Original Article

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

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(Received 15 Apr 2015; accepted 3 Oct 2015)

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 mite 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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Published Online: January 06, 2016
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 transstadiial transmission routes (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 (Wenren 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
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 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 a now number of loci including wsp, 16S rDNA, coxA, ftsZ, hcpA, gatB, groEL, hbpA, 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 resolve 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 °C 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
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 ATAAATGATGAAGAAAC–3’ and 691R: 5’–AAAAATTAACGTACTCCA–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 MEGAS 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 (χ²) 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
found to harbor a single wPip 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 (wPipB) (Zhou et al. 1998, Pidiyar et al. 2003), we can conclude that the *Wolbachia* strains from Iranian *Cx. pipiens* specimens belongs to wPipB 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, Thrip-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 com-
posed 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 supergroup 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 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 Wolbachia. 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).
Table 1. Description of *Wolbachia* strains used for phylogenetic analysis in this study

| No | Wolbachia Strain | Host | Common name | Accession number | References |
|----|------------------|------|-------------|------------------|------------|
| 1  | wPip/B           | Culex pipiens | Mosquito | KM401552         | This study |
| 2  | wPip/B           | Cx. pipiens | Mosquito | KM401553         | This study |
| 3  | wPip/B           | Cx. pipiens | Mosquito | KM401556         | This study |
| 4  | wPip/B           | Cx. pipiens | Mosquito | JX474753         | Direct Submission |
| 5  | wPip/B           | Cx. pipiens (syn. pallens) | Mosquito | AF216860 | Direct Submission |
| 6  | wPip/B           | Cx. pipiens form molestus | Mosquito | HG428761 | (Pinto et al. 2013) |
| 7  | wPip/B           | Cx. quinquefasciatus | Mosquito | AF020060 | (Zhou et al. 1998) |
| 8  | wPip/B           | Cx. quinquefasciatus | Mosquito | AJ110400 | (Ricci et al. 2002) |
| 9  | wAlbA/B          | Ae. albopictus | Mosquito | AF020059 | (Zhou et al. 1998) |
| 10 | wAlbB/B          | Ae. puncior | Mosquito | AF020059 | (Zhou et al. 1998) |
| 11 | wNo/B            | Drosophila simulans | Fruit Fly | AF020074 | (Zhou et al. 1998) |
| 12 | wMel/B           | D. melanogaster | Fruit Fly | AF020072 | (Zhou et al. 1998) |
| 13 | wMors/A          | G. morsitans | Mosquito | AF020077 | (Zhou et al. 1998) |
| 14 | wNo/B            | G. morsitans | Mosquito | AF020077 | (Zhou et al. 1998) |
| 15 | wPak-B1          | Hydrellia paxianae | Leaf mining fly | AF217718 | (Jayaprakash and Hoy, 2000) |
| 16 | Turk 07          | Ph. mongolensis | Sand Fly | KC56916 | (Varzi et al. 2013) |
| 17 | wCon/B           | Tribolium confusum | Flour Beetle | AF020083 | (Zhou et al. 1998) |
| 18 | N.S              | Chelymorpha alternans | Leaf Beetle | DQ842458 | (Baldo et al., 2006) |
| 19 | wOri/B           | Tagosodes orizicolus | Plant hopper | AF020085 | (Zhou et al. 1998) |
| 20 | wStri/B          | Laodelphax striatellus | Plant hopper | AF020080 | (Zhou et al. 1998) |
| 21 | F                | Cimex lectularius | Bed Bug | DQ842459 | (Baldo et al. 2006) |
| 22 | wDei/B           | Trichogramma deion | Wasp | AF020084 | (Zhou et al. 1998) |
| 23 | wTde-HEB         | T. dendrolimi | Wasp | JX027991 | (Parvizi et al. 2013) |
| 24 | N.S              | Encarsia formosa | Wasp | DQ842471 | (Baldo et al. 2006) |
| 25 | wNPan/A          | Nomada panzeri | Red Wasp | KC798315 | (Gerth et al. 2013) |
| 26 | A                | Solenopsis invicta | Fire Ant | DQ842483 | (Baldo et al. 2006) |
| 27 | A                | Formica truncorum | Ant | AF326978 | (Wenseleers et al. 2002) |
| 28 | wCauB/B          | Euphyes cautella | Moth | AF020076 | (Zhou et al. 1998) |
| 29 | wCau/A/A         | Euphyes cautella | Moth | AF020075 | (Baldo et al. 2006) |
| 30 | B                | Ostrinia scapulalis | Moth | DQ842481 | (Baldo et al. 2006) |
| 31 | N.S              | Eurema hecabe | Butterfly | AB285478 | (Varzi et al. 2007) |
| 32 | N.S              | Udaupes folius | Butterfly | JN236179 | (Salunke et al. 2012) |
| 33 | N.S              | Agrionemis femin | Damsel Fly | AJ173939 | (Thupakorn et al. 2003) |
| 34 | N.S              | Gryllus firmus | Cricket | DQ842474 | (Baldo et al. 2006) |
| 35 | A                | Incisitermes sniderii | Termite | DQ842475 | (Baldo et al. 2006) |
| 36 | F                | Coptotermes acinaciformis | Termite | AJ833931 | (Baldo et al. 2006) |
| 37 | N.S              | Hercinotrips femoralis | Thrips | AB245521 | Direct Submission |
| 38 | N.S              | Nephila clavata | Spider | EF612772 | Direct Submission |
| 39 | N.S              | Oxyopes sertatus | Spider | EF612771 | Direct Submission |
| 40 | N.S              | Eriovixia canaliculata | Spider | DQ778738 | Direct Submission |
| 41 | N.S              | Tetranuchus urticae | Two-spotted spider mite | AJ437290 | Direct Submission |
| 42 | N.S              | Bryobia berlesei | Mite | JN572865 | (Ros et al. 2012) |
| 43 | N.S              | Armadillidium vulgare | Pill woodlouse | DQ842457 | (Baldo et al. 2006) |
| 44 | Outgroup         | Dirofilaria immitis | Nematode | AJ252062 | (Bazzocchi et al. 2000) |

NS: Not stated.
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)  | Duzicheh | 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) | Shamul   | 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* form molestus  | JX474753         | Turkey  | Direct Submission          |
|                 | *Culex pipiens* (Syn. pallens)| HG428761         | NS      | (Pinto et al. 2013)         |
|                 | *Cx. quinquefasciatus*         | AF216860         | China   | Direct Submission          |
|                 | *C. quinquefasciatus*         | KJ140126         | China   | Direct Submission          |
|                 | *C. quinquefasciatus*         | EU194447         | India   | Direct Submission          |
|                 | *C. quinquefasciatus*         | AF397413,        | India   | Direct Submission          |
|                 | *C. quinquefasciatus*         | AE462861         | Taiwan  | (Tsai et al. 2004)         |
|                 | *C. quinquefasciatus*         | AM999887         | NS      | (Klasson et al. 2008)      |
| Butterfly       | *Aedes punctor*               | AJ311040         | Italy   | (Ricci et al. 2002)        |
|                 | *Udaspes folus*               | JN236179         | India   | (Salunke et al. 2012)      |
|                 | *Hypolimnas bolina*           | JN236180         | India   | (Salunke et al. 2012)      |
|                 | *Castalia rosimon*            | JN236182         | India   | (Salunke et al. 2012)      |
|                 | *Eurema hecabe*               | JN236189         | India   | (Salunke et al. 2012)      |
|                 | *Yphima asterope*             | JN236192         | India   | (Salunke et al. 2012)      |
|                 | *Papilio demoleus*            | JN236193         | India   | (Salunke et al. 2012)      |
|                 | *Zizeeria kaysera*            | JN236194         | India   | (Salunke et al. 2012)      |
|                 | *Colotis amata*               | JN236195         | India   | (Salunke et al. 2012)      |
|                 | *Pseudocizeeria 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      | (Kandaramaiah et al. 2011) |
| Moth            | *Hypolimnas bolina*           | AJ307076         | Fiji    | (Dyson et al. 2002)        |
|                 | *Corcyra cephalonica*         | KC844060         | China   | Direct Submission          |
|                 | *Epiprita autumata*           | 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          |
|                 | *Agrionemis femina*           | AX173939         | NS      | (Thipakson et al. 2003)    |
| Damselfly       | *Coenagrionidae sp*           | KC161926         | China   | Direct Submission          |
**Table 3.** Continued...

| Fruit fly           | Leaf-mining fly          | Mite                      | Accession | Country      | Reference                        |
|---------------------|--------------------------|---------------------------|-----------|--------------|----------------------------------|
| Bactocera diversa  | Hydrellia pakistanae    | Bryobia berlesei          | AF295353  | NS (Jammongluk et al. 2002)       |
|                     | (Jamnongluk et al. 2002)|                           | AF217718  | NS (Jeyaprakash and Hoy 2000)     |
|                     |                          |                           | 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 (Protocoliphora sialia), P.p (Phlebotomus papatasi), P.m (Phlebotomus mongolensis), T.c (Tricholium confusum), C.a (Chelomyopa alternans), L.s (Laodelphax striatellus), T.o (Trogosodes orizicolus), C.l (Cimex lectularius), C.t (Cicada lectularia), T.d (Trichogramma deion), T.d (T.dendrolimi), S.c (Spalangia cameroni), E.f (Encarsia formosana), N.p (Nomadina panzeri), S.i (Solenopsis invicta), F.t (Formica truncorum), E.c1–2 (Ephesia cautella), O.s (Ostrinia scapulalis), E.h (Eurema hecabe), G.f (Grallus firmus), L.s (Incisitermes snyderii), C.a (Coptotermes acinaciformis), N.c (Nephele clavata), O.x.s (Oxyopes sertatus), E.c (Eroicovisia calvareia), T.u (Tetranychus urticae), A.v (Armadillium vulgare), A.f (Agriocnemis femina), H.f (Hercinthrips femoralis), B.b (Bryobia berlesei), A.p (Aedes punctor), U.f (Udaspes fulus), H.p (Hydrellia pakistanae), and D.i (Dirofilaria immitis). The bootstrap values are shown as numbers on the nodes.

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Published Online: January 06, 2016
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. maculipennis 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 Kitta-yapong 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-endo symbiont 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 transferred 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
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 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 wPip 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).

**Acknowledgements**

Tehran University of Medical Sciences (TUMS) financially supported this work (Grant No. 22738). Also it is noteworthy that this research has been done by support of the Babol University of Medical Sciences (BUMS). Special thanks to Roghayeh Pourbagher, Seyyedeh Narges Mousavi Kani, Zeinab Abedian, and Seyed Mohsen Aghajanpour Mr for helping in Cellular and Molecular Laboratory. The authors appreciate Mr Bagheri and Mr Hosseintabar for their cooperation in the laboratory. We would like to thank Mr Pakari, Mr Salari and Mr Arandian for helping in field collections.

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Published Online: January 06, 2016