting human health.[1, 2] A wide range of arboviruses are transmitted by this family, including *dengue virus, dengue hemorrhagic virus,* and *West Nile virus*.[2] Some mosquitoes of the Culicidae family are extremely active in terms of blood-feeding in urban environments.[3] The role of these species in the harassment caused by the bite, along with the transmission of pathogens, has doubled the medical importance of these species.[1-3] *West Nile virus* has attracted attention due to its rapid spread throughout much of the world. This virus was first detected in 1937 in Uganda in a 37-year-old woman. Currently, it is known as one of the most common arbovirus diseases that cause severe complications.[4, 5] Since 1970, when this disease was first reported in Iran, many studies have

been conducted throughout the country.[6, 7] In general, some different species of the genera *Aedes* and *Culex* have been reported as the main vectors of the *West Nile virus* from different regions of the world.[8] Potential vectors of the *West Nile virus* are distributed throughout most of Iran from north to south.[2] The presence of *Ae. Caspius,* along with *Cx. Pipiens,* in Shiraz can indicate the possibility of a complete disease transmission cycle.

Aedes caspius causes significant disturbance and harassment and is sometimes able to transmit pathogenic agents to humans in tropical latitudes. For example, we can mention the endemicity of the West Nile virus in the USA and the spread of the Zika virus in Italy by this mosquito.[8, 9] In most regions of Europe, sympatric dispersal between *Ae. caspius* and *Ae. dorsalis* has been reported.[10] *West Nile virus* (WNV), *Tahyan virus,* and *Francisella tularensis* bacteria, the causative agent of tularemia, have been isolated from the natural population of this species.[11] Probably, *Ae. Caspius* plays an important role in the spread of tularemia, the transmission of the *Tahyan virus,* and the rabbit *myxoma virus.*[12, 13]

Culex mosquitoes constitute a wide range of mosquitoes involved in the transmission of arboviral diseases, including *West Nile virus, Sindbis virus, equine encephalitis, St. Louis, Oro-pouch fever, avian malaria, lymphatic filariasis,* and *Rift Valley fever.*[14] The genus *Culex* is one of the most important groups of the *Cx. pipiens* complex, which consists of six members: *Cx. quinquifasciatus, Cx. pallens, Cx. australicus, Cx. globocoxitus, Cx. Molestus,* and *Cx. pipiens.*[15]

Diseases transmitted by arthropods impose a great health burden on the inhabitants of tropical and subtropical regions. Many efforts have been made over the years to fight these diseases. However, the prevalence and geographical distribution of these diseases are expanding, and the available methods to fight them are limited. Controlling mosquitoes with the endosymbiont bacterium *Wolbachia* is a new approach in this field.[16] Presently, in Australia, Brazil, Colombia, and Southeast Asia, the strategy of deploying mosquitoes infected with *Wolbachia* is being employed to control Dengue and chikungunya within their natural habitats.[17] This study aims to determine *Wolbachia* infection in two species of *Ae. caspius* and *Cx. pipiens*, in Shiraz City, Fars Province. The results of this study provide basic information for better control of arbovirus vectors and the possibility of using new environmentally friendly biological methods to control the transmission of dangerous arbovirus diseases such as dengue fever and West Nile in the country.

Methods

Study Area

Shiraz is the capital of Fars province, situated at a latitude of 29.61 and longitude of 52.53, with an elevation of 1545 meters above the sea level. The total average annual precipitation has been reported to be 305.6 mm. Within the scope of this study, sampling sites were selected based on various factors, including the type of larval habitats, water availability, sunlight penetration, water flow, and water transparency. The selected sites with geographical coordinates are shown in Figure 1 and Table 1.

To determine the *Wolbachia* infection, we collected Culicidae samples from April to September 2022. Larvae were collected using the dipping method in selected locations of larval habitats. Subsequently, they were transported to the laboratory to determine their abundance and identity. Adult samples were caught during the day to determine the abundance and *Wolbachia* infection in each of the studied locations. To catch samples from methods such as Aspiration inside human dwellings, Landing catches on human baits, Animal baited trap, CO2-baited CDC light traps, Sticky trap, Total catch, and Shelter pits were used.[18] The samples collected were identified based on morphological identification keys and registered in standard forms.[19, 20] Finally, they were kept in a freezer at minus 20 degrees Celsius until molecular investigations.

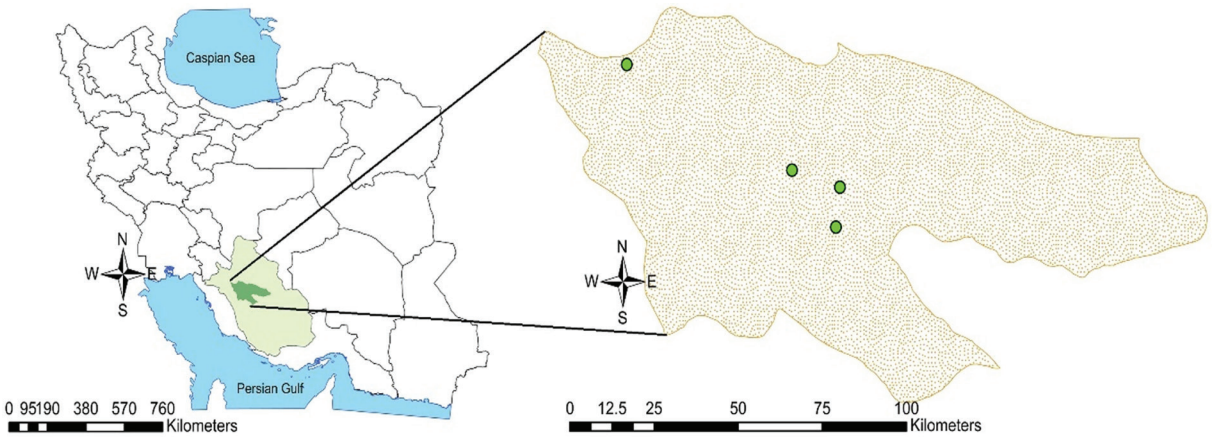

Figure 1: Sampling locations in the study area, Shiraz City, Fars Province, Iran. (Generated using ArcMap10.8)

Table 1: Geographical characteristics of the study areas, Shiraz City, Iran, 2022

No.	Locations	Latitude (N)	Longitude (E)	Altitude (m)
1	Shiraz industrial park	29°26'40.9"	52°35'58.7"	1472
2	Haft Barm	29°49'04.8"	52°02'26.6"	2170
3	Mian rood	29°34'31.9"	52°28'56.0"	1543
4	Airport	29°32'11.0"	52°36'37.2"	1482

N: Northing, E: Easting, M: Metro

Table 2: The thermal program was used to carry out Polymerase Chain Reaction (PCR) reactions to amplify a part of the wsp gene, in the study of *Wolbachia* infection in selected populations of adults *Aedes caspius* and *Culex pipiens*

Step	Temperature (degrees Celsius)	Time(minute)	Number of cycles
Initial denaturation	95	5	1
Denaturation	94	1	35
Annealing	55	1	
Extension	72	1	
Final extension	72	7	1

Table 3: First and second-stage primers specifications

Primer	Primer sequence	Function	Primer length (bp)	Melting temperature (C)	PCR Steps
81F	TGG TCC AAT AAG TGA TGA AGA AAC	Forward	24	66	First-Round
691R	AAA AAT TAA ACG CTA CTC CA	Reverse	20	52	
183F	AAG GAA CCG AAG TTC ATG	Forward	18	52	Second-Round
691R	AAA AAT TAA ACG CTA CTC CA	Reverse	20	52	

Table 4: Larval and adult mosquitoes collected from Shiraz City, Iran, 2022

Species	Sample collection location								Total specimens
	Airport		Haft Barm		Mian-rood		Shiraz industrial park		
	Adult	Larvae	Adult	Larvae	Adult	Larvae	Adult	Larvae	
Culex pipiens	211	126	56	11	217	187	9	0	817
Culex theileri	0	0	0	0	0	19	0	0	19
Culex. sinaiticus	0	0	0	0	0	27	0	0	27
Culex quinquefasciatus	0	0	0	0	28	18	0	0	46
Ae. caspius Aedes caspius	47	55	0	37	0	0	65	0	204
Culiseta longiareolata	0	5	64	201	0	0	0	0	270
Anopheles hyrcanus	0	0	0	4	0	0	0	0	4
Anopheles dthali	0	0	0	17	0	0	0	0	17
Total specimens	258	186	120	270	245	251	74	0	1404

Molecular Study

Given that *Cx. pipiens* and *Ae. caspius* mosquitoes were the predominant species and significant vectors in the study area, they were subjected to investigation for *Wolbachia* infection. Based on the level of *Wolbachia* infection, 20% of *Cx. pipiens* mosquitoes and 50% of *Ae. caspius* mosquitoes were included in the study sample. DNA extraction from adult mosquitoes was done to determine *Wolbachia* infection using the Collins method.[21] The *Wolbachia* surface protein (WSP) genes were amplified by nested Polymerase Chain Reaction (PCR) to investigate the presence and genetic diversity of *Wolbachia* strains inside the body of *Ae. caspius* and *Cx. pipiens* mosquitoes. The specifications of primers and PCR thermal cycles are as follows.[22, 23]

To perform the reaction in both PCR steps, we used Maxime PCR Premix Kit (i-Taq), Cat. No. 25025 manufactured by INtRON Company. In both steps, 2.5 microliters of forward and reverse primers were used, and 5 microliters of DNA were used in the first reaction, and 2.5 microliters of the PCR product of the first step were used in the second reaction. The PCR products were run on a 1% agarose gel, and then the bands were visualized under UV light. Each sequence obtained was compared with the sequences registered in GenBank using the NCBI database. MEGA6 and BioEdit software were used to investigate phylogenetic relationships. Finally, the sequences were recorded in GenBank (Tables 2 and 3).

Results

In this study, a total of 1404 mosquito samples, including 697 adults and 707 larvae, were collected from the study area in Shiraz City, Fars Province, Iran. In this study, eight species of four Culicidae genera were identified, including *Cx. pipiens, Cx. theileri, Cx. sinaiticus, Cx. quinquefasciatus, Ae. caspius, Culiseta longiareolata, Anopheles Hyrcanus,* and *An. dethali*. The results show that the most species caught was *Cx. Pipiens,* and the least species caught was *An. hyrcanus* (Table 4).

Table 5: *Wolbachia* infection in *Culex pipiens*, Shiraz City, Iran, 2022

Location	The total number of samples collected	Number of tested samples (M)	Percentage of infection (M)	Number of tested samples (F)	Percentage of infection (F)	Total number of tested samples (M&F)	Percentage of infection
Airport	93	10	4 (40%)	20	12 (60%)	30	16 (53.3%)
Mianrood	204	20	7 (35%)	20	18 (90%)	40	25 (62.5%)
Haft Barm	260	20	14 (70%)	20	20 (100%)	40	34 (85%)
Total	557	50	25 (50%)	60	50 (83.3)	110	75 (68%)

M: Male; F: Female

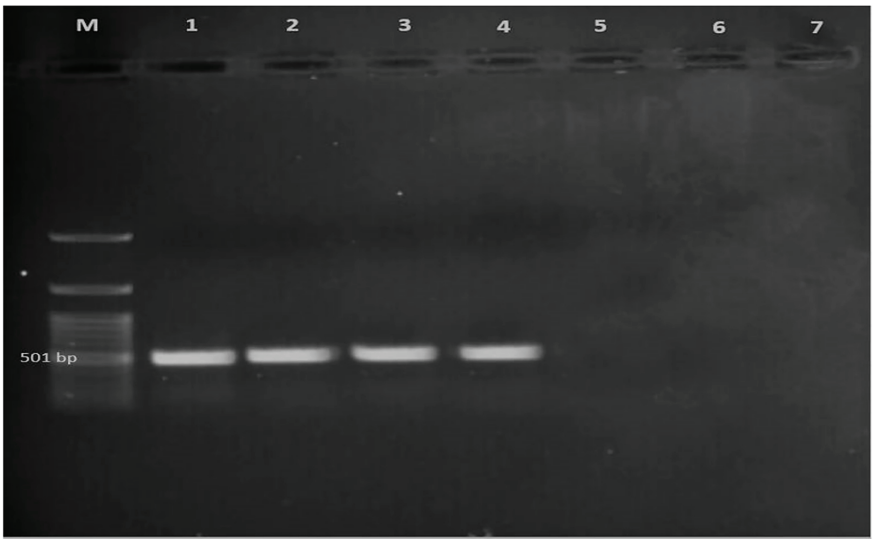

Figure 2: Electrophoresis of the second stage of Nested-Polymerase Chain Reaction (PCR) for a part of *Wolbachia* surface protein (wsp) gene of 501 bp length in different populations of *Aedes caspius* and *Culex pipiens* collected from different parts of Shiraz City, Fars Province (M marker 100bp, 1: *Drosophila melanogaster* (positive control), 2-4: *Culex pipiens* populations, 5-6: *Aedes caspius* populations, 7: negative control)

Table 6: *Wolbachia* infection in *Aedes caspius*, Shiraz City, Iran, 2022

Location	Total number of collected samples	Number of tested samples (M)	Number of tested samples (F)	total number of tested samples (M&F)	Percentage of infection
Airport	102	20	40	60	0
Shiraz industrial park	65	10	20	30	0
Haft Barm	37	10	20	20	0
Total	204	40	80	110	-

M: Male; F: Female

In this study, we screened 110 *Ae. caspius* and 110 *Cx. pipiens* species for *Wolbachia* infection by PCR assays with wsp gene primers. From 110 adult *Cx. Pipiens* screened, 75 (68%) *Cx. Pipiens* mosquitoes were infected with *Wolbachia*. The molecular results showed that the level of *Wolbachia* infection in the female population of *Cx. pipiens* was between 60-100 percent; in the male population, it was between 35-70 percent (Table 5).

Wolbachia sequences were obtained from infected individuals in each of the infected *Cx. Pipiens* species in this study (sequences were registered in the GenBank; accession numbers OR478431, OR478432, OR478433, OR478434, OR478435). The results show that there is no *Wolbachia* infection in the population of *Ae. caspius* (Figure 2 and Table 6). In addition, the wsp gene phylogeny analysis indicated *Cx. Pipiens* specimens were infected with strains of B *Wolbachia* supergroup (Figure 3).

Discussion

Aedes caspius is found in most regions of the world and transmits many arbovirus diseases to humans.[24] This species has been reported from most parts of Iran, such as West Azarbaijan and East Azarbaijan, Central, Semnan, Khorasan, Fars, Kurdistan, Chahar Mahal and Bakhtiari, Kohgiluyeh and Boyer Ahmad, Isfahan, and Hormozgan.[2, 5] In the present study, *Ae. caspius* and *Cx. Pipiens* were caught from all sampling sites in Shiraz City. Therefore, the results of the present study are similar to the results of previous studies in terms of the distribution of *Ae. caspius*. *Culex pipiens* is widely distributed throughout the world.[24] These species were collected from all the places under study, so in terms of distribution, the results of the present study are similar to those of previous research.[24]

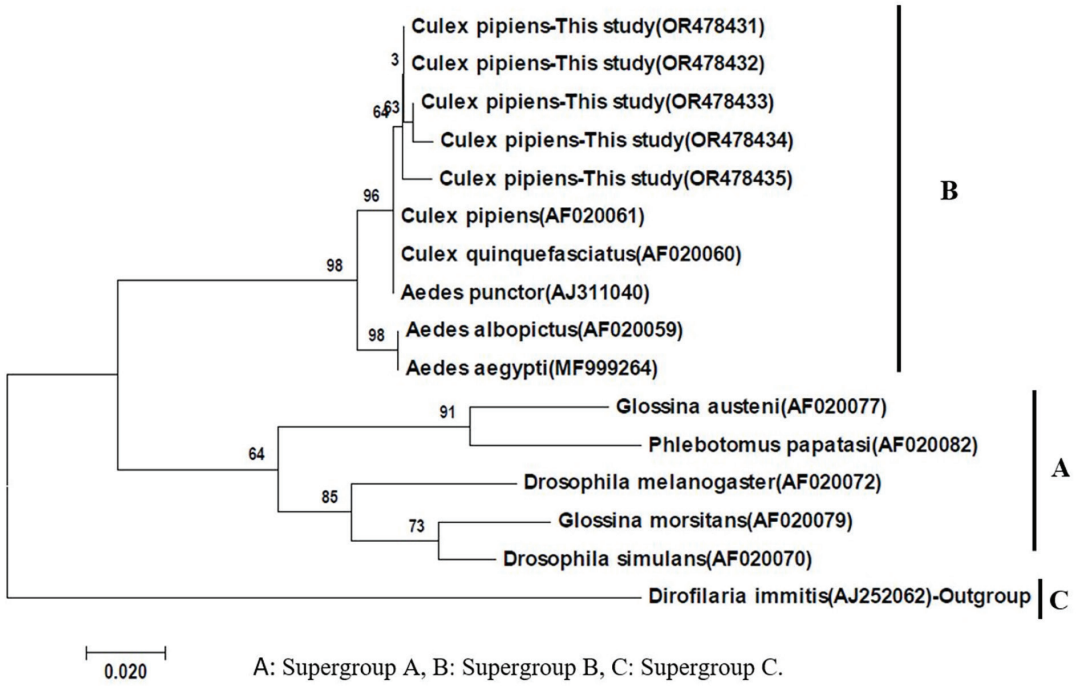

Figure 3: Phylogenic analysis based on the *Wolbachia* surface protein gene gene. Sample sequences of *Culex pipiens* collected in Shiraz City, Fars Province in Iran. *Wolbachia* sequences in the samples were done to show the degree of similarity (99–100%). Supergroup B was identified in this research, and Supergroup C was used as an outgroup.

This study showed that *Ae. caspius* are not infected with *Wolbachia* bacteria, while *Cx. pipiens* are highly infected with the *Wolbachia* bacterium (Tables 5 and 6). Indeed, the level of *Wolbachia* infection in *Cx. Pipiens* mosquitoes can vary across different locations, and environmental factors such as temperature, vegetation, and humidity may play a role in influencing this variation. Studies have suggested that higher temperatures could potentially reduce the presence of *Wolbachia* within the host mosquitoes. This relationship between environmental conditions and *Wolbachia* infection highlights the complexity of vector biology and the importance of considering ecological factors in understanding vector-borne diseases and their control.[25] In addition, the rates of infection with *Wolbachia* bacteria were between 60-100% among female populations and between 35-70% in males. The findings of this study are in the same line with previous research indicating that female mosquitoes tend to exhibit higher levels of *Wolbachia* infection compared to male mosquitoes. This observation is consistent with the fact that *Wolbachia* bacteria are often vertically transmitted from mother to offspring through the eggs, resulting in higher infection rates in female mosquitoes.[26] Understanding the sex-specific patterns of *Wolbachia* infection in mosquitoes is important for elucidating the biology of both the bacteria and their mosquito hosts, as well as for informing strategies for vector control and disease management. Previous studies show that the rate of *Wolbachia* infection in different populations of *Cx. pipiens* is between 80-90%; also, a study conducted in California reported an infection rate between 93-100%, which indicates that the present study aligns with the aforementioned studies.[27] The identification of *Wolbachia* strains in our study as belonging to supergroup B and their presence in *Cx. pipiens* mosquitoes are consistent with the findings reported in previous studies. Supergroup B is one of the major clades of *Wolbachia* bacteria, and its association with *Cx. Pipiens* mosquitoes has been documented in various geographic regions. This consistency across studies suggests a stable relationship between *Cx. Pipiens* mosquitoes and *Wolbachia* supergroup B strains.[28-30] A wide variety of *Wolbachia* strains including strains from supergroups A and B have been identified in *Aedes albopictus* and various *Drosophila* species.[22, 31, 32] Generally, mosquitoes are commonly infected by *Wolbachia* strains from supergroups A and B. Therefore, previous studies confirm the results of our study.[33]

In a study in Italy, *Wolbachia* infection was reported in *Ae. cinereus*, *Ae. punctor*, and *Ae. detritus*, but there were no reported infections in *Ae. caspius* and *Ae. vexans*.[34] During this study, there was no infection with *Wolbachia* in *Ae. caspius.* Therefore, the results of the present study are completely consistent with those of the previous study. In a study in Portugal, researchers detected *Wolbachia* in *Cx. Pipiens* but did not find any infection with *Wolbachia* in *Ae. caspius*, *Ae. Detritus,* and *An. maculipennis*.[35] Therefore, the present study confirms the results of the previous study. The higher prevalence of *Wolbachia* infection in *Culex* mosquitoes compared to *Aedes* mosquitoes is influenced by ecological, evolutionary, and biological

factors. *Culex* mosquitoes, found in urban areas, have a higher infection rate due to prolonged co-evolution with *Wolbachia* and strong reproductive interactions. In contrast, *Aedes* mosquitoes inhabit different ecological niches with lower infection rates, possibly due to limited evolutionary history with *Wolbachia* and distinct biological traits.[36]

The results of a study that was conducted to determine *Wolbachia* infection in the population of Culicidae mosquitoes in Egypt showed that in *Cx. pusillus*, *Cx. pipiens*, *Cx. antennatus*, *An. Pharoensis,* and *Ae. caspius*, which were infected with *Wucheraria bancroftii*, *Wolbachia* infection was also reported from these species.[37] Based on previous studies, it seems that when *Anopheles pharoensis* and *Ae. caspius* mosquitoes were not infected with *Wucheraria bancrofti*, bacterial infection was not reported in these species. However, when they were infected with *Wucheraria bancrofti*, bacterial infection was observed. From this observation, it is hypothesized that *Wucheraria bancrofti*, the filarial nematode, likely contains *Wolbachia* bacteria. This is consistent with previous findings where certain species of filarial nematodes, including *Wucheraria bancrofti*, have been shown to harbor endosymbiotic *Wolbachia* bacteria.[37] Some studies conducted in Europe showed that *Cx. Pipiens*, *Cx, Ae. Punctor,* and *Coquillettidia richiardii* are infected with *Wolbachia*.[38] It appears that the present study findings align with previous research, indicating that *Wolbachia* infection is predominantly reported in the *Cx. Pipiens* complex. This trend could potentially be attributed to the compatibility of *Wolbachia* bacteria with the *Cx. Pipiens* complex.[5, 39] While infection with *Wolbachia* was not reported in *Ae. Caspius* in our study, *Cx. Pipiens* showed a high rate of *Wolbachia* infection. This observation supports and corroborates the results of previous studies, which have also highlighted the prevalence of *Wolbachia* infection within the *Cx. Pipiens* complex. This consistency across studies further strengthens our understanding of *Wolbachia* distribution among mosquito species. It underscores the importance of continued research to elucidate the factors driving *Wolbachia* prevalence and its implications for mosquito biology, vector competence, and disease transmission dynamics.

The results of our study and previous research suggest that *Wolbachia* infection varies among different species of Culicidae mosquitoes. This highlights the species-specific nature of *Wolbachia* infection in mosquitoes. Such factors as the mosquito's biology, ecology, and evolutionary history may influence its susceptibility to *Wolbachia* infection. Understanding these species-specific patterns of *Wolbachia* infection is crucial for developing targeted vector control strategies and leveraging *Wolbachia*-based interventions for disease control.

Conclusion

The findings of the current study revealed that the *Wolbachia* infection detected in *Cx. Pipiens* belonged to supergroup B. Given the absence of *Wolbachia* infection in *Ae. Caspius* observed in our study, there's an opportunity to artificially infect this species with *Wolbachia*. This can be achieved through laboratory-based methods, such as microinjection of *Wolbachia*-infected mosquito embryos or introduction of *Wolbachia*-infected adult mosquitoes into the wild.

Ethics Approval and Consent to Participate

This study was approved by the ethical committee of Shiraz University of Medical Sciences (IR.SUMS.SCHEANUT.REC.1400.102).

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Authors' Contribution

Kourosh Azizi and Saideh Yousefi analyzed and interpreted the data and drafted the manuscript. Narjes Moezi and Mozaffar Vahedi performed laboratory experiments. Reza Sadeghi and Saeed Shahabi reviewed and edited the manuscript. Azim Paksa conceived and designed the study and reviewed the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

None declared.

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