Applied Entomology and Zoology (2020) 55:105–113
https://doi.org/10.1007/s13355-019-00658-6

ORIGINAL RESEARCH PAPER

Wolbachia infection in West Nile Virus vectors of northwest Iran

Faramarz Bozorg-Omid[1,2] · Mohammad Ali Oshaghi[3] · Mozaffar Vahedi[1] · Fateh Karimian[3] · Seyyed Javad Seyyed-Zadeh[1] · Ali Reza Chavshin[1,4]

Received: 25 July 2019 / Accepted: 26 November 2019 / Published online: 13 December 2019

Abstract
Mosquito-borne viral diseases are serious health problems in many countries. Various methods have been used for controlling the vectors of these diseases. Among symbiotic bacteria, the members of the genus *Wolbachia* are the most ubiquitous symbionts of arthropods and play key roles in their host biological characteristics with various effects on their hosts. The identification of these bacteria in Iranian mosquitoes is limited to a few studies. The current study was carried out to determine (1) the *Wolbachia* infection of probable arbovirus vectors (*Aedes caspius*, *Culex pipiens*, *Culex theileri* and *Culiseta longiareolata*), (2) the *Wolbachia* strain(s) infecting the mosquitoes, and (3) the geographical distribution of the *Wolbachia* strain(s) in the northwest of Iran. Eight species including *Ae. caspius*, *Anopheles hyrcanus*, *An. maculipennis*, *Cx. hortensis*, *Cx. modestus*, *Cx. pipiens*, *Cx. theileri*, and *Cs. longiareolata* were identified, amongst which *Ae. caspius* with 63.1% and *An. hyrcanus* with 0.3% were the most and the least abundant species, respectively. The results of semi-nested PCR using *Wolbachia* surface protein (*wsp*) fragment assays showed that *Wolbachia* infection was present in three out of the four above mentioned arboviral vector species (*Aedes caspius*, *Culex pipiens*, *Culex theileri* and *Culiseta longiareolata*), where the highest infection rate was seen in *Cx. pipiens*. The infection rates of mosquitoes with *Wolbachia* in the species of *Cx. pipiens, Cs. longiareolata*, *Cx. theileri,* and *Ae. caspius* were 96.9%, 11.5%, 5.2% and 0%, respectively.

Keywords West Nile Virus · Arbovirus · Cytoplasmic incompatibility · Endosymbiont

Introduction

Mosquito-borne viral diseases, such as yellow fever, dengue fever, chikungunya, Sindbis, Zika, and West Nile Virus (WNV), are serious health problems in many countries around the world and the wide distribution of their vectors has had a great impact on the transmission and spread of these diseases (Weaver and Reisen 2010). Numerous methods have been implemented to control mosquito-borne diseases. However, mosquito-borne viral diseases remain frequent and deadly due to emerging insecticide resistance in mosquitoes, lack of treatment or resistance to drugs in some pathogens, and unavailability of effective vaccines for the majority of these diseases. Accordingly, discovering new methods for controlling mosquitoes and the diseases transmitted by them is of particular importance (Iturbe-Ormaetxe and Walker 2011; Medlock et al. 2012).

One of the new methods in this regard is the use of symbiotic bacteria to interfere with the pathogen transmission, reduce the vector's lifespan and eventually, reduce or stop the transmission of the diseases (Douglas 2007). *Wolbachia pipientis,* as an endosymbiont bacterium, which its wWil strain mainly infects their host's ovaries (Hertig and Wolbach 1924; Kozek and Rao 2007) and other strains such as wMel, wMelPop and wRi infect a much broader range of somatic and germline tissues, play a key role in the host's biological characteristics and have several effects including feminization (Bouchon et al. 1998; Kageyama et al. 2002), parthenogenesis (Pannebakker et al.

✉ Ali Reza Chavshin
chavshin@umsu.ac.ir; chavshin@gmail.com

1 Department of Medical Entomology and Vector Control, School of Public Health and Social Determinants of Health Research Center, Urmia University of Medical Sciences, Urmia, Iran

2 Student Research Committee, Urmia University of Medical Sciences, Urmia, Iran

3 Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

4 Social Determinants of Health Research Center, Urmia University of Medical Sciences, Urmia, Iran

2004; Weeks and Breeuwer 2001), male killing (Hurst and Jiggins 2000), and sperm–egg incompatibility known as cytoplasmic incompatibility (CI) (Atyame et al. 2014; Dobson et al.2004; Sinkins 2004; Tortosa et al. 2010). These actions probably can disrupt the mosquito-borne diseases (Ahmad et al. 2017; Aliota et al. 2016; Ant and Sinkins 2018; Bull and Turelli 2013; Dodson et al. 2017; Frentiu et al. 2010; Schultz et al. 2017, 2018a, b; Silva et al. 2017, Sinkins 2013).

Based on the results of the interaction of different strains of *Wolbachia* with arthropod-host and pathogens, different strategies can be used to reduce or stop the disease transmission (Blagrove et al. 2012; Calvitti et al. 2012, 2015; Hughes and Rasgon 2014; Rao 2005; Sabesan and Jambulingam 2012; Schnettler et al. 2016; Schultz et al. 2018a, b; Telschow et al. 2017). It seems that oxidative stress induction by *Wolbachia* spp. and consequently the reactivation of oxygen species in response to WNV infection lead to activation of the Toll pathway and production of defensins and cecropins which inhibit the replication of flavivirus in mosquito's organs (Pan et al. 2012). The presence of *Wolbachia* suppresses the density of the dengue virus to the level incapable of being transmitted to mammalian hosts (Frentiu et al. 2010). *Wolbachia* has been reported to prevent the replication of WNV in its vectors (Glaser and Meola 2010). However, a different situation, even a negative effect of *Wolbachia* on WNV transmission has been reported (Dodson et al. 2014).

Apart from sometimes contradictory laboratory findings regarding the effect of *Wolbachia* on WNV, on a practical and field scale, *Wolbachia*-induced cytoplasmic incompatibility (incompatibility insect technique: IIT) in combination with (sterile insect technique: SIT) nearly eliminated two field populations of *Aedes albopictus* over a 2-year period (Zheng et al. 2019).

Since most of the effects of *Wolbachia* are due to the introduction of new or different strains of Wolbachia into wild populations, as the first step, it is important to identify the infection of *Wolbachia* in disease vectors. The use of molecular markers has been raised as a reliable method. Among the various fragments of *Wolbachia* genome, *Wolbachia surface protein* (*wsp*) gene has been widely used for macro (supergroup designation) and micro (subgroup designation) taxonomy (Karimian et al. 2018).

West Nile Virus has been reported from various epidemiological rings in Iran, e.g., in humans (prevalence 1.3–95.8% in different areas) (Chinikar et al. 2012, 2013; Meshkat et al. 2015; Naficy and Saidi 1970; Saidi et al. 1976; Shah-Hosseini et al. 2014; Sharifi et al. 2010) and different species of birds from Fars, Guilan, Mazandaran and Tehran Provinces (15.0% overall prevalence; 54.2% in common coots) (Fereidouni et al. 2011). Finally Bagheri et al. (2015) and Shahhosseini et al. (2017) isolated the virus from *Ae. caspius* s.l. from West Azerbaijan Province, northwestern Iran, and *Cx. pipiens* from Guilan Province (northern Iran).

Although several studies have been conducted on infections with *Wolbachia* in different arthropods in Iran, e.g., fruit flies (Karimi and Darsouei 2014), Trichogrammatidae (Karimi et al. 2012), *Phlebotomus spp.* sandflies (Karimian et al. 2018; Parvizi et al. 2013a, b), and some other arthropods and nematodes (Pourali et al. 2009), the identification of these bacteria in Iranian mosquitoes is limited to a few studies despite their medical importance. One of these studies on *Culex* mosquitoes of southwest Iran (Behbahani 2012) included three species of *Culex theileri*, *Cx. tritaeniorhynchus*, and *Cx. quinquefasciatus*, while in another study, the infection of *Culex pipiens* populations in the northern, central, and southern parts of Iran was evaluated (Karami et al. 2016).

Entomological studies in the northwest of Iran indicate the presence of different species of mosquitoes (Abai et al. 2007; Azari-Hamidian et al. 2009; Bagheri et al. 2015; Khoshdel-Nezamiha et al. 2014, 2016), some of which are vectors of arboviral diseases. Considering the specific geographic features of northwestern Iran, where the country borders four countries of Azerbaijan, Turkey, Iraq, and Armenia, with diverse climate conditions, the high diversity of mosquito species, the insecticide resistance of mosquitoes of the region (Chavshin et al. 2015; Naseri-Karimi et al. 2015) and the history of mosquito-borne diseases, the identification of mosquito species and their possible infection with *Wolbachia* is very important to provide basic information to use *Wolbachia*-based vector control strategies in the future. Accordingly, the current study was carried out to determine (1) the species composition of mosquitoes of the northwest of Iran, (2) the *Wolbachia* infection of probable arbovirus vectors (*Ae. Caspius*, *Cx. pipiens*, *Cx. theileri*, and *Cs. longiareolata*) and, (3) the *Wolbachia* strain(s) infecting the mosquitoes.

Materials and methods

Study area, collection, and identification of samples

West Azerbaijan province is located in the northwest of Iran, bordering Turkey, Iraq, Armenia, and Azerbaijan, and the provinces of East Azerbaijan, Zanjan, and Kurdistan within Iran (Fig. 1).

Adult and larval sample collections were done using conventional methods (Silver 2007) from May to November 2017. Sampling was performed in a total of 10 regions of West Azerbaijan province in three districts of Makoo in the north, Urmia in the center, and Mahabad in the south of the province (Table 1). The collected specimens were transferred to the laboratory and identified based on

Fig. 1 The location of study areas and collection localities. 1: Makoo, 2: Urmia and 3: Mahabad

Table 1 The collected species among different counties across West Azerbaijan Province

Species	County			
	Makoo	Urmia	Mahabad	Total *N* (%)
Aedes caspius	186	119	545	850 (63.1)
Anopheles maculipennis	62	71	57	190 (14.1)
Anopheles hyrcanus	4	0	0	4 (0.3)
Culex pipiens	17	83	0	100 (7.4)
Culex theileri	23	26	23	72 (5.4)
Culex modestus	11	17	43	71 (5.2)
Culex hortensis	3	2	0	5 (0.4)
Culiseta longiareolata	23	31	0	54 (4.1)
Total	329	349	668	1346

morphological characteristics to species level using the standard keys (Azari-Hamidian and Harbach 2009).

DNA extraction and polymerase chain reaction

The genomic DNA of all caught specimens in different regions of West Azerbaijan Province was extracted individually using the Bioneer AccuPrep® Genomic DNA Extraction Kit (Daejeon, South Korea). The extracted DNAs were kept at + 4 °C for amplification of *Wolbachia* surface protein (*wsp*) gene, using semi-nested PCR by two specific primer pairs (81F: 5′–TGGTCCAATAAGTGATGAAGAAAC–3′ and 691R: 5′–AAAAATTAAACGCTACTCCA–3′) and (183F: 5′–AAGGAACCGAAGTTCATG–3′ and 691R) (Zhou et al. 1998), to increase the PCR sensitivity. The amplicon of the first primer pair was 632 bp of the partial sequence of the *wsp* gene and was used as a template for the second PCR. Finally, using the second pair of primers, a 501-bp fragment was amplified. The PCR conditions were as follows: 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 46 °C for 50 s, 72 °C for 1 min, and 72 °C for 10 min. Double-distilled water and DNA of *Anopheles maculipennis* have been used as negative controls. Also, DNA of confirmed samples from the previous study (Karimian et al. 2018) was used as positive controls.

The resultant amplicons were examined using a 1.5% agarose gel and visualized by UV trans-illuminator after staining with Yekta Tajhiz® safe stain (Tehran, Iran). High-quality *wsp* amplicons of the desired size were sequenced using Sanger sequencing by Microsynth^{R} (Balgach, Swiss).

Sequence and phylogenetic analyses

The acquired sequences were edited and assembled using Bioedit software and analyzed using NCBI (Nucleotide collection) database (https://www.ncbi.nlm.nih.gov/). The confident sequences were aligned with other *Wolbachia* sequences available in GenBank (www.ncbi.nlm.nih.gov) using CLUSTAL OMEGA (www.ebi.ac.uk/Tools/msa/clustalo).

For phylogenetic analysis, the evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). All positions containing gaps and missing data were eliminated. There was a total of 351 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Results

In the present study, a total of 1346 mosquitoes were caught from different regions of West Azerbaijan province. After being identified based on morphological characteristics, they were categorized based on species and collection sites (Table 1). The eight species included *Aedes caspius*, *Anopheles hyrcanus*, *An. maculipennis*, *Culex hortensis*, *Cx. modestus*, *Cx. pipiens*, *Cx. theileri*, and *Culiseta longiareolata* of four genera. *Aedes caspius* with $n = 850$ (63.1%) and *An. hyrcanus* with $n = 4$ (0.3%) were, respectively, the most and the least abundant species in the study area.

Among all collected mosquitoes across West Azerbaijan province, 195 specimens belonging to four species with more important roles in the WNV transmission including *Ae. caspius*, *Cx. pipiens*, *Cx. theileri*, and *Cs. longiareolata* were evaluated individually for *Wolbachia* infection (Table 2). The results of PCR amplification against the *wsp* gene confirmed the presence of *Wolbachia* in 68 (34.8%) out of the 195 tested specimens.

The results of semi-nested-PCR assays showed that *Wolbachia* infection is present in three out of the four species, where the highest infection rate was related to *Cx. pipiens*. In general, infection with *Wolbachia* in the species *Ae. caspius*, *Cx. pipiens*, *Cx. theileri* and *Cs. longiareolata*, and was 0%, 97%, 5.3%, and 12%, respectively (Table 3).

The amplified fragments of the *wsp* gene of *Wolbachia* in infected specimens, belonging to three mosquito species, were subjected to sequencing and the resulting sequences were analyzed and deposited in GenBank (accession no. MH368736-51).

Table 3 The total number of samples tested and their infection rate with *Wolbachia* by species

Total infection rate (%)	No. of infected specimens	Total tested no.	Species
0	0	66	*Aedes caspius*
97	63	65	*Culex pipiens*
5.3	2	38	*Culex theileri*
12	3	26	*Culiseta longiareolata*
35	68	195	Total

The results of the sequence and phylogenetic analyses indicated a variation in the structure of the *wsp* gene in the mosquitoes of West Azerbaijan province. The distribution of

Table 2 The number of probable arbovirus vectors *Aedes caspius*, *Culex pipiens*, *Culex theileri* and *Culiseta longiareolata* and the percentage of their infection with *Wolbachia* by region

Infection rate (%)	No. of infected specimens	No. of tested for *Wolbachia* infection	No. of collected specimens	Species	Geographical properties (latitude, longitude)	Region	County
0	0	2	2	*Aedes caspius*	39° 20′ 38.35″ N, 44° 26′ 10.32″ E	Milan	Makoo (north of Province)
0	0	15	184	*Aedes caspius*	39° 18′ 59.73″ N, 44° 25′ 53.99″ E	Sangar	
100	15	15	17	*Culex pipiens*			
0	0	8	23	*Culex theileri*			
20	3	15	23	*Culiseta longiareolata*			
0	0	9	9	*Aedes caspius*	37° 36′ 8.01″ N, 45° 8′ 57.94″ E	Jarchloo	Urmia (Center of Province)
100	10	10	10	*Culex pipiens*			
13.13	2	15	26	*Culex theileri*			
100	9	9	9	*Culex pipiens*	37° 44′ 31.92″ N, 45° 11′ 39.96″ E	Gajin	
0	0	15	101	*Aedes caspius*	37° 39′ 10.78″ N, 45° 12′ 11.81″ E	Ghahraman-Loo	
87	13	15	46	*Culex pipiens*			
0	0	7	9	*Aedes caspius*	37° 43′ 50.12″ N, 44° 39′ 33.78″ E	Koraneh	
100	7	7	9	*Culex pipiens*			
100	9	9	9	*Culex pipiens*	37° 39′ 24.39″ N, 44° 59′ 0.39″ E	Nazloo	
0	0	11	31	*Culiseta longiareolata*			
0	0	2	2	*Aedes caspius*	37° 1′ 35.70″ N, 45° 44′ 1.47″ E	Kanibarazan	Mahabad (south of Province)
0	0	15	542	*Aedes caspius*	36° 59′ 14.29″ N, 45° 43′ 3.22″ E	Khor-Khoreh	
0	0	15	23	*Culex theileri*			
0	0	1	1	*Aedes caspius*	36° 45′ 42.49″ N, 45° 42′ 23.85″ E	Mahabad	
35	68	195	1076	Total			

sequences in the analysis in the form of branches and clades represented the possibility for the distribution of *Wolbachia* beyond the species that was geography dependent. In addition, determining the *Wolbachia* sub- and supergroups in the current study showed that they belonged to Supergroup B and Subgroup Pip (Fig. 2). Also, depending on how the sequences of the current study have been placed in a clade, genetic variation among *Wolbachia* could be supposed in different species in the present study. Blast analysis showed that all *Wolbachia* strains found in the current study were 99% similar to other *Wolbachia* strains isolated from other mosquito species, such as *Cx. pipiens* complex, including *Cx. pipiens*, *Cx. pallens*, *Cx. quinquefasciatus*, and *Cx. pipiens molestus* from other geographic regions of the world.

Discussion

The present study is one of the few studies on the detection of *Wolbachia* infection in Iranian mosquitoes and the infection of one of the species (*Cs. longiareolata*) is new to science. On the other hand, focusing on the potential vectors of WNV could highlight the results.

As the notable climate variation in West Azerbaijan province can provide a diverse environment for the growth and distribution of various species of mosquitoes, including important arbovirus vectors in the region, and according to reports indicating the presence of WNV in this province and surrounding regions (Ahmadnejad et al. 2011, 2016; Chinikar et al. 2013; Naficy and Saidi 1970; Saidi et al. 1976), the presence of these species should be considered more and there is a need for more extensive research.

Among the identified species (eight species belonging to four genera), four species (*Ae. caspius*, *Cx. pipiens*, *Cx. theileri*, and *Cs. longiareolata*), as potentially important vectors of WNV compared to other species, have been reported previously from the northwest of Iran (Abai et al. 2007; Bagheri et al. 2015; Khoshdel-Nezamiha et al. 2014, 2016) and actually WNV has been detected in one of these species (*Ae. caspius*) recently from this region (Bagheri et al. 2015). Accordingly, the infection of these four species with *Wolbachia* was investigated in the present study.

Three out of the four study species were infected with *Wolbachia;* however, this infection did not exist among all of their different populations and in the central part of the province, they had the highest infection. Nevertheless, in the southern part of the province, infection with *Wolbachia* was not detected. The difference in the infection rates between the southern part and the north and center of the province seems to be related to the abundance of different species in different regions (e.g., the absence of *Cx. pipiens* in the southern part of province, with the highest infection rate in other areas). Also, perhaps part of this infection rate difference may also be related to important environmental factors, such as temperature, as previous reports showed the temperature probably affects the presence of *Wolbachia* in hosts (Ross et al. 2017; Ulrich et al. 2016; Van Opijnen and Breeuwer 1999).

Since the significant differences of environmental and climatic conditions in the study area have been reported previously (Amini et al. 2019), the assumption of the effect of environmental and temperature conditions on the infection rate of *Wolbachia* seems to be more acceptable.

The results of a recent study determining the infection of different populations of *Cx. pipiens* in the north, center, and south of Iran showed that all populations of the mentioned species were infected with *Wolbachia* (Karami et al. 2016), which is in agreement with the results of the present study.

Other studies have reported the infection of *Wolbachia* in different species, e.g., *Cx. pipiens* and *Cx. theileri* in Portugal (de Pinho et al. 2016), *Culex* spp. in Egypt (Dyab et al. 2016), and different mosquito species in India (Ravikumar et al. 2011), which generally confirms the presence of *Wolbachia* in a wide range of important disease vectors. In addition to the intensification of the need for further research on various effects of *Wolbachia* in the host body, it is hoped to increase the probability of the use of these bacteria in controlling diseases transmitted by their hosts.

Wolbachia, found in the current study belonged to supergroup B. Among the 17 supergroups of *Wolbachia*, supergroups A and B have been described to often cause changes in the reproductive system of the host and are broadly spread in many arthropod groups (Werren et al. 2008). This should also be considered regarding the potential role of these bacteria in speciation in host mosquitoes and the possible use of these bacteria in the discontinuation of the transmission cycle.

Given the fact that the use of multiple markers can make identification and isolation of strains more reliable, use of other markers such as *groE*, multilocus sequence typing (MLST) and 16S rRNA in future studies is recommended. However, recently relying on a few genes for Wolbachia characterization has been criticized (Bleidorn and Gerth 2018).

Conclusion

Although the results of this study regarding the infection of some medically important mosquitoes, especially the vectors of WNV, are important in one of the provinces prone to the spread of the disease, future research should compensate for the limitations of the present study, especially with the coverage of wider areas, the use of other useful molecular markers (such as *groE*, MLST or 16S rRNA, providing a better picture of the presence of *Wolbachia* in mosquitoes in

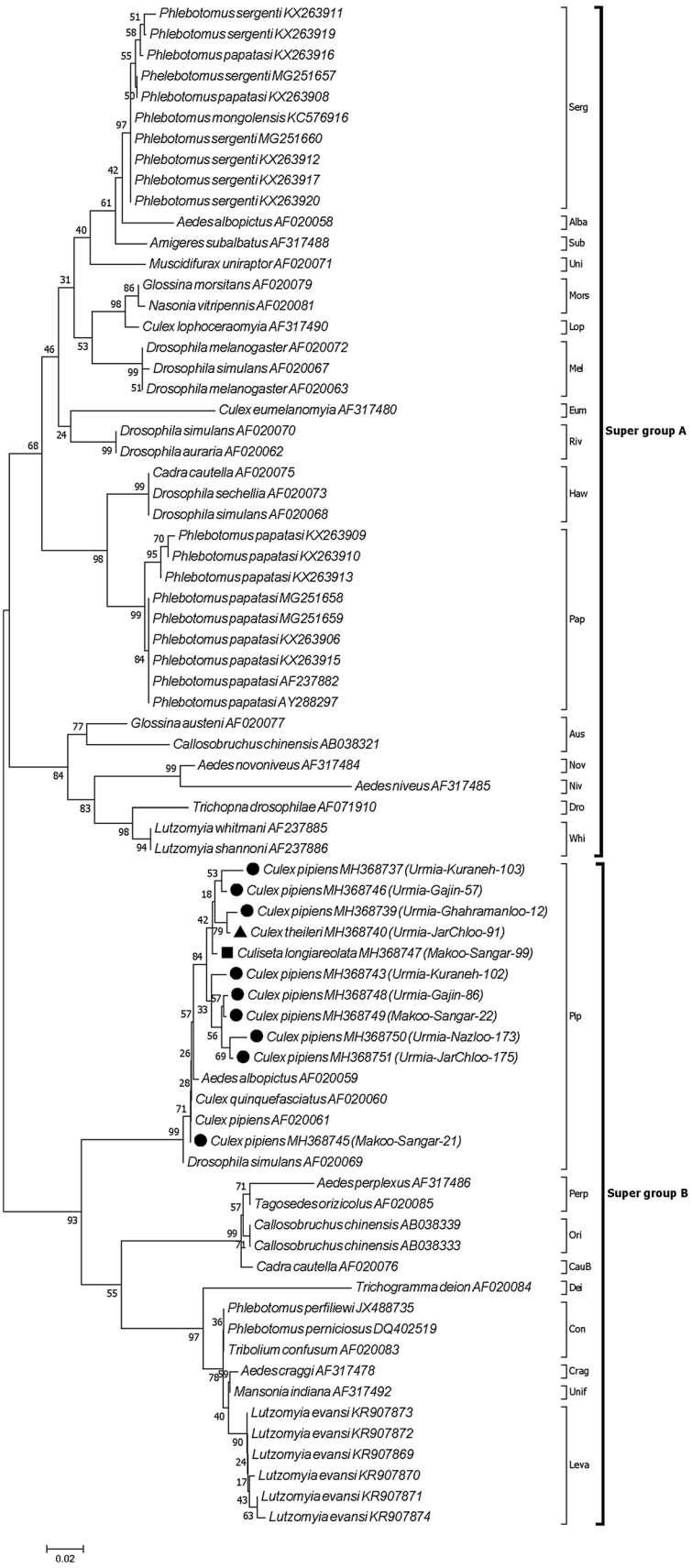

Fig. 2 Neighbor joining analyses of DNA sequences of *wsp* sequences of *Wolbachia* strains isolated from mosquito species of northwest of Iran and other insects in the super/subgroups levels. The scale bar indicates genetic distance. The isolates of the current study have been indicated by filled square: for *Cs. longiareolata*, filled circle: *Cx. pipiens* and filled triangle: *Cx. theileri*

the region, the probable variation among present *Wolbachia,* and finally its effects on the different biological aspects of vectors.

According to the results of this study and the confirmation of *Wolbachia* infection in potential vectors of WNV in the region, continuous research on this path is emphasized.

Acknowledgements This article is part of the results of the first author's dissertation for the fulfillment of MSc degree in Medical Entomology and Vector Control from the Department of Medical Entomology and Vector Control, School of Public Health, Urmia University of Medical Sciences, Urmia, Iran. This study was supported by the Urmia University of Medical Sciences (UMSU), Urmia, Iran.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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