wsp-based analysis of *Wolbachia* strains associated with *Phlebotomus papatasi* and *P. sergenti* (Diptera: Psychodidae) main cutaneous leishmaniasis vectors, introduction of a new subgroup wSerg

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ABSTRACT

Sand flies of *Phlebotomus papatasi* and *P. sergenti* are the main vectors of cutaneous leishmaniasis (CL) in the old world. We aimed to screen Iranian *P. papatasi* and *P. sergenti* for their natural infections with *Wolbachia* and to determine their phylogenetic association with other species. *Wolbachia* surface protein (wsp) gene was PCR amplified from DNA extracted from *Phlebotomus* species, sequenced, and were analysed in combination with *wsp* sequences related to Phlebotominae and other insects. All *Wolbachia*-infecting Iranian sand flies of *P. papatasi* and *P. sergenti* were classified in the Supergroup A., *Wolbachia* isolated from *P. sergenti* were clustered in a new subgroup within Supergroup A so-called wSreg. The *Wolbachia* strains identified from the *P. papatasi* clustered mainly in the subgroup wPap and partly in wSerg. Multiple *Wolbachia* infection within a single population of *P. papatasi* warrants investigation on existence and intensity of cytoplasmic incompatibility between the wPap and wSerg subgroups.

KEYWORDS

Wolbachia; sand fly;
Phlebotomus papatasi;
Phlebotomus sergenti; sub/
supergroup

Introduction

Neglected tropical diseases (NTDs) are a diverse group of infectious diseases that affect more than one billion people in tropical and subtropical areas in 149 countries [1]. Leishmaniasis is one of the thirteen NTDs that is caused by *Leishmania* parasites and transmitted to vertebrate hosts by the infective bites of female Phlebotomine (Diptera: Psychodidae) sand flies. Phlebotomine sand flies are also vectors of several viral, bacterial, and other protozoal diseases of humans and other animals [2,3]. Historically both cutaneous and visceral leishmaniasis have been endemic diseases in Iran [4]. Currently there are two forms of cutaneous leishmaniasis in Iran namely Anthroponotic Cutaneous Leishmaniasis (ACL) which is caused by *L. tropica* and transmitted mainly by *Phlebotomus sergenti* [5,6] and Zoonotic Cutaneous Leishmaniasis (ZCL) which is caused by *L. major* and transmitted primarily by *P. papatasi* [7,8]. Both *P. papatasi* and *P. sergenti* were incriminated as proven vectors of ACL and ZCL respectively around the old world and they are often sympatric in their distribution ranges [9–12]. *Wolbachia* are maternally transmitted Alphaproteobacteria that are found in most of arthropod (up to 75%) and nematode

species [13]. *Wolbachia* is known a very good model of manipulation of the host biology by the symbiont [14]. These bacteria can distort a number of reproductive phenotypes in their hosts, such as cytoplasmic incompatibility, parthenogenesis, feminization, and male-killing. Recently, it was revealed that *Wolbachia* infection makes *Drosophila* fruit flies more resistant against various RNA viruses [13, 15]. This antiviral effect is also effective in other dipterans like mosquitoes of *Culex quinquefasciatus* or *Aedes aegypti* and might therefore be recruited to reduce their competence for carrying viruses like Dengue, West Nile, Yellow fever, Zika, and Chikungunya [16–20]. Aside from viruses, different *Wolbachia* strains can reduce *Plasmodium* development in *Anopheles* species or filarial nematodes in *Aedes* mosquitoes [21–23]. The *Wolbachia* strain of wMelPop which drastically shortens the lifespan of *D. melanogaster*, were successfully introduced to *Ae. aegypti*. It could halve the average lifespan of laboratory populations and created hopes for reducing the efficacy of dengue fever vector in field conditions [24–27]. Although there is no published data about use of *Wolbachia* as a vector control agent in sand flies yet, however, *Wolbachia*-based distortions might become a

new tool to develop novel control strategies for sand fly-borne diseases.

Various genes have been used by scientists to study *Wolbachia* for various purposes. The *Wolbachia* surface protein gene (*wsp*) has been widely used for macro-taxonomy (Supergroup designation) and micro-taxonomy (subgroup designation) and of the genus *Wolbachia*, as well as for detection of new lineages. So far seventeen Supergroups of *Wolbachia* have been known, named A-Q (except G) plus a new un-named strain from cave spider species [28]. The majority of insect *Wolbachia* strains belong to Supergroups A and B. A general naming system for *Wolbachia* was established by Zhou, Rousset [29] based on *wsp* gene. Currently, Super group A including fourteen subgroups of Mel, AlbA, Mors, Riv, Uni, Haw, Pap, Aus, Whi, Lop, Eum, Nov, Niv, and Sub and Super group B including ten Con, Dei, Pip, CauB, Prn, Dro, Unif, Crag, Perp, and Leva subgroups [30]. Each of these subgroups may include strains or similar types, for example, the *Wolbachia* isolated from *Culex fuscocephala* (wFus), *Drosophila simulans* (mauritiana), *Ephesia kuehniella* (wMa), *D. simulans* (Noumea) (wNo), *Aedes albopictus* (wAlbB), and *Ae. pseudalbopictus* (wPseu) are categorized as members of wPap subgroup [30]. It is expected that the more sequence information becomes available the number of such groups to increase. As *wsp* shows relatively high genetic divergence among strains, the gene has been used widely in phylogenetic studies and for micro-taxonomic subdivision [31,32]. Literature has shown some Phlebotominae sand fly species are known to be infected with *Wolbachia pipientis* including *Lutzomyia*, *Sergentomyia* and *Phlebotomus* genera. In fact *Wolbachia* were detected in 27–78.6% of the sand fly species sampled [33–35]. However phylogenetic structure of *Wolbachia* in sand flies has not been undertaken in previous studies. Also previous studies reflected gaps of knowledge regarding geographical distribution of *Wolbachia* Supergroups and subgroups in *P. papatasi* and *P. sergenti*, the two main vectors of CL across Iran [5,7,36], where leishmaniasis is endemic [8]. To address this issue, in a cross sectional descriptive study, we have screened *P. papatasi* and *P. sergenti* sand flies occurring in endemic foci of Leishmaniasis in Iran and the obtained sequences along with those available in GenBank were compared with representative reference sequences of other subgroups of Supergroups A and B.

Material and methods

Study area and collection of specimens

Sand flies were collected from five different districts (Badroud, Mamasani, Germi, Bojnord and Bam) respectively belong to the provinces (P) of Esfahan in center, Fars in south center, Ardebil in north west, North Khorasan in north east and Kerman in southeast of Iran (Figure 1). These locations are the main endemic foci for either

or both ACL and ZCL in the country where *P. papatasi* and *P. sergenti* sand flies present often sympatric. Sand fly specimens were captured monthly using either CDC light traps, moth aspirator, and or car traps from indoors and outdoors between the hours of 18:00 PM to 4:00 AM next day during the spring and summer of 2015 and 2016 [37,38]. Car traps involved a just parked vehicle used to attract sand flies in the vicinity of rodent burrows at night time. By this method the sand flies landing on the car were collected using a mouth aspirator and battery operated torch. Sampling was conducted during three to four consecutive days per month. Traps were placed adjacent to animal shelters, gardens, rest rooms, baths, and toilets of the houses. Traps were set by the noon (before sunset) and collected following day before the sunrise. Mouth aspirator also was used to sample sand flies from animal shelters and toilets and bathrooms within human houses. Wild-caught sand flies were transferred to micro centrifuge tubes containing 96% ethanol and moved to laboratory in cold chain condition and stored in freezer (−20 °C) for further investigations. Samples were washed first with 70% ethanol for 3–4 min then rinsed three times with sterile PBS and at last with double-distilled water. After washing steps, sand flies were gently dissected under stereomicroscope using single use sterile insect needles. Before and between dissections, insect needles were sterilized by flaming. Dissections were done on sterilized single use slide covers and heads and terminal abdominal segments of the collected sand flies were mounted with Pouri solution on glass slides for morphological identification to species level. Specimens were identified using available morphological keys [39,40].

Phlebotomus sergenti is morphologically very similar to *P. similis* [41] where both species have shared geographical distribution in Iran [10]. Both species are present in northwest, north, northeast, center, southeast, and south of the country. Specimens of *P. sergenti* s.l. were distinguished using morphological characters of spermatheca, pharyngeal teeth, pharyngeal armature, length of third segment of antenna (A-III), number and level of setae. *P. sergent* and *P. similis* can be separated by the shape of basal lobe of coxite and the length of the lateral lobe [6,40]. The ambiguous specimens were discarded from the study.

The rest of body were transferred to 1.5 ml centrifuge tubes after species identification and stored individually at −20 °C for further molecular investigations. Badroud strain had been colonized in the Phlebotominae insectary of School of Public Health-Tehran University of Medical Sciences (SPH-TUMS), and some F1-F2 generation laboratory specimens were used for this study. DNA of a *Wolbachia* infected *Culex pipiens* from insect molecular biology laboratory [42] as positive control and double distilled water and DNA of adult *Anopheles stephensi* specimen as negative controls were used in both semi nested-PCR steps.

Figure 1. Map of study areas. Source: Fateh Karimian and Naseh Maleki.

PCR amplification and sequencing

Following identification of the collected sand fly specimens, *P. papatasi* and *P. sergenti* specimens selected for molecular assays. Genomic DNA of all specimens individually was extracted using the method already described by Collins [43]. A set of primers introduced by Zhou, Rousset [29] and semi nested PCR assay explained by Karami, Moosa-Kazemi [42], were used to detect *Wolbachia* strains in sand flies. As much as possible, a subset of specimens representing different locations, seasons, habitats (indoor and outdoor), and species with clear and sharp *wsp* gene amplicons randomly were selected for sequencing via the same amplification forward and reverse primers used for the second step of semi nested PCR by Bioneer Company (S. Korea). The acquired sequences in this study were edited and assembled using Chromas and Bioedit softwares to construct consensus

sequences and analyzed using NCBI (Nucleotide collection) database (<https://www.ncbi.nlm.nih.gov/>). The consensuses of confident sequences were aligned with other *Wolbachia* infected sand fly sequences available in GenBank using CLUSTALW2. Also available gene sequences of *Wolbachia* subgroups of Supergroups A and B were obtained from Genbank and *Wolbachia* MLST database (<http://pubmlst.org/wolbachia/>) and compared with the *Wolbachia* infected sand fly sequences. All DNA sequences used for alignment were cut to get a consistent region. The alignment of the *wsp* gene sequences were used for phylogenetic tree construction for the *Wolbachia* Supergroups A and B and their subgroups. The obtained sequences in the current study (KX263906-KX263920 and MG251657-MG251660) were submitted to GenBank. Double distilled water and DNA of *Anopheles stephensi* have been used as negative controls.

Phylogenetic analysis

Phylogenetic analysis were inferred using the Neighbor Joining, maximum parsimony, and maximum likelihood methods implemented on MEGA7[44], to infer phylogenetic relationships of *Wolbachia* sequences distributed among sand fly vectors and the ones deposited in GenBank. 69 representative sequences including all available subgroups of A ($n = 15$ subgroups) and B ($n = 9$ subgroups) Super groups were set as classification references. Details of *wsp* sequences used for this study have been shown in figure 2. Statistical support for internal branches of the trees was evaluated by bootstrapping with 1000 iterations. The sequences were also analyzed to evaluate number of haplotypes, nucleotide composition, variable sites, and genetic distances using Bioedit v.7.2.5 software.

Results

Sand fly identification

In total, 2953 sand flies were collected from the study areas and identified to species level. These comprised species of subgenera *Adlerius* (*P.halepensis*), *Larrousius* (*P.kandelakii*, *P.perfliewi*, *P.major*), *Paraphlebotomus* (*P.sergenti*, *P.alexandri*, *P.caucasicus*, *P.mongolensis*), *Phlebotomus* (*P.papatasi*), and *Sergentomyia* (*S.sintoni*, *S.dentata*, *S.sumbarica*). Out of 2953 specimens, 912 sand flies were identified as *P. papatasi* (59%) and *P. sergenti* (41%) respectively (Table 1).

Wolbachia infection

Wsp gene of *Wolbachia* was successfully amplified by PCR in 80 percent of specimens (Table 1). The length of PCR products were about 600 bp and were obtained from both *P.papatasi* and *P.sergenti* specimens. Relative infection rates were found to be higher in *P.papatasi* (83%) than *P.sergenti* (76%) (Table 1). Sequencing and blast analysis showed that the amplicons were 94–100% homology with the *Wolbachia* strains described from other insects or sand flies. The *wsp* gene sequence from the Iranian *P.papatasi* showed 98–100% nucleotide sequence homology with *Wolbachia* sp in *P.papatasi* from Egypt (AY288297), and India (AF237882). The *wsp* gene sequence from the *P.sergenti* showed 98–100% nucleotide sequence homology with *Wolbachia* sp in *P.mongolensis* (KC576916) and 72–83.1% with *Wolbachia* sp in *P.papatasi* (AY288297, AF237882, AF020082) from Genbank.

Wsp sequence analysis

Sequence analysis was performed on 585 bp long fragments of *wsp* gene. The alignment of *wsp* sequences of the Iranian *P.papatasi* and *P.sergenti* showed an

Figure 2. Phylogenetic relationships inferred from Neighbor Joining analyses constructed by MEGA7 based on 585 bp DNA sequences of *wsp* sequences of *Wolbachia* strains isolated from sand flies of *P. papatasi* and *P. sergenti* and other insects in the Super/sub groups levels.

Bootstrap values are indicated ant nodes. The scale bar indicates genetic distance. The Iranian isolates are bold.

enormous number of substitutions and indels ($n = 145$, 25.17%) among the strains (Table 2). This value was increased to 453 variable sites when Genbank available *wsp* sequences of other *Wolbachia* strains isolated from other sand fly species (*Phlebotomus* spp, *Lutzomyia* spp, and *Sergentomyia* spp) included in the analysis. There were 106 parsimony-informative sites between *wsp* sequences of *Wolbachia* obtained from the Iranian *P.papatasi* and *P.sergenti*. The genetic variation at amino acid level was 32.6% (including 21.7% indels and 78.3 substitutions) among the sequences of *Wolbachia* infected sand flies of this study which was higher than

Specie	Location	N	Wol- bachia infection rate %	Leish- maniasis foci	Latitude, longitude
<i>P. papatasi</i>	Badroud	123	83	ZCL	33.6912° N, 52.0083° E
	Mamasani	109	90	ZVL	30.1151° N, 51.5413° E
	Germi	90	89	ZVL	39.0394° N, 48.0803° E
	Bojnord	120	75	ZCL/ZVL	37.4702° N, 57.3143° E
	Bam	95	80	ACL/ZCL	29.0985° N, 58.3375° E
Subtotal		537	83		
<i>P. sergenti</i>	Badroud	0	0	ZCL	33.6912° N, 52.0083° E
	Mamasani	102	83	ZVL	30.1151° N, 51.5413° E
	Germi	70	72	ZVL	39.0394° N, 48.0803° E
	Bojnord	100	80	ZCL/ZVL	37.4702° N, 57.3143° E
	Bam	103	69	ACL/ZCL	29.0985° N, 58.3375° E
Subtotal		375	76		
Total		912	80		

(32.60 v. 25.17%) the mutation rate at DNA level, indicating that most of the mutations were nonsynonymous.

Totally 9 haplotypes of the *wsp* gene were found among the DNA sequences of the Iranian *P.papatasi* and *P.sergenti*, which *P.papatasi* harbored most haplotypes. The genetic distances values among the haplotypes

Phylogenetic analysis

Sixty-nine (69) *Wolbachia* sequences belong to super groups A and B related to Phlebotominae (22 species) and other insect species ($n = 47$) were subjected to molecular phylogenetic analysis. Due to the different lengths of the sequences, they were trimmed to obtain a consistent region for phylogenetic analysis. Phylogeny was reconstructed by using 585 nucleotides of *wsp* gene. All three methods of neighbor joining, maximum parsimony, and maximum likelihood analysis revealed similar topology. The phylogenetic analysis scattered the *Wolbachia* sequences of this study among the obtained sequences from Genbank (Figure 2). Representatives of *Wolbachia* Super groups A and B clustered in two main separate clades. Twenty-five known subgroups of Super groups A and B resolved well and formed separate clusters supported by a high bootstrap values. The bootstrap support values for clusters were more than 50%. More than 85 percent of the subgroup' clusters were supported

[illegible][illegible]

Notes: Vertical numbers in three top lines refer to the position of substitutions or indels throughout the 585 bp sequences. The wsp sequence alignment has been constructed using first line (wSerg, KX263917) as reference and other sequences were compared with the first line. Similar nucleotides are shown as dot (.), and deletions as dash (-).

by more than 80% bootstrap values (Figure 2). *Wolbachia* wsp sequences in *P.sergenti* with 94% bootstrap value formed new independent subgroups in Supergroup A, and have a close relationship with wAlbA and wMel subgroups of Supergroup A. In addition the wsp sequences of *Wolbachia* in *P.mongolensis* and some *P.papatasi* specimens were positioned within the new subgroup wSerg.

The phylogeny analyses showed that *Wolbachia* strains infected *P.papatasi* are divided into two different subgroups of wPip and wSerg within A Supergroup with robust bootstrap values. Of nine *Wolbachia* sequences obtained from Iranian *P.papatasi*, two (KX263908 and KX263916) were in subgroup wSerg and seven (KX263906–KX263907–KX263909–KX263910–KX263913–KX263919 and MG251658–98) were in subgroup wPap (Figure 1).

Discussion

In the present study PCR-direct sequencing was used for detecting and identifying *Wolbachia* among *P.papatasi* and *P.sergenti* originated from five endemic CL foci of Iran. We also determined their phylogenetic relationship with other recognized strains using the nucleotide sequence of the bacterium surface protein (wsp) gene. Results of wsp gene sequence analysis and phylogeny revealed two divergent types of *Wolbachia* species within *P. papatasi*, i.e. one belonging to the wPap subgroup of Super group A while the other to the novel wSerg subgroup of Super group A.

In this study we have focused on *P.papatasi* and *P.sergenti* the vector of CL in Iran. However, *P.sergenti* s.l. includes *P.sergenti* and *P.similis* which are morphologically similar. *Phlebotomus sergenti* is an incriminated vector whereas *P.similis* is not. Due to the role of *P.sergenti* in transmission of *L.tropica*, only the well-identified *P.sergenti* specimens were tested for *Wolbachia* infection. In this study we also found some specimens with intermediate forms of *P.sergenti* and *P.similis*. Previous studies have shown that intermediate morphotypes and even *P.similis* have 100% identical genotypes with *P.sergenti*, suggesting there is a gene flow between the two taxa, and presumably they are a single taxon. We recommend to test *Wolbachia* strain of *P.similis* type specimens and to compare with the *Wolbachia* strains identified from the *P.sergenti*. These studies may provide information for better understanding of the host-symbiont coevolution, host resistance to *Leishmania* parasite, ecology, evolution, and distribution of these two closely taxa.

Although, in general, each sand fly species like *P.sergenti* or other insect species seemingly harbors a single *Wolbachia* subgroup within a single Supergroup, *P.papatasi* and some other insect species including sand flies, fruit flies, beetles, wasps and mosquitoes present a polyphyletic species [45–49]. For examples, *Wolbachia* endosymbionts (wLcy and wLev) occurred in

the population of *Lutzomyia c. cayennensis* [30], *Aedes albopictus* presented two divergent *Wolbachia* groups A and B (wAlbA and wAlbB) [46,50], and *D.simulans* grants five different *Wolbachia* variants [51] including wMel, wRiv, and wHaw of Supergroup A and wAu, and wMa of Supergroup B [29,52]. Parvizi, Bordbar [53] found two *Wolbachia* strains in sand flies, one strain (Turk 07, accession KC576916) in the two sister taxa of *P.mongolensis* and *P.caucasicus* and another strains in *P.papatasi* (Turk 54, accession EU780683). They concluded that one species of sand fly can be infected with different *Wolbachia* strains and that different species of sand flies can be infected with a common strain. The presence of two or more *Wolbachia* strains in a single species may be explained by either shared ancestral origin or interspecific hybridization (horizontal transmission) between two closely species that led to introgression of a different *Wolbachia* strain [47,54–58]. One strain of *Wolbachia* in particular (designated Adm) appears to have undergone extensive ‘recent’ horizontal transmission. Mechanisms of intertaxon transmission are unknown, although there is indirect evidence for exchange between parasitoids and their host insects [47]. The two *Wolbachia* subgroups (wSerg and wPip) were found simultaneously in the TUMS/SPH lab and Ardabil strains of *P.papatasi*. *Wolbachia* multiple infection in a population with identical ecological region has been already reported in some insects [52]. Cytoplasmic Incompatibility (CI) is a known phenomenon happening in crosses between males *Wolbachia* infected and uninfected females or females infected with other type of *Wolbachia*, resulted in intense decrease in the amount of viable eggs produced. However, different level of CI intensity has been observed between host species. For example, CI is fully expressed in *D. simulans* but is fully or partially permissive in *D. melanogaster* [51]. These authors concluded that host factors are involved in the control of CI. In another study by James, Dean [52] the dynamics associated with double and single *Wolbachia* infections in *D. simulans* was investigated. They showed that the CI is higher when a singly infected male is mated with a female carrying the other strain (1.00–0.30) in comparison with an uninfected female (0.33–0.08), suggesting that both sexes (male and female) fund to the expression of CI. They suggested that either the DNA of these bacterial isolates have diverged from a shared ancestral ones, host genetic factors has led to lessening in the CI phenotype, or some *Wolbachia* may display different levels of incompatibility.

This study revealed that the infection rates of *P.papatasi* and *P.sergenti* to *Wolbachia* were 83% and 76% respectively. Benlarbi and Ready [59], reported a high infections rates of *Wolbachia* in the thorax and abdomen of sandflies. Thorax is a vital site for metacyclogenesis of *Leishmania* and abdomen is an important location for vertical transmission of *Wolbachia* in sand

flies. They conclude that this endosymbiont could be useful for driving transgenes through wild populations of sandflies.

In this study we found two subgroups of wSerg and wPap within lab strain of *P.papatasi* originated from Badroud of Esfahan and a field strain from northwest of Iran. However, we have no document indicating CI phenotype between wSerg and wPap subgroups. Also we do not know what CI strength presents between these two subgroups in *P.papatasi*. In our sand fly insectary, we are normally facing problems in maintenance of the colony and have observed reduction of population size of *P.papatasi* in cages, which often has led to release new wild individuals to the cages to boost the population. Although this reduction may be caused by fungal infection, mite infection, and diapause, but it may also be caused by incompatible crossings induced by multiple infection of the *P.papatasi* population with two *Wolbachia* strains. Study of Kassem and Osman [60] showed lack of CI between the crosses between uninfected females and infected males of a laboratory *P.papatasi* population from Egypt. However, they used an unknown *Wolbachia* strain with a low maternal transmission (five out of the 23 (21.7%) matings). Further investigation warrant to elucidate presence of CI phenotype as well as its strength in *P.papatasi*. We suggest to study CI phenotype by cross mating between wPap infected males with females carrying wSerg and vice versa in the *P.papatasi* lab strain. This may lead to innovate new tools using cytoplasmic incompatibility for population substitution and control of sand fly vectors as developed for other vector diseases [18,61]. Also presence of *Wolbachia* in the main CL vector populations encourage further investigation on the possible effects of these strains in *Leishmania* transmission by the vectors.

In conclusion, endosymbiotic *Wolbachia* was found in two species of Iranian *P.papatasi* and *P.sergenti*. Two *Wolbachia* strains (wPap and wSerg subgroups) belong to the A Super group were found in these sand flies. The wSerg subgroup was new in dipteran insects. The wPap and wSerg were found in *P.papatasi* and the wSerg was found only in *P.sergenti*. Improved understanding of the role of these subgroups in vector biology such as cytoplasmic incompatibility and competence for transmitting the parasites and viruses by sand flies, are warranted for developing alternative control tools.

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Disclosure statement

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