Original Article

Wolbachia Endobacteria in Natural Populations of *Culex pipiens* of Iran and its Phylogenetic Congruence

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Abstract

Background: *Wolbachia* are common intracellular bacteria that infect different groups of arthropods including mosquitoes. These bacteria modify host biology and may induce feminization, parthenogenesis, male killing and cytoplasmic incompatibility (CI). Recently *Wolbachia* is being nominated as a bio-agent and paratransgenic candidate to control mosquito borne diseases.

Methods: Here we report the results of a survey for presence, frequency, and phylogenetic congruence of these endosymbiont bacteria in *Culex pipiens* populations in Northern, Central, and Southern parts of Iran using nested-PCR amplification of *wsp* gene.

Results: *Wolbachia* DNA were found in 227 (87.3%) out of 260 wild-caught mosquitoes. The rate of infection in adult females ranged from 61.5% to 100%, while in males were from 80% to 100%. The Blast search and phylogenetic analysis of the *wsp* gene sequence revealed that the *Wolbachia* strain from Iranian *Cx. pipiens* was identical to the *Wolbachia* strains of supergroup B previously reported in members of the *Cx. pipiens* complex. They had also identical sequence homology with the *Wolbachia* strains from a group of distinct arthropods including lepidopteran, wasps, flies, damselfly, thrips, and mites from remote geographical areas of the world.

Conclusion: It is suggested that *Wolbachia* strains horizontally transfer between unrelated host organisms over evolutionary time. Also results of this study indicates that *Wolbachia* infections were highly prevalent infecting all *Cx. pipiens* populations throughout the country, however further study needs to define *Wolbachia* inter-population reproductive incompatibility pattern and its usefulness as a bio-agent control measure.

Keywords: *Culex pipiens*, *Wolbachia*, cytoplasmic incompatibility, nested-PCR, Iran

Introduction

Mosquitoes including *Culex pipiens* complex with global distribution are vectors of arboviral pathogens and parasites such as West Nile, St Louis, Sindbis, *Wuchereria bancrofti*, *Dirofilaria immitis*, *D. repens*, *Plasmodium relictum*, and *P. gallinaceum* (Vinogradova

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2000, Pawelek et al. 2014). Among the 'neglected' mosquito-borne diseases, lymphatic filariasis continues to be a hazard to over a billion people in 83 countries (O'Connor et al. 2012). *Culex pipiens* is a species complex and comprise *Cx. quinquefasciatus* and *Cx. pipiens* in South and North America, Asia and Africa, as well as *Cx. globocoxitus* and *Cx. australicus* in Australia (Farajollahi et al. 2011). *Culex pipiens* and *Cx. quinquefasciatus* are distributed in most parts of Iran ranging from north to south (Zaim 1986, Azari-Hamidian 2007, Nikookar et al. 2010, Khoshdel-Nezamiha et al. 2013, Banafshi et al. 2013, Dehghan et al. 2013, 2014).

The raising of resistance to current insecticides by insect vectors (Hemingway and Ranson 2000), the progress of drug resistance in parasites (Talisuna et al. 2004) and lack of clinical cures or vaccines for many vector borne diseases have led researchers to develop urgently new and advanced approaches to control of the diseases. Paratransgenesis, as a new approach, direct towards reducing vector competence through genetically manipulated symbionts (Coutinho-Abreu et al. 2010). Transformed symbionts are distributed across the insect population via transovarial or transstadial transmision routs (Durvasula et al. 1997, Chavshin et al. 2012, 2014, 2015, Maleki-Ravasan et al. 2015). Symbionts currently aimed at in paratransgenesis include fungi (Rasgon 2011), symbiont bacteria of triatomine bugs (Durvasula et al. 1997, Durvasula et al. 1999, Durvasula et al. 2008), tsetse flies (Cheng and Aksoy 1999), sandflies (Maleki-Ravasan et al. 2015) and mosquitoes (Favia et al. 2007, Chavshin et al. 2014), and densoviruses infecting *An. gambiae* and *Ae. aegypti* mosquitoes (Ward et al. 2001, Ren et al. 2008). Recently, paratransgenesis have been successfully employed to reduce vector competence of the triatomine bug, *Rhodnius prolixus*, vector of *Trypanosoma cruzi*, the causative agent of Chagas disease (Durvasula et al. 1997), and *Anopheles gambiae* and *An. stephensi*, two main malaria vectors (Rasgon 2011, Wang and Jacobs-Lorena 2013). These data showed that the genetically manipulated symbionts could interfere with the development of the parasites in the vectors and provide the groundwork for the use of genetically modified symbionts as a potent tool to battle vector borne diseases.

The bacterium of *Wolbachia pipientis* is an intracellular organism and inherited maternally. It is established in more than 20% of all insects and a vast majority of other arthropods as well as filarial nematodes (Werren 1997a, Dobson 2004, Lo and Evans 2007). Recent studies imply that 20–76% of investigated insects give shelter to *Wolbachia* (Hilgenboecker et al. 2008), as well as many arachnids, terrestrial crustaceans, and mites (Cordaux et al. 2001, Gotoh et al. 2003, Rowley et al. 2004). This unique endosymbiont species was originally found in *Cx pipiens* but later molecular studies have discovered a number of phylogenetically diverse strains within the species (Lo et al. 2007). This endosymbiont bacterium has significant effects on its arthropod hosts and nominated as a bioagent to control important arthropod pests.

Wolbachia is the cause of various modifications in insect reproductive arrangement, comprising male-killing, feminization, cytoplasmic incompatibility (CI), and parthenogenesis (Werren et al. 2008). When CI occurs, sperm and eggs are not able to produce feasible progeny (Werren 1997b, Clark et al. 2003, Beckmann and Fallon 2013). Infected females relative to uninfected ones, participate more in offspring production, which permit *Wolbachia* to take up by all of host individuals even if it cases fitness costs (Field et al. 1999). The bacterium also can be used as a vector for delivering desirable genetic modifications in insect populations (Werren 1997b). As reviewed by Werren (1997a), *Wolbachia* have potential roles in

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the rapid speciation of their hosts. Also as a pandemic endosymbiont, *Wolbachia* can be recruited to control of a large number of human infectious diseases (Slatko et al. 2014). In filarial nematodes comprising *Wuchereria bancrofti*, *Brugia malayi*, *Brugia timori* and *Onchocerca volvulus* that infect humans, *Wolbachia* are obligated for proper development, fertility and survival, whereas in arthropods, although they can affect development and reproduction, but are not required for host survival. So *Wolbachia* have been a target for drug discovery against filariasis. In vivo/ vitro experiments indicate that antibiotics such as doxycycline and tetracycline can kill both adults and immature nematodes through depletion of *Wolbachia* (Foster et al. 2013, Taylor et al. 2014). It is also shown that, *Wolbachia* spp where naturally infected or artificially introduced into vector population can affect and decrease the mosquitoes competence carrying of viruses, such as Yellow Fever, Chikungunya, Dengue, West Nile, as well as ones transmitting of the *Plasmodium* protozoans and filarial nematodes (Bourtzis et al. 2014).

Due to the fact that *Wolbachia* is an obligate endosymbiont that cannot be cultured exterior their hosts, recognition of infection has been based vastly on amplification of *Wolbachia* DNA using PCR. Until now a number of loci including *wsp*, 16S rDNA, coxA, ftsZ, hcpA, gatB, groEL, fbpA, gltA and dnaA genes have been studied and evaluated in the phylogenetic studies (Zhou et al. 1998, Ravikumar et al. 2011). The sequences from *Wolbachia* surface protein (*wsp*) gene were extremely mutable and could be used to recognition and to re solve the phylogenetic relationships of different *Wolbachia* strains (Zhou et al. 1998).

In the present study we used a nested PCR assay to detect and investigate the prevalence of *Wolbachia* endobacteria using the partial genomic nucleotide sequence of *wsp* gene in twelve field populations of *Culex pipiens* in various geographical regions across Iran ranging from north to south. Results of this study will provide fundamental background for understanding ecology, distribution, and potential utility of *Wolbachia* as bio-control agent of *Cx. pipiens*.

Materials and Methods

Study areas

The study was conducted in twelve locations belong to three provinces of Iran, Mazandaran in the North (six locations), Isfahan in the center (3 locations) and Hormozgan in the South (3 locations) of the country (Fig. 1). Live larvae, pupae, and adult mosquitoes were collected from different biotypes including plane, jungle, riverside, rice field and human dwellings.

Mosquito collection

Adult mosquitoes were collected in human dwellings monthly for a period of five months (June to late October, 2014) by hand-catch collection method using mouth aspirator. Also live larvae and pupae were collected from mosquito breeding sites locating in plane, jungle, riverside and rice field using dipping method, transferred to insectary, and allowed them to grow till adult emergence. Adult specimens were keyed to species level using standard morphological keys (Zaim 1986, Azari-Hamidian and Harbach 2009). The male and female mosquito specimens belong to *Cx. pipiens* were selected and stored individually at -20 ºC for further molecular investigations. Double distilled water and mix of 10 adult male and female specimens of *Anopheles maculipennis* were collected from Mazanderan Province and used as negative controls.

DNA extraction and PCR

Totally 260 (120 males and 140 females) *Cx. pipiens* specimens originated from different biotopes from north to south of Iran

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were randomly subjected to genomic DNA extraction. Genomic DNA of *An. maculipennis ss* was extracted and used in all PCR assays as negative control. Total DNA of individual mosquitoes was extracted using Collins DNA extraction method (Collins et al. 1987). Previously a PCR based method for the classification of *Wolbachia* has been described (Zhou et al. 1998). In that method, group-specific wsp PCR primers have been used to identify *Wolbachia* strains without the need to clone and sequence individual *Wolbachia* genes. Here in detection of *Wolbachia* infection in the mosquitoes was performed by a nested-PCR assay on the basis of Zhou introduced primers. Initially, a set of primers including 81F: 5'–TGGTCCA ATAAGTGATGAAGAAAC–3' and 691R: 5'– AAAAATTAAACGCTACTCCA–3' were recruited to amplify 632 bp of partial sequence of the *wsp* gene. The PCR product of the first step was applied as a template for second step. In the second step, another pairs of the primers, 183F: 5'–AAGGAACCG AAGTTCATG–3' and 691R: 5'–AAAAA TTAAACGCTACTCCA–3', were used to amplify a 501 bp fragment.

The PCR amplification was performed using Maxime PCR PreMix Kit (i-Taq) Cat. No. 25026 in 20 µl reaction mixtures containing 2.5 µl of 10 µM both forward and reverse primers and 5 µl (~0.5 µg) of genomic DNA and 2.5 µl PCR product for the first and second step of nested-PCR reactions respectively. An individual specimen of *Anopheles maculipennis* s.s. was used as DNA extraction and PCR negative controls. The PCR conditions were set as an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were visualized on a 1% agarose gel containing ethidium bromide and using an UV transilluminator.

Wsp gene sequencing and analyzing

Representative specimens with clear and sharp *wsp* gene amplicons of the twelve *Cx. pipiens* populations were sequenced via the same amplification primers by Bioneer Company (S. Korea). The consensus of confident sequences was analyzed using NCBI (Nucleotide collection) database.

The *wsp* gene sequences determined in this study were subjected to molecular phylogenetic analysis together with 44 *wsp* gene sequences of *Wolbachia* from various arthropod host species retrieved from the Genbank database (Table 1). A multiple alignment of the *wsp* sequences was generated by the program package Clustal W (Thompson et al. 1994). Phylogenetic trees were constructed using the neighbor-joining method embedded in MEGA5 software. Bootstrap tests were performed with 1,000 replications.

Statistics analyzing

Wolbachia infection data in *Culex pipiens* specimens were analyzed using SPSS 22.0 and Chi square (χ^2) test to make comparisons and evaluate variation in infection rates between the males and females and among the twelve populations. The P-value more than 5% was considered as significant.

Results

Wolbachia detection in *Cx. pipiens*

The infection of *Wolbachia* in different *Cx. pipiens* populations was detected by the nested-PCR assay using *wsp* gene. The *amplicons* of first and second runs of nested-PCR assay were ~ 650 and 500 bp respectively (Fig. 2).

Wolbachia infection rate

Results of the study demonstrated that in total, 227 (87.3%) out of 260 individual adult mosquitoes belonged to 12 distinct populations were positive against *wsp* gene (Table 2). All the infected mosquitoes were

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found to harbor a single *w*Pip strain. Infection rate in adult females and males were 61.5–100% and 80–100% respectively. There were no significant differences between total infection rates of either sexes (Female= 89.2%, Male = 85.7%, df= 1, P> 0.05) or zones (df= 3, P> 0.05).

Wolbachia wsp sequences

Seven nested–PCR products the *wsp* gene of *Wolbachia* found in different Iranian populations of *Cx. pipiens* were successfully sequenced and submitted to Genbank (Accession Numbers (ANs): KM401551–7). The nested primers we used were only able to amplify fragments from infected specimens and not from uninfected *An. maculipennis ss* hosts. The sequences were A-T rich (61%) with only 39% GC content. The BLAST results indicated that all the *wsp* sequences of *Wolbachia* detected from the Iranian Cx. *pipiens* were 100% identical to each other and to the *Wolbachia* strains found in other members of the *Cx. pipiens* complex including *Cx. pipiens*, *Cx. pipiens* form *molestus*, *Cx. pipiens* (syn. *pallens*), and *Cx. quinquefasciatus* from remote geographical areas of the world (Table 3). Since the *Wolbachia* strain that infects *Cx. pipiens* complex belongs to Pip group of B supergroup (*w*PipB) (Zhou et al. 1998, Pidiyar et al. 2003), we can conclude that the *Wolbachia* strains from Iranian *Cx. pipiens* specimens belongs to *w*PipB strain. In addition, the sequences of *Wolbachia wsp* gene of Iranian *Cx. pipiens* were 100% identical to the *wsp* gene of *Wolbachia* strains found in divers insect or arthropod groups particularly to the order of Lepidoptera comprising 18 different butterfly and moth species, as well as to wasps, thrips, damselflies, *Aedes* mosquito, Three-striped fruit fly, leaf-mining fly, and mite. These *Wolbachia* host species belong to geographically remote regions of Asian, European, and African countries (Table 3). A comparison of the *wsp* sequences from the arthropod hosts showed up to 30.67% genetic diversity between taxa, in which the *wsp* sequence from bedbug was the most diverged one.

Phylogenetic analysis

For phylogenetic analysis a subset of the *Wolbachia* strains identified in this study were combined with a 44 available sequence data of other *Wolbachia* strains from Genbank. These sequences belonged to twenty different arthropod hosts of *Wolbachia* including mosquitoes (*Culex* and *Aedes*), fruit flies, blow flies, sand flies, tsetse flies, leaf mining flies, bed bugs, thrips, damselflies, plant hoppers, crickets, termites, butterflies, moths, wasps, ants, beetles, pill woodlouse, spiders, and mites (table 1). Phylogenetic tree was constructed using neighbor-joining method, based on the 445–511 bp of *wsp* sequences (Fig. 3). The length variation between sequence data was due to insertion or deletion (indels) events. We also used *Dirofilaria immitis wsp* sequence as an out-group in the analysis. Phylogenetic analysis showed that *Wolbachia* strains from Iranian *Cx. pipiens* specimens were clustered with *Wolbachia* strains of other members of the *Cx. pipiens* complex such as *Cx. pipiens*, *Cx. pipiens* (syn. *pallens*), *Cx. pipiens* form *molestus* and *Cx. quinquefasciatus* (Fig. 3). They also associated with *Wolbachia* strains found in distinct groups of arthropods not obtained from the same insect genus, family, or even order. In other word, *Wolbachia* strains obtained from the same insect genus or families were not clustered into distinct groups but were scattered throughout the phylogenetic tree. Except for the congenic clusters of mosquitoes, sand flies, and tsetse flies, there were no other congenic clusters indicating little congruence between *Wolbachia* phylogeny and host systematics. The phylogenetic analysis revealed six main clades for the *wsp* sequences of *Wolbachia* strains analysed (Fig. 3). The first clade was composed of all mosquitoes (eight *Culex* spp and two *Aedes* spp) and ten *wsp* sequences from lepidopteran, wasp, Thrips, damselfly, Three-striped fruit fly, leaf-mining fly, leaf beetle, and mite, all belonged to the known super-group B of *Wolbachia*. The second lineage was composed of nine *wsp* sequences from blowfly, plant hopper, cricket, moth, wasp, fire ant, flour beetle, and mite. Eleven *wsp* sequences from fruit flies, sand flies (2 species), tsetse flies (2 species), termite, moth, wasps (2 species), ant, and spider, constituted an isolated lineage. The *wsp* sequences from one of each wasp, plant hopper, and moth formed a distinct clade. Most of strains of second and third clades belong to the known supergroup A of *Wolabachia*. Notably the bedbug and one termite *wsp* sequences associated together and formed a well-defined clade, and finally pill wood louse constituted a diverse clade well separated from other five clades. Except for four nodes with 57–71% support, all of the nodes had very high (82–100) bootstrap support values (Fig. 3).

Fig. 1. Map of study areas for collection of *Culex pipiens* specimens in Iran. Nos, 1–2: Ramsar, 3–4: Amol, 5–6: Behshahr in Mazandaran Province, 7: Vinicheh, 8: Dizicheh, 9: Dorcheh in Isfahan Province, 10: Hormodar, 11: Siahoo, and 12: Shamil in Hormozgan Province

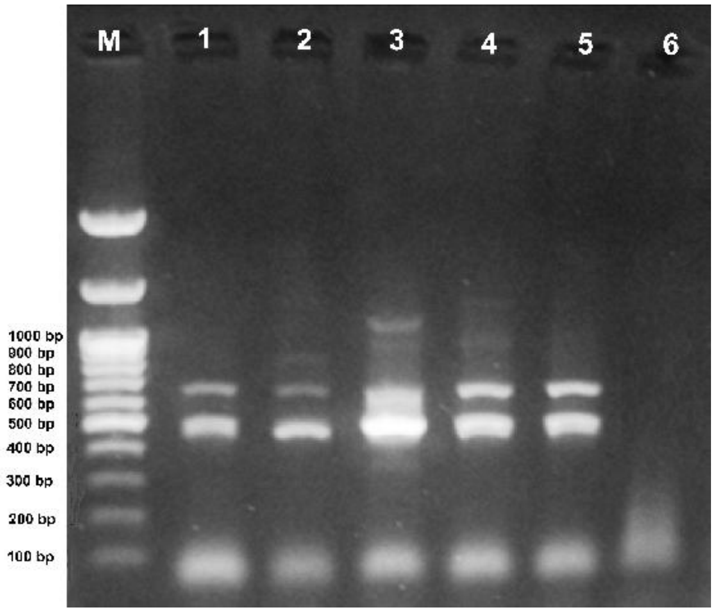

Fig. 2. Species-specific nested-PCR products (~ 500 bp) of *Wolbachia wsp* gene of *Culex pipiens* specimens. Lanes: M, 1 Kbp molecular weight marker (Fermentas), 1–2: Mazandaran Provine, 3: Isfahan Provine, 4–5: Hormozgan Provine, 6: *Anopheles maculipennis* as negative control

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Table 1. Description of *Wolbachia* strains used for phylogenetic analysis in this study

No	*Wolbachia* Strain	Host	Common name	Accession number	References
1	*w*Pip/B	*Culex pipiens*	Mosquito	KM401552	This study
2	*w*Pip/B	*Cx. pipiens*	Mosquito	KM401553	This study
3	*w*Pip/B	*Cx. pipiens*	Mosquito	KM401556	This study
4	*w*Pip/B	*Cx. pipiens*	Mosquito	JX474753	Direct Submission
5	*w*Pip/B	*Cx. pipiens* (syn. *pallens*)	Mosquito	AF216860	Direct Submission
6	*w*Pip/B	*Cx. pipiens* form *molestus*	Mosquito	HG428761	(Pinto et al. 2013)
7	*w*Pip/B	*Cx. quinquefasciatus*	Mosquito	AF020060	(Zhou et al. 1998)
8	*w*Pip/B	*Cx. quinquefasciatus*	Mosquito	KJ140126	Direct Submission
9	*w*AlbB/B	*Aedes albopictus*	Mosquito	AF020059	(Zhou et al. 1998)
10	*w*Pip/B	*Ae. punctor*	Mosquito	AJ311040	(Ricci et al. 2002)
11	*w* AlbA/A	*Ae. albopictus*	Mosquito	AF020059	(Zhou et al. 1998)
12	*w*No/B	*Drosophila simulans*	Fruit Fly	AF020074	(Zhou et al. 1998)
13	*w*Mel/A	*D. melanogaster*	Fruit Fly	AF020072	(Zhou et al. 1998)
14	*w*Aus/A	*Glossina austeni*	Tsetse fly	AF020077	(Zhou et al. 1998)
15	*w*Mors/A	*G. morsitans morsitans*	Tsetse fly	AF020079	(Zhou et al. 1998)
16	N.S	*Protocalliphora sialia*	Blow fly	DQ842482	(Baldo et al. 2006)
17	*w*Pak-B1	*Hydrellia pakistanae*	Leaf mining fly	AF217718	(Jeyaprakash and Hoy, 2000)
18	papa01/A	*Phlebotomus papatasi*	Sand fly	EU780683	(Parvizi et al. 2013)
19	Turk 07	*Ph. mongolensis*	Sand Fly	KC576916	(Parvizi et al. 2013)
20	*w*Con/B	*Tribolium confusum*	Flour Beetle	AF020083	(Zhou et al. 1998)
21	N.S	*Chelymorpha alternans*	Leaf Beetle	DQ842458	(Baldo et al., 2006)
22	*w*Ori/B	*Tagosodes orizicolus*	Plant hopper	AF020085	(Zhou et al. 1998)
23	*w*Stri/B	*Laodelphax striatellus*	Plant hopper	AF020080	(Zhou et al. 1998)
24	F	*Cimex lectularius*	Bed Bug	DQ842459	(Baldo et al. 2006)
25	*w*Dei/B	*Trichogramma deion*	Wasp	AF020084	(Zhou et al. 1998)
26	*w*Tde-HEB	*T. dendrolimi*	Wasp	JX027991	Direct Submission
27	*w*kue/A	*Spalangia cameroni*	Wasp	AF289668	Direct Submission
28	N.S	*Encarsia formosa*	Wasp	DQ842471	(Baldo et al. 2006)
29	*w*NPan/A	*Nomada panzeri*	Red Wasp	KC798315	(Gerth et al. 2013)
30	A	*Solenopsis invicta*	Fire Ant	DQ842483	(Baldo et al. 2006)
31	A	*Formica truncorum*	Ant	AF326978	(Wenseleers et al. 2002)
32	*w*CauB/B	*Ephestia cautella*	Moth	AF020076	(Zhou et al. 1998)
33	*w*CauA/A	*Ephestia cautella*	Moth	AF020075	(Baldo et al. 2006)
34	B	*Ostrinia scapulalis*	Moth	DQ842481	(Baldo et al. 2006)
35	NS	*Eurema hecabe*	Butterfly	AB285478	(Narita et al. 2007)
36	NS	*Udaspes folus*	Butterfly	JN236179	(Salunke et al. 2012)
37	NS	*Agriocnemis femina*	Damselfly	AY173939	(Thipaksorn et al. 2003)
38	NS	*Gryllus firmus*	Cricket	DQ842474	(Baldo et al. 2006)
39	A	*Incisitermes snyderii*	Termite	DQ842475	(Baldo et al. 2006)
40	F	*Coptotermes acinaciformis*	Termite	AJ833931	(Baldo et al. 2006)
41	NS	*Hercinothrips femoralis*	Thrips	AB245521	Direct Submission
42	NS	*Nephila clavata*	Spider	EF612772	Direct Submission
43	NS	*Oxyopes sertatus*	Spider	EF612771	Direct Submission
44	NS	*Eriovixia cavaleriei*	Spider	DQ778738	Direct Submission
45	NS	*Tetranychus urticae*	Two-spotted spider mite	AJ437290	Direct Submission
46	NS	*Bryobia berlesei*	Mite	JN572865	(Ros et al. 2012)
47	NS	*Armadillidium vulgare*	Pill woodlouse	DQ842457	(Baldo et al. 2006)
48	Outgroup	*Dirofilaria immitis*	Nematode	AJ252062	(Bazzocchi et al. 2000)

NS: Not stated.

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Table 2. Prevalence of *Wolbachia pipientis* infection in the *Culex pipiens* collected from North, Center and South of Iran, 2014

Province	Location	Biotope	Males tested (% P +)	Females tested (% P+)	Total (% P+)
Mazandaran (North)	Amol 1	Plane	10(90)	13(61.5)	74
	Amol 2	Jungle	10(80)	10(100)	90
	Behshar 1	Plane	10(100)	10(100)	100
	Behshar 2	Jungle	10(90)	10(90)	90
	Ramsar 1	Plane	10(90)	10(80)	85
	Ramsar 2	Jungle	10(100)	14(100)	100
Isfahan (Center)	Dizicheh	Rice fields	10(90)	10(90)	90
	Vinicheh	Rice fields	10(80)	10(70)	75
	Dorcheh	Rice fields	10(100)	15(100)	100
Hormozgan (South)	Shamil	Date Groves	10(80)	13(61.5)	70
	Siahoo	Riverside	10(80)	10(90)	85
	Hormoodar	Date Groves	10(90)	15(86.7)	88
Total			120(89.2)	140(85.7)	87.3 (260)

Table 3. Details of arthropods have identical *Wolbachia wsp* sequences with the Iranian *Culex pipiens*

Arthropod group	Species	Accession Number	Country	Reference
Mosquito	*Culex pipiens*	JX474753	Turkey	Direct Submission
	Cx. pipiens form *molestus*	HG428761	NS	(Pinto et al. 2013)
	Cx. pipiens (Syn. *pallens*)	AF216860	China	Direct Submission
	Cx. quinquefasciatus	KJ140126	China	Direct Submission
	Cx. quinquefasciatus	EU194487	India	Direct Submission
	Cx. quinquefasciatus	AF397413,	India	Direct Submission
	Cx. quinquefasciatus	AF397412	India	Direct Submission
	Cx. quinquefasciatus	AY462861	Taiwan	(Tsai et al. 2004)
	Cx. quinquefasciatus	AM999887	NS	(Klasson et al. 2008)
	Aedes punctor	AJ311040	Italy	(Ricci et al. 2002)
Butterfly	*Udaspes folus*	JN236179	India	(Salunke et al. 2012)
	Hypolimnas bolina	JN236180	India	(Salunke et al. 2012)
	Castalius rosimon	JN236182	India	(Salunke et al. 2012)
	Eurema hecabe	JN236189	India	(Salunke et al. 2012)
	Ypthima asterope	JN236192	India	(Salunke et al. 2012)
	Papilio demoleus	JN236193	India	(Salunke et al. 2012)
	Zizeeria knysna	JN236194	India	(Salunke et al. 2012)
	Colotis amata	JN236195	India	(Salunke et al. 2012)
	Pseudozizeeria maha	JN236205	India	(Salunke et al. 2012)
	Leptidea sinapis	KC137222	NS	(Russell et al. 2012)
	Pararge aegeria	KC137224	NS	(Russell et al. 2012)
	Polygonia calbum	JN093149	NS	(Kodandaramaiah et al. 2011)
	Hypolimnas bolina	AJ307076	Fiji	(Dyson et al. 2002)
Moth	*Corcyra cephalonica*	KC844060	China	Direct Submission
	Epirrita autumnata	JX310335	NS	(Kvie et al. 2012)
	Spodoptera exempta	JN656943	Tanzania	Direct Submission
	Corcyra cephalonica	AY634679	China	Direct Submission
	Acraea encedon	AJ271198	Tanzania	Direct Submission
Wasp	*Trichogramma chilonis*	AY311486	China	Direct Submission
	T. dendrolimi	JX027991	China	Direct Submission
	T. brassicae	AF452646	China	Direct Submission
	T. dendrolimi	DQ017751	China	Direct Submission
	T. japonicum	KC161917	China	Direct Submission
	Tropobracon schoenobii	AF481194	NS	(Kittayapong et al. 2003)
Thrips	*Hercinothrips femoralis*	AB245521	Japan	Direct Submission
Damselfly	*Agriocnemis femina*	AY173939	NS	(Thipaksorn et al. 2003)
	Coenagrionidae sp	KC161926	China	Direct Submission

Table 3. Continued…

Fruit fly	*Bactocera diversa*	AF295353	NS	(Jamnongluk et al. 2002)
Leaf-mining fly	*Hydrellia pakistanae*	AF217718)	NS	(Jeyaprakash and Hoy 2000)
Mite	*Bryobia berlesei*	JN572865	France	(Ros et al. 2012)

NS: Not stated.

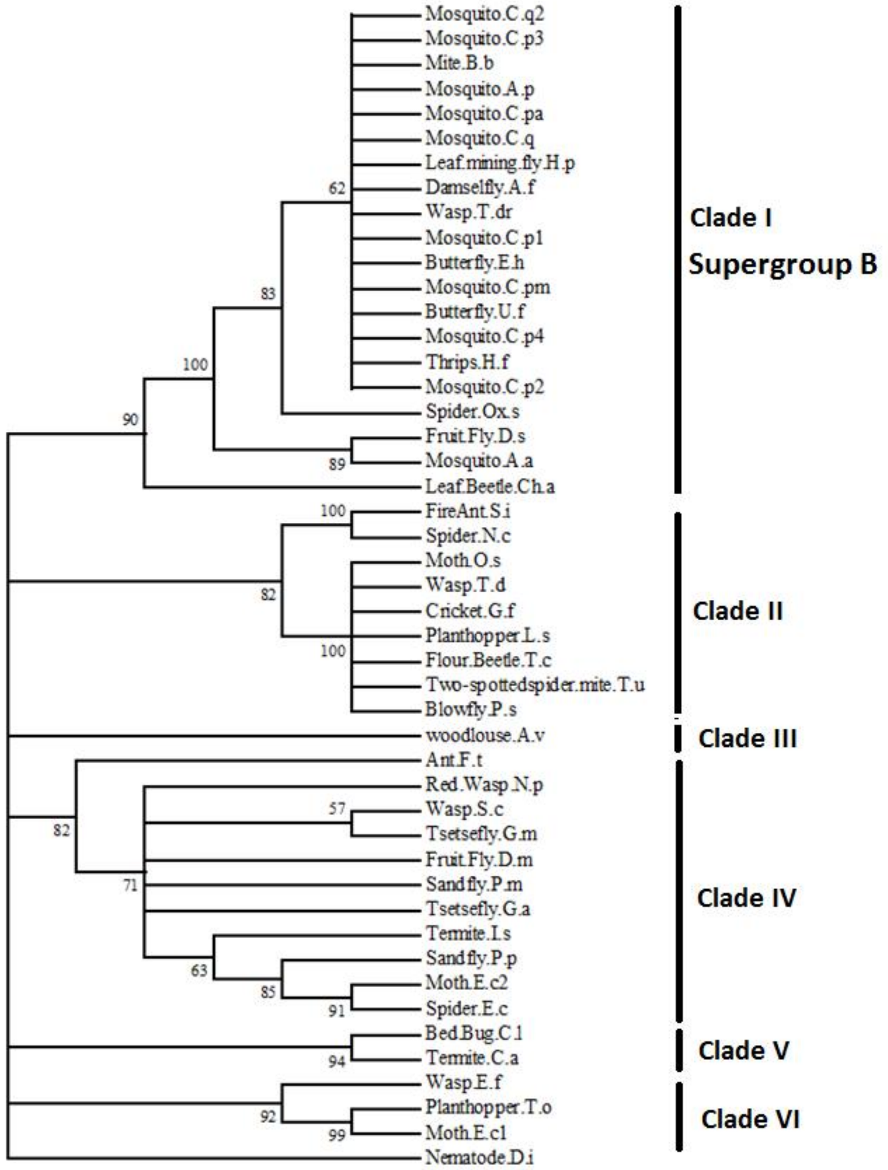

Fig. 3. The phylogenetic tree inferred from 445–511 bp of *wsp* sequences of *Wolbachia pipientis* hosts using the neighbor-Joining method embedded in MEGA 5.0. C.p1–3 (*Culex pipiens* from this study), C.p4 (*Culex pipiens*), C.pm (*Culex pipiens* form *molestus*), C.q and C.q2 (*Culex quinquefasciatus*), C.pa (*Culex pipiens, syn.: pallens*), A.a (*Aedes albopictus*), D.m (*Drosophila melanogaster*), D.s (*Drosophila simulans),* G.m (*Glossina morsitans morsitans*), G.a (*Glossina austeni*), P.s (*Protocalliphora sialia*), P.p (*Phlebotomus papatasi*), P.m (*Phlebotomus mongolensis*), T.c (*Tribolium confusum*), Ch.a (*Chelymorpha alternans*), L.s (*Laodelphax striatellus*), T.o (*Tagosodes orizicolus*), C.l (*Cimex lectularius*), T.d (*Trichogramma deion*), T.dr (*T.dendrolimi*), S.c (*Spalangia cameroni*), E.f (*Encarsia formosa*), N.p (*Nomada panzeri*), S.i (*Solenopsis invicta*), , F.t (*Formica truncorum*), E.c1–2 (*Ephestia cautella*), O.s (*Ostrinia scapulalis*), E.h (*Eurema hecabe*), G.f (*Gryllus firmus*), I.s (*Incisitermes snyderii*), C.a (*Coptotermes acinaciformis*), N.c (*Nephila clavata*), Ox.s (*Oxyopes sertatus*), E.c (*Eriovixia cavaleriei*), T.u (*Tetranychus urticae*), A.v (*Armadillidium vulgare*), A.f (*Agriocnemis femina*), H.f (*Hercinothrips femoralis*), B.b (*Bryobia berlesei*), A.p (*Aedes punctor*), U.f (*Udaspes folus*), H.p (*Hydrellia pakistanae*), and D.i (*Dirofilaria immitis*). The bootstrap values are shown as numbers on the nodes

Discussion

This is the first report on *Wolbachia* infection from *Cx. pipiens* populations of Iran. In our study, 260 specimens of *Cx. pipiens* collected from the 12 villages were individually assayed for *Wolbachia*, and the overall rate of infection was determined to be 87.3%. This result is in agreement with previous study conducted in South West Iran revealed 100 percent *Wolbachia* infection in *Cx. quinquefasciatus* specimens (Behbahani 2012). In California, *Wolbachia* infection frequency in *Cx*. *pipiens* complex during 1999 and 2000 was 99.4% (Rasgon and Scott, 2003). Also Sunish et al. (2011) found an overall prevalence of 91.2% *Wolbachia* infections in *Cx. quinquefasciatus* mosquitoes from south India. Study of Chen et al (2013) revealed that three *Cx. pipiens* (Syn. *pallens*) populations of China were all infected with *Wolbachia*. This rate was reported between 10–100% in members of *Cx. pipiens* complex mosquitoes from the Upper Rhine Valley in Germany and Cebu City in Philippines (Mahilum et al. 2003).

In this study we found no *Wolbachia* infection in *An. maculippenis* ss specimens which is in concurrence of study of Rasgon and Scott (2004) who tested five genera of mosquito (*Aedes*, *Anopheles*, *Culiseta*, *Culex*, and *Ochlerotatus*) for *Wolbachia*, and infections was only detected in members of the *Cx. pipiens* complex. Also study of Kittayapong et al. (2000) detected *Wolbachia* infection in all main disease vector genera excluding *Anopheles*. In our study, the percentage prevalence in adult males was 80–100%, while in females were 61.5–100%. However the difference was not significant between males and females. In contrast, in the study of Sunish et al (2011) the rate of *Wolbachia* infection in females of *Cx. quinquefasciatus* was found slightly higher than in males but like our study it was not statistically significant.

This study showed no sequence variation in *wsp* gene of *Wolbachia* from *Cx. pipiens* populations across geographical regions of Iran, which is similar to the results of Morais et al. (2012) which showed that both *Cx. quinquefasciatus* and *Cx. pipiens* × *Cx. quinquefasciatus* hybrids collected Brazil and Argentina were infected with a single *Wolbachia* strain. The genetic similarity detected among *Wolbachia* samples in the *Culex* mosquitoes from geographically scattered regions may be explained by either *Wolbachia* host-endosymbiont specificity (Werren et al. 2008) or recently Wolbachia infection in *Culex* populations (Morais et al. 2012).

High sequence homology and close phylogenetic relationships of *Wolbachia* strains from mosquitoes, spider, wasp, mite, damselfly, butterfly, thrips, fruit fly, and leaf mining fly indicate that *Wolbachia* endosymbionts not only are maternally transmitted through host generations by vertical transmission but also horizontally transfer between unrelated host organisms (i.e. shift host species or “jumping”) (Van Meer et al. 1999, Baldo et al. 2005). Although the mechanisms of jumping are still unclear, it is believed that parasitoids may involve (Heath et al. 1999, Huigens et al. 2000, Noda et al. 2001, Kikuchi and Fukatsu 2003). Recombination in *wsp* gene of *Wolbachia* strains has been evidenced by other researchers (Werren and Bartos 2001, Jiggins 2002, Reuter and Keller 2003). For example, Werren and Bartos (2001) reported recombination within supergroup B, occurring between the two *Wolbachia* strains of a parasitoid wasp and the fly it parasitizes. More recently it is shown that hypervariable regions of *wsp* gene of *Wolbachia* strains have got a complex mosaic structure, suggesting a clear intragenic recombination of segments among several divergent strains, both within and

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between the arthropod supergroups (Baldo et al. 2005).

The phylogenetic analysis of *wsp* sequences of *Wolbachia* from 20 different arthropod hosts scattered the sequences into five main clades that in some parts, topographically matched well with the tree of Zhou et al. (1998). Based on *Wolbachia* ftsZ gene sequences, two major supergroups A and B were reported within the *Wolbachia* strains (Werren and Jaenike 1995) where the type strain from *Cx. pipiens* was placed within supergroup B. In the tree we obtained in this study, two main clades represent supergroups A and B (Fig. 3). In addition to the *Wolbachia* strains from mosquitoes, the strains from spider, wasp, mite, damselfly, butterfly, thrips, fruit fly, and leaf mining fly also placed in supergroup B. Interestingly the *Wolbachia* strain from bedbug was associated with the one from termite of supergroup F or H. As reviewed by Lo et al. (2007), currently the genus *Wolbachia* was divided into eight taxonomic supergroups (A to H) where A and B are the two major groups established in arthropods, C and D are found in filarial nematodes, E infecting springtails and F contains *Wolbachia* bacteria that infect termites and filarial species. Supergroup G and H were reported in spiders and termites respectively. In addition other divergent lineages, such as those from various flea species and the filarial nematode *Dirofilaria repens*, might be added to the list of supergroups. Therefore, as more sequence information becomes available the number of clades, groups, or supergroups might be increased. For example, in our analysis the *Wolbachia* from woodlouse construct a single clade and might be considered as a separate clade.

Conclusion

In this study we found a single *Wolbachia* strain from *Cx. pipiens* populations across the country. Although it is suggested that a large set of compatible *Wolbachia* strains are always locally dominate within mosquito populations (Duron et al. 2011), however, several studies have showed that some *w*Pip strains are reciprocally incompatible but also that some others, although genetically distinct, are fully compatible (Duron et al. 2006, Duron et al. 2007, Atyame et al. 2011). Therefore, it is worth to test cytoplasmic incompatibility (CI) between the Iranian populations. In case of having CI, it can be used as a form of sterile-insect technique (SIT), to suppress, to replace, or to reduce the survival of mosquito populations and thereby control them or reduce their ability to transmit the infection (Townson 2002).

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