Detection of *Wolbachia pipientis*, including a new strain containing the *wsp* gene, in two sister species of *Paraphlebotomus* sandflies, potential vectors of zoonotic cutaneous leishmaniasis

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Individual, naturally occurring Phlebotomus mongolensis *and* Phlebotomus caucasicus *from Iran were screened for infections with the maternally inherited intracellular* Rickettsia*-like bacterium* Wolbachia pipientis *via targeting a major surface protein gene (*wsp*). The main objective of this study was to determine if* W. pipientis *could be detected in these species. The sandflies were screened using polymerase chain reaction to amplify a fragment of the* Wolbachia *surface protein gene. The obtained sequences were edited and aligned with database sequences to identify* W. pipientis *haplotypes. Two strains of* Wolbachia *were found. Strain Turk 54 (accession EU780683) is widespread and has previously been reported in* Phlebotomus papatasi *and other insects. Strain Turk 07 (accession KC576916) is a novel strain, found for first time in the two sister species. A-group strains of* W. pipientis *occur throughout much of the habitat of these sandflies. It is possible that* Wolbachia *is transferred via horizontal transmission. Horizontal transfer could shed light on sandfly control because* Wolbachia *is believed to drive a deleterious gene into sandflies that reduces their natural population density. With regard to our findings in this study, we can conclude that one species of sandfly can be infected with different* Wolbachia *strains and that different species of sandflies can be infected with a common strain.*

Key words: *Wolbachia pipientis* - *wsp* gene - *Paraphlebotomus* - *L. major* - Iran

The prevalence of zoonotic cutaneous leishmaniasis (ZCL), caused by *Leishmania major*, is increasing in many parts of Iran. ZCL originates as a disease of gerbils and *Leishmania* parasites are transmitted by sandflies that live and breed in gerbil burrows (Mohebali et al. 2004, Mirzaei et al. 2011). This form of leishmaniasis is of great public health importance in rural areas of 15 out of 32 provinces of Iran (Yaghoobi-Ershadi et al. 2005, Parvizi et al. 2010a). The disease is generally restricted to areas that are heavily infested by sandflies, which are the vectors of ZCL. The Turkmen Sahara region is one of the known focal areas of ZCL in Iran and includes two important ZCL foci (Gonbad Kavous and Maraveh Tapeh) in rural areas. *Phlebotomus papatasi* (Diptera: Psychodidae) is the main vector and *Paraphlebotomus* species (*Paraphlebotomus mongolensis* and *Paraphlebotomus caucasicus*) are secondary principal vectors of ZCL in this district (Nadim & Faghih 1968, Yaghoobi-Ershadi et al. 1996, Parvizi & Ready 2008).

Wolbachia pipientis is a maternally inherited endoparasitic bacterium belonging to the α-proteobacteria family that infects 20-75% of all insect species, including sandflies (Werren et al. 1995, 2008, West et al. 1998, Jeyaprakash & Hoy 2000, Werren & Windsor 2000, Hilgenboecker et al. 2008, Azpurua et al. 2010).

This bacterium has attracted attention because it induces a number of intriguing abnormalities in the host's reproductive system (O'Neill et al. 1997, Stouthamer et al. 1999). These intracellular microorganisms affect the biology of their invertebrate hosts in many ways, ranging from mutualistic effects to the establishment of reproductive isolation and thus, speciation (Werren 1997, Bordenstein et al. 2001, Telschow et al. 2005, Werren et al. 2008).

A wide range of phenotypes are expressed under *W. pipientis* infection, associated with processes ranging from evolution to speciation (Breeuwer & Werren 1990, Hurst & Schilthuizen 1998) to mechanisms involved in the genetic modification and biological control of pest arthropods (Sinkins et al. 1997, Bourtzis & O'Neill 1998), but these bacteria are commonly considered to be reproductive parasites (Moran et al. 2008, Werren et al. 2008).

It is has been noted that most *Wolbachia*-induced phenotypes increase the proportion of females among the offspring of infected females and pose unique threats to host populations (Werren et al. 2008).

The phenotypes associated with these threats include (i) induction of parthenogenesis in the host, (ii) transformation of males into functional daughters (feminisation), (iii) provoking the selective abortion of sons of infected mothers (male killing) and (iv) cytoplasmic incompatibility (CI), which is the most common phenotype occurring in the mosquito (Hoffmann & Turelli 1997, Werren et al. 2008). The reproductive distortions caused by *Wolbachia* infection are known to result in embryonic death and subsequent egg hatching failure due to disruptions during

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the early events of fertilisation. Thus, a *Wolbachia* strain can promote its rapid vertical transmission throughout a population of the *Phlebotomus* genus (Benlarbi & Ready 2003). *Wolbachia* are vertically transmitted from mothers to daughters, but can occasionally be horizontally transmitted between individuals, including individuals belonging to different species (Pascal et al. 2006).

Wolbachia display tropism for the reproductive tissue of their hosts and are transmitted vertically from insect to insect through the ovules. Interspecific transmission appears to occur horizontally, with the possible aid of parasitoids (Stouthamer et al. 1999, Charlat et al. 2002, Kondo et al. 2002, Ahrens & Shoemaker 2005, Baldo et al. 2005, Espino et al. 2009).

The *Wolbachia* surface protein (*wsp*) is commonly used as a marker for strain typing (Schaub et al. 1989, Van Meer et al. 1999, Pintureau et al. 2000, Shoemaker et al. 2002, Kyei-Poku et al. 2005) (Fig. 1) and a strain typing system utilising *wsp* has been developed (Zhou et al. 1998).

The *wsp* gene is analogous to the antigens employed for serotyping pathogenic bacteria (Stouthamer et al. 1999, Baldo et al. 2006). It is approximately 10 times more variable in its DNA sequence than 16S rRNA (Zhou et al. 1998).

Traditionally, *Wolbachia* spp detected in arthropods have been divided in two groups (A and B) based on the sequences of their 16S rRNA, *ftsZ* and *wsp* genes (Werren et al. 1995, Zhou et al. 1998). Both groups contain *Wolbachia* spp that have been detected in several genera of sandflies. Group A includes the *Wolbachia* spp detected in *Sergentomyia* and from *Phlebotomus*. Group B contains the *Wolbachia* spp detected in sandflies belonging to the *Phlebotomus* and *Lutzomyia* genera (Werren et al. 1995, Zhou et al. 1998, Ono et al. 2001).

These bacteria have been detected using molecular tools and reported as "*Wolbachia* species" from both arthropod and nematode hosts and only a single species has thus far been properly isolated and bears a valid name, *W. pipientis* (Lo et al. 2002).

Wolbachia may have an influential effect on the control of disease vectors based on its potential role in reducing the ability of the host to reproduce, thus decreasing its population size (Beard et al. 1993). Therefore, it is worthwhile to investigate the occurrence and distribution of *Wolbachia* in sandflies in nature.

The first objective of this study was to determine if *W. pipientis* could be detected in two sister species of *Paraphlebotomus* sandflies in which the bacterium has not been previously reported. In these two sandfly species, *P. mongolensis* and *P. caucasicus*, the external genitalia of males provide the only diagnostic morphological characteristics. The females of these sister species cannot be separated morphologically or molecularly based on the structure of the spermathecae, the weakly developed pharyngeal armature or the mitochondrial cytochrome *b* gene (Theodor & Mesghali 1964, Killick-Kendrick 1999, Esseghir et al. 2000, Parvizi et al. 2010a, b). The second objective of this study was to improve our knowledge regarding the detection of one or more strains of *W. pipientis* in one or both sister species of sandfly. Its third objective was to determine if sandflies can be simultaneously infected by two different strains of *W. pipientis*. Finally, the fourth objective of this study was to demonstrate the presence of *Wolbachia* in *Paraphlebotomus* sandflies, which would have a significant impact in assessing the diversity of *Wolbachia* strains, particularly in *L. major* vectors.

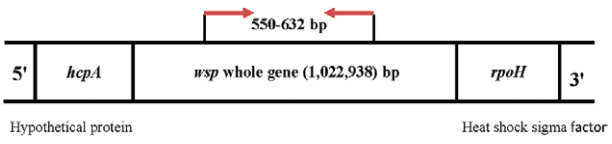

Fig. 1: schematic presentation of the *Wolbachia* surface protein (*wsp*) gene, an appropriate candidate as an antigenic serotyping for strain typing of pathogenic bacteria of *Wolbachia*.

MATERIALS AND METHODS

Origin and identification of sandflies - The Turkmen Sahara villages in northeastern Iran are a focus of ZCL, where *L. major* parasites are the major causative agent of ZCL. Two sister *Paraphlebotomus* species were collected regularly from 18 villages of Gonbad Kavous and Maraveh Tapeh in late June and the middle of September in 2008 and 2009. There were also collections performed in September 2010 during peak sandfly activity in the transmission seasons of two successive years (Table I). Additionally, sticky paper and CDC traps were used to sample sandflies in the ruins of mud-walled outhouses adjacent to animal shelters, around houses, at the entrance of gerbil burrows and in houses surrounded by gerbil burrows at the edge of rural areas.

The specimens were identified as *P. mongolensis* males, *P. caucasicus* males or *P. mongolensis*/*P. caucasicus* females, based on morphological characters of the head and the abdominal terminalia (Lewis 1982).

DNA extraction, gene amplification and sequencing - DNA was extracted from the thorax and attached anterior abdomen of each sandfly. The samples were screened for the presence of *W. pipientis* using the general (or non-strain specific) primer pair *wsp* 81F and 691R (Zhou et al. 1998) (Fig 1). Polymerase chain reaction (PCR) amplification was carried out according to the protocol of Benlarbi and Ready (2003).

Each 20 µL PCR mixture consisted of 1X Promega buffer B, 25 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM each primer, one unit of Taq DNA polymerase (Promega) and 2 µL of sandfly genomic DNA. PCR amplification was carried out as follows: 2 min denaturation at 94ºC, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 50ºC for 30 sec and extension at 72ºC for 1 min 30 sec, with a final extension at 72ºC for 10 min.

Following amplification, the samples were fractionated via horizontal submerged gel electrophoresis using 1.5% agarose gels with DNA size markers (Promega PCR markers G316A or Bioline Hyperladder IV). The obtained DNA fragments were visualised by ethidium bromide staining and then excised and purified using

the Geneclean II Kit (BIO 101 Inc), prior to sequencing each strand (Testa et al. 2002). The sequences were then edited and aligned with database sequences using Sequencher™ version 4.4 software (Gene Codes Corp) to identify unique sequences (= haplotypes), which were analysed phylogenetically using MEGA software.

RESULTS

P. mongolensis and *P. caucasicus* were identified based on morphological characteristics of their external genitalia. A total of 203 *P. mongolensis* and *P. caucasicus* sandflies were trapped at two sites in Turkmen Sahara in the province of Golestan.

The wild sandflies were identified as belonging to two sister species of the *Paraphlebotomus* subgenus. In total, 74 out of the 203 sandflies examined were infected with *Wolbachia*. The *Wolbachia wsp* gene was detected for the first time in all three groups and was distributed as follows: in 33/96 males of *P. mongolensis*, 10/15 males of *P. caucasicus* and 31/92 females of *P. mongolensis/P. caucasicus* (31/92), corresponding to infection rates of 34.37%, 66.66% and 33.69%, respectively (Table I).

We attempted sequencing in all 74 sandflies with *Wolbachia* infections. However, only 25 of the sandflies presented sufficient DNA for sequencing and/or showed readable sequences demonstrating the presence of *Wolbachia* strains.

Two strains of *Wolbachia* were found in *P. mongolensis* and *P. caucasicus* sandflies (Fig. 2). The common strain, Turk 54 (accession EU780683) and a novel strain, Turk 07 (accession KC576916), were found for first time in *P. mongolensis* and *P. caucasicus* sandflies from Turkmen Sahara. Following phylogenetic analyses, these strains appeared to belong to the A-group. A total of 19 of the *P. mongolensis* and *P. caucasicus* sandflies exhibited the Turk 54 strain haplotype, while six of the sandflies were infected with strain Turk 07 (Fig. 3, Table II).

Fig. 2: alignments of the two *Wolbachia* surface protein gene sequence of *Wolbachia pipientis* isolated from Iranian sandflies strain Turk 54 isolated first from *Phlebotomus papatasi* and then most different sandfly species with the GenBank sequence EU780683, Turk 07 isolated from *Paraphlebotomus mongolensis* and *Paraphlebotomus cacusicus*. Nucleotide differences are marked by a star.

TABLE I

Paraphlebotomus caucasicus and *Paraphlebotomus mongolensis* detected *Wolbachia* surface protein (*wsp*) gene in different villages in two locations, Turkmen Sahara, Iran

Genus Subgenus	Species	Location	Gonbad Kavous							Maraveh Tapeh						Total *wsp* gene Ve per species n (%)	Total *wsp* gene +Ve n (%)
		Villages	Dashlibroun	Shourdegesh	Kheyrkhajeolia	Inchebroun	Dozalum Fadavi	Daneshmand	Okhitapeh	W-Gharagol	E-Gharagol	Makhtumgholi	Souzesh	Khajekaldi	Jafarbay		
Phlebotomus Paraphlebotomus	*P. mongolensis* (M)		4	3	1	2	1	15	4	0	0	1	1	0	1	33/96 (34.37)	33/203 (16.26)
	P. caucasicus (M)		2	0	0	1	1	2	2	0	0	1	0	1	0	10/15 (66.66)	10/203 (4.92)
	P. mongolensis and *P. caucasicus* (F)		6	2	2	5	1	2	1	2	1	2	3	1	3	31/92 (33.69)	31/203 (15.27)
Total			12	5	3	8	3	19	7	2	1	4	4	2	4	74/203	
(%)			5.91	2.46	1.48	3.94	1.48	9.35	3.45	0.99	0.49	1.97	1.97	0.99	1.97	(36.45)	
			28.08							8.37							

F: female; M: male; +Ve: *Wolbachia* positive.

All of the *wsp* gene fragments amplified from *P. mongolensis* and *P. caucasicus* were sequenced directly. The 564-bp Turk 54 haplotype (minus primers) (GenBank accession EU780683) was indistinguishable from that of the A-group strain of *W. pipientis* (wPap), which was previously isolated from *P. papatasi* originating from Israel/West Bank (AF237883) (Ono et al. 2001), India (GenBank accession AF237882) (Ono et al. 2001), Spain and Iran (Benlarbi & Ready 2003, Parvizi et al. 2003). All of these sandflies from Spain and Iran were wild caught, as was the pool of sandflies possessing the AF237883 sequence. The other sequences were isolated from *P. papatasi* bred in laboratory colonies. Using the same *wsp* gene primers, Cui et al. (1999) amplified a fragment of approximately 600 bp from a colony of *P. papatasi* originating from Israel/West Bank, North Sinai in Egypt and Saudi Arabia. However, these authors did not report any sequence data. Haplotype wPap was originally reported as presenting an ambiguous base (C/T) at nucleotide position 102 (GenBank accession AF020082) (Zhou et al. 1998).

The new Turk 07 haplotype differed in a number of point mutations and insertion-deletion events from the previously described haplotypes (Fig 2, Table II). The new sequence was aligned with the available *wsp* sequences of *Wolbachia* from sandflies and other insects and phylogenetic relationships were inferred. The phylogram generated via neighbour-joining analysis placed the sequences from *P. papatasi* on the same terminal branch within the A-group of strains of *W. pipientis* (Fig. 3).

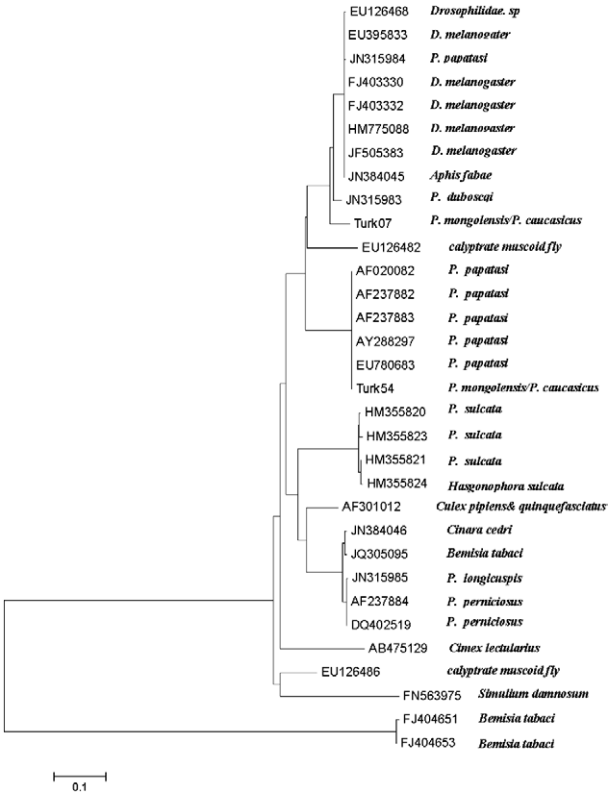

Fig. 3: unrooted neighbour-joining tree showing the relationships of the haplotypes of the *Wolbachia* surface protein gene fragment for the isolates of *Wolbachia* listed in Table II.

DISCUSSION

Here, we report the detection of *Wolbachia* in two potential vectors of ZCL (*P. mongolensis* and *P. caucasicus)* for the first time. *Wolbachia* had previously been isolated from various insects including sandflies, but infection of *P. mongolensis* and *P. caucasicus* had not been detected. A new *W. pipientis* strain (Turk 07) was found in both *P. mongolensis* and *P. caucasicus.* Additionally, the widespread common strain (Turk 54) was found in these two sister species.

The infection of the two *Paraphlebotomus* species with the same strain of *Wolbachia* may have been due to recent horizontal transmission of *Wolbachia* across host species. Alternatively, in the case of the closely related species *P. mongolensis* and *P. caucasicus*, infection may be due to co-divergence and co-evolution of *Wolbachia* strains and their hosts.

Another intriguing finding of this study was that one *Paraphlebotomus* species can be infected with different *Wolbachia* strains.

The *wsp* gene was used for phylogenetic analysis of the *Wolbachia* strains from which the sequences originated based on a previous study that detected *Wolbachia* in sandflies with the *wsp* gene (Werren et al. 1995, Zhou et al. 1998, Ono et al. 2001, Wu & Hoy 2012).

The *wsp* sequences obtained from the *P. mongolensis* and *P. caucasicus* Turk 54 strains showed a high similarity to sequences deposited in GenBank as "incompatibility symbiont of *P. papatasi*" (Fig. 3, Table II).

However, the Turk 54 haplotype *wsp* sequences isolated from *Paraphlebotomus* were identical to a sequence previously reported from *P. papatasi* from Tunisia and Iran (Benlarbi & Ready 2003). In contrast, the Turk 07 sequences obtained from *P. mongolensis* and *P. caucasicus* were unique.

Phylogenetic studies using 16S rDNA have established that strains of *W. pipientis* from highly divergent hosts form a monophyletic clade related to the *Ehrlichia* assemblage within the α-subdivision of proteobacteria (O'Neill et al. 1992, Rousset & Solignac 1995). The examination of 16S rDNA sequences is a conservative approach that has allowed not only the phylogenetic placement of bacterial species, but also the phylogenetic resolution of *W. pipientis* into two strain groups: A and B (O'Neill et al. 1992). More recently, the more rapidly evolving *wsp* gene has been used to improve the phylogenetic resolution within the *W. pipientis* species clade, which was divided into four groups (A-D) and 12 subgroups (Zhou et al. 1998, Ono et al. 2001). The A and B groups are consistent with those identified via 16S rDNA for *W. pipientis* strains from insects, mites and crustaceans, whereas groups C and D are consistent with the strains from filarial nematodes.

Wolbachia is normally maintained in nature through vertical transmission (Werren 1997). Although horizontal transfer appears to be infrequent within host populations, it has previously occurred between two host sisters

species. This demonstrates that the phylogeny of *Wolbachia* strains differs noticeably from that of their hosts (O'Neill et al. 1992, Werren et al. 1995). This conclusion is supported by experiments revealing the transfer of symbionts between host individuals (Huigens et al. 2000).

The demonstrated horizontal transmission of *Wolbachia* could shed light on sandfly control because *Wolbachia* is believed to drive a deleterious gene into mosquitoes that reduce their natural population density (Beard et al. 1993, Sinkins et al. 1997).

The results of a detailed phylogenetic analysis including characterisation of the limitations of such an approach could serve as a foundation for understanding the evolution of *Wolbachia* bacteria.

Because genetically similar strains are often found in similar insect hosts, ecological interactions among hosts may mediate horizontal transfers. In particular, it appears that the horizontal transmission of *Wolbachia* has occurred quite recently and frequently within the genus *Phlebotomus*.

TABLE II

Wolbachia strains isolated from different hosts in various geographical origins and Iran

Host	Strain	Geographic origin	GenBank accession	*Wolbachia* surface protein gene fragment (bp)
Drosophilidae sp.	*A_PanPNM_Droso12b*	Panama	EU126468	513
Drosophila melanogaster	*wMel*	China	EU395833	632
Phlebotomus papatasi	*wPhleb31f*	France	JN315984	568
D. melanogaster	wMel	Wuhan	FJ403330	638
D. melanogaster	*wMel*	Yunnan	FJ403332	637
D. melanogaster	-	Ukraine	HM775088	600
D. melanogaster	-	India	JF505383	590
Aphis fabae	wsp_GRA4	Greece	JN384045	620
Phlebotomus duboscqi	*wPhlebM4*	France	JN315983	524
Paraphlebotomus mongolensis *Paraphlebotomus caucasicus*	(Turk 07)	Iran	KC576916	551
Calyptrate muscoid fly	*A_MexSon_Calyp150671*	Mexico	EU126482	486
P. papatasi	Wpap	Israel	AF020082	564
P. papatasi	*wPap*	India	AF237882	563
P. papatasi	*wPap*	Israel	AF237883	563
P. papatasi	-	Egypt	AY288297	561
P. papatasi *P. mongolensis* *P. caucasicus*	*papa01* (Turk 54)	Tunisia/Iran	EU780683	563
Phasgonophora sulcata	-	Canada	HM355820	596
P. sulcata	-	Canada	HM355823	596
P. sulcata	-	Canada	HM355821	596
Hasgonophora sulcata	-	Canada	HM355824	596
Culex pipiens quinquefasciatus	*Wpip*	United States of America	AF301012	558
Cinara cedri	*wsp_BS_Salamanca(CCeS)*	Spain	JN384046	585
Bemisia tabaci		Bangladesh	JQ305095	490
Phlebotomus longicuspis	wPhleb54d	France	JN315985	517
P. perniciosus	*wPrn*	Italy	AF237884	554
P. perniciosus	-	France	DQ402519	510
Cimex lectularius	*TIH*	Japan	AB475129	562
Calyptrate muscoid fly	*A_NY_Calyp150743b*	United States of America	EU126486	501
Simulium damnosum	-	Ghana	FN563975	565
Bemisia tabaci	*wBtab ch2*	China	FJ404651	601
B. tabaci	*wBtab ch4*	China	FJ404653	607

Wolbachia is also interesting in an evolutionary context due to the diversity and elegance of its interactions with its hosts and because the barriers to cross crossmating between populations may reinforce the genetic divergence between them or speciation (Laven 1967, Bordenstein 2003).

The presence of *W. pipientis* is not strictly associated with ZCL endemicity, as this bacterium has been found in populations of *P. papatasi* from non-endemic areas of Iran (Parvizi et al. 2003).

Independent of their mitochondrial haplotype, geographical origin and habitat, wild *P. papatasi* have been found to be either uninfected with *W. pipientis* or infected with one common widespread strain. This raises the possibility of using a genetically modified strain of *W. pipientis* to drive transgenes through wild sandfly populations to intervene in the transmission of *Leishmania*.

CI is a recognised phenotype caused by natural *Wolbachia* infection in sandflies. It is believed that *Wolbachia* might prevent the transmission of virus and parasite infections (Dobson et al. 2002).

Until now, it was believed that individual strains of *Wolbachia* could only be assigned to a particular species of sandfly. However, in the present study, it has been shown that one species of sandfly can be infected with different *Wolbachia* strains and that different species of sandflies can be infected either with a common haplotype or common strain. While *Wolbachia* strains were also found in two species of the *Paraphlebotomus* subgenus for the first time in the present study, the first two observations are more significant. The *wsp* gene is a very useful tool for typing different *Wolbachia* strains. The frequent incidence of *Wolbachia* strains and their ability to manipulate host reproduction has led to *Wolbachia* being proposed as important agents in the evolution of arthropod hosts. *Wolbachia* provides a starting point for inducing changes in host sex or sexuality. By manipulating *Wolbachia* via transgenes, it is hoped that these bacteria may be able to be used as a system to decrease vector-borne-diseases and to reduce the transmission of diseases in the future. As a main vector of *L. major*, these bacteria can be employed as a gene-driving system for spreading anti-pathogen transgenes through wild populations of sandflies.

The infection rates of *Wolbachia* among the observed Iranian populations of *P. mongolensis* and *P. caucasicus* were low and the reason for such low infection rates could be that there are different densities of the infecting bacteria in different locations. The low detection rates of *Wolbachia* in *P. caucasicus* males in comparison with *P. mongolensis* males and the females of both species can be explained by the fact that most of the *P. mongolensis* males and the females of both species were caught in gerbil burrows. Therefore, it is possible that *Wolbachia* might be spread by transgenes in gerbil burrows.

Further studies examining *Wolbachia* species in sandflies are needed to reveal the relationship between *Wolbachia* endosymbionts and phlebotomine hosts.

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