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Polymorphism of *Cytb* gene and *Leishmania* infection in different populations of *Phlebotomus papatasi*

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Abstract
Phlebotomus papatasi is the main vector of leishmaniasis in the Middle East and Iran. Molecular analysis of population and evaluation of sensitivity of different groups of this vector to *Leishmania* parasite are essential for choosing the vector control measures. Sand fly samples were collected from different parts of Iran and *P. papatasi* species were identified and their partial *Cytb* of mtDNA genes was amplified and analyzed to identify different haplotypes. Also, *Leishmania* infection of samples was tested by partial kDNA. Out of 1922 collected sandflies, 1191 (97/67%) were *P. papatasi* containing 450 females. *Leishmania* parasite was detected in 4(1%) of the samples. Sequence analysis of *Cytb* fragments revealed the existence of 5 haplotypes with haplotype I having the highest frequency. Health related significance of *P. papatasi* requires further attention by policy makers. Further studies concerning the host preference of this species and monitoring of haplotype I are suggested.

Keywords: *Phlebotomus papatasi, Cytb* gene, *kDNA, Leishmania*

1. Introduction
Phlebotomus papatasi is the most important sand fly species that causes numerous health problems through transmission of leishmaniasis and sand fly fever in different parts of the world. This species has a wide distribution in the Middle East, occurring in Iran, Iraq, Turkey, Afghanistan, Pakistan, Uzbekistan, Tajikistan, Azerbaijan, Saudi Arabia, Jordan and Morocco [1, 2]. Geographic distribution of this vector, encompasses a wide range climatic and ecological conditions. It is a pre-domestic species and has a high human preference for blood feeding compared to other *Phlebotomus* species. Anthropophilic index, and vector competence of this species vary with its habitats [3]. Therefore, knowledge of the genetic relationships and biogeography among *P. papatasi* populations will lead to better understanding of the current geographic distribution and improve the design of appropriate control measures for this species.

The control of *P. papatasi* is the main approach in the prevention and control of cutaneous leishmaniasis and sand fly fever. Meanwhile, planning the control programs depend on the identification of the population structure and the vector competence in different population groups of this species. Since *P. papatasi* is a monotypic species, traditional and classical morphological methods are not able to separate its population groups. Therefore, the introduction of novel systematics methods and appropriate markers which explain the ecology of vector populations and epidemiological future of the transmitted diseases are necessary.

Various molecular methods including multilocus enzyme electrophoresis, sequencing of mitochondrial, ribosomal and housekeeping genes, and multilocus microsatellite typing have been used previously in population genetic studies [4]. Comparison of *Cytochrome b (Cytb*) sequences has been proven useful for the detection of intra-species variation in *P. papatasi* and consistent significant level of genetic differentiation has been observed by using this tool [5]. High proportion of silent mutations and recombination free of *Cytb* make it as appropriate discriminatory power in exhibit of population variations and their phylogenetic analysis. Various *Cytb* haplotypes have been identified in *P. papatasi* populations from different areas of the Old World [6, 7]. Despite the wide distribution of these haplotypes, their relative frequency and ability in disease transmission compared to the main haplotype is unclear. Therefore, identification of the main vector among different haplotypes needs comprehensive

investigation. Moreover, the genetic structure of *P. papatasi* populations in North West of Iran and the new focus of cutaneous leishmaniasis, particularly in Fars province is unclear.

Regarding the outbreaks of cutaneous leishmaniasis and sand fly fever, and widespread distribution of *P. papatasi* populations in the Middle East, and the necessity to identify the structure of these populations in the vector control and prevention programs of transmitted diseases, and sensitivity of *Cytb* in fallow-up of population dynamics, this study was conducted to determine the molecular identity and the haplotype infectivity with *Leishmania* parasite in different populations of this species in Iran.

2. Materials and Methods

2.1. Collection of samples

Sand flies were collected through sticky traps from indoors, rodent burrows and pyrethroid spray catch from human resident is and animal shelters [8] during 2013-2014 from Zanjan, Teheran, Isfahan, Fars and Hormozgan provinces of Iran. Following the stereomicroscopic observation (under 30X magnification) probable *P. papatasi* samples were separated and the head and posterior section of abdomen were mounted in Berles medium and the remaining parts preserved individually in ethanol and stored at -20 ^{O}C. The mounted samples were examined under the microscope observation and *P. papatasi* samples identified through standard keys [9] and their preserved sections selected for molecular studies.

2.2. Molecular survey

The preserved specimens were washed with distilled water, dried at room temperature and stored at -80 ^{O}C for an hour. Samples were homogenized completely in 100 µl TEN lysing buffer (100 mM TrissCl pH 8.0, 0.5 mM NaCl, 10 mM EDTA, 1% W/V SDS), and a few pieces of glass powder with electric homogenizer then 100 µl TEN buffer and 20 µg Proteinase K (SinaClon) were added to the homogenized samples The samples were incubated at 55 ^{O}C for 12 hrs, then 100µl potassium acetate 5M was added and vortexed gently. The samples were centrifuged at 4000gX for 5 minutes, the supernatant was transferred to a new vial and equal volume cooled isopropanol was added to it and centrifuged at 8000gX for 10 minutes. Sediments of samples were washed twice with cold 75% ethanol and final pellet was dried at room temperature and resuspended in 20 µl TE buffer.

Total volume of PCR reactions was 50µl. PCRs contained 2 µl genomic DNA, 10 pM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 U *Taq* DNA polymerase.

The PCR degenerated primers to amplify the fragment of 545 bp end of the mitochondrial *Cyt b* gene were CB1F, 5'-TATGTACTACCCTGAGGACAAATATC-3', and CB1R, GCTATTACTCCYCCTAACTTRTT-3 [10].

PCR consisted of 5 cycles of denaturation at 94 °C for 30 s, annealing at 40 °C for 30 s and extension at 72 °C for 1 min followed by 35 similar cycles but with annealing at 44 °C and a final extension at 72 °C for 10 min.

For kDNA gene of *Leishmania* parasite amplification, the total volume of reaction was 50 µl. This reaction included 5 µl of template, 10 pM pf each primer, dNTPs 10 mM, $Mgcl_2$ 50 mM, and 1u of *Taq* DNA polymerase. Forward (5' GGGGTTGGTGTAAAATAGGG 3') and reverse (5' TTTGAACGGGATTTCTG 3') primers were designed within the conserved area of *L major* minicircle kDNA. Reference strain of *L. major* (MHOM/IR/54/LV39) was used as standard.

Temperature profile of the reactions included a cycle of predenaturation at 94 ^{O}C for 5 minutes, 30 cycles of denaturation at 94 ^{O}C for 30 seconds, annealing at 52 ^{o}C for 30 seconds, extention at 72 ^{o}C for 1 minute and final extention at 72 ^{o}C for 10 minutes.

A 5µl of PCR products was subjected to electrophoresis in 1.5% agarose gel. The amplified fragments were stained with Ethidium bromide and run at 80V for 30 minutes and observed under UV transilluminator.

A number of samples were purified and sequenced bi-directionally by Source Bioscience (Cambridge UK) using the forward and reverse specific primers.

2.3. Data analysis and bioinformatics

The nucleotide sequences of samples were aligned and edited by means of MUSCLE, BioEdit and MEGA software. The consensus of sequences was BLAST (http://www.ncbi.nlm.gov/BLAST) searched for fragment identification and compared with other submitted alignments in NCBI GenBank.

3. Results

A total of 1972 sand fly samples were collected among which 1191 (87.3%) were *P. papatasi*, 76 (5.5%) *P. halepensis*, 44 (3.2%) *P. sergenti*, 43 (3.13%) *P. caucasicus*, 4 *P. major* group, and 1 each of *P. simici, P. alexandri, P. brevis, and P. jacusieli*. In addition, 540 *Sergentomyia sintoni*, 4 *Se. baghdadis*, 2 *Se. theodori* and 1 *Se. palestinensis* were identified among the samples (fig. 1).

Out of 1191 *P papatasi* samples, 899 (71.28) were collected from indoors (55% of which were females) and 342 samples (28.7%) were captured from outdoors and rodent burrows (34.5% of which were female)

Out of 405 female *P. papatasi* 288, 69, 6 and 6 samples were collected from Fars, Zanjan, Hormozgan, Tehran, and Isfahan provinces respectively. Among the collected samples *Leishmania* infection was observed in 4 cases (1%). In these cases the amplified product of PCR reaction was 760 bp in size (fig. 2).

In 1191 samples of *P. papatasi* (786 female (64%), and 405 male (34%); partial of *Cyt b* was amplified and the PCR production had 439 bp in size (fig 3). Eighteen samples of products were selected randomly and sequenced. Multiple alignment of the samples indicated polymorphism in 6 locations of sequences and all point mutations were transition (fig. 4 and table 1).

Phylogenic analysis of the samples showed the existence of 5 haplotypes in study groups, among which haplotype I had high relative frequency compared with other haplotypes (fig. 5). Haplotype I is distributed across all study areas. This haplotype was captured from indoors and rodent burrows. All *Leishmania* infections belonged to this haplotype. The sequence of haplotype I shared 96% similarity with IRNO2 haplotype reported in previous studies (Accession Number HM992927). Haplotype II was sister group of haplotype I. This haplotype was collected from Hormozgan province. The other haplotypes (3-5) were only distributed in Fars province.

Table 1: Nucleotide polymorphisms of mitochondrial *Cytb* gene in different haplotypes of *P. papatasi* populations in studied areas.

Haplotype group	Origin	Sampling method	Position of nucleotide substitution					
			138	150	171	270	330	393
Ref			A	A	T	C	C	T
IRNO2			.	.	.	.	.	.
HI	Zanjan, Esfahan, Teheran, Fars, Hormozgan	IDS, PSC, RBS	.	.	.	.	.	.
HII	Hormozgan	IDS, PSC	.	.	.	.	T	.
HIII	Fars (Hasan Abad and Sadatshahr districts)	RBS	.	G	G	T	.	G
HIV	Fars (Eslamabad district)	RBS	.	G	G	.	.	G
HV	Fars (Marvdasht district)	IDS, PSC	G	G	G	T	.	G

IDS= Indoor Sticky trap PSC= Pyrethroid spray catch RBS=Rodent burrow sticky trap
A dot indicates identity with the reference sequence
IRNO2 and Ref are presented haplotypes in Parvizi 2006 and Hamarsheh *et al*., 2007.

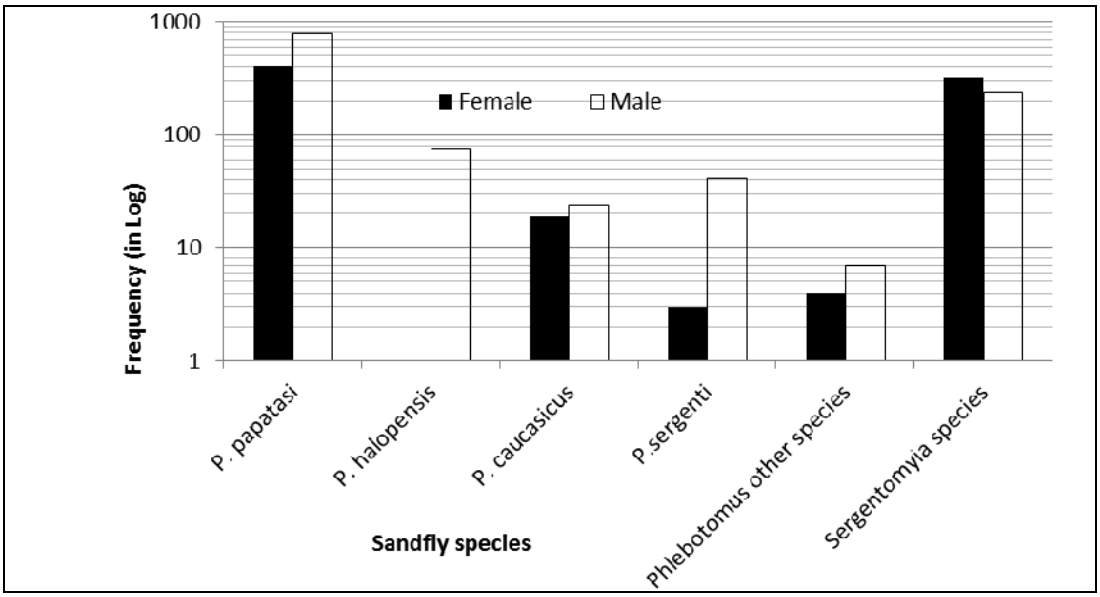

Fig 1: Frequency of different species of sandflies collected from different areas of Iran.

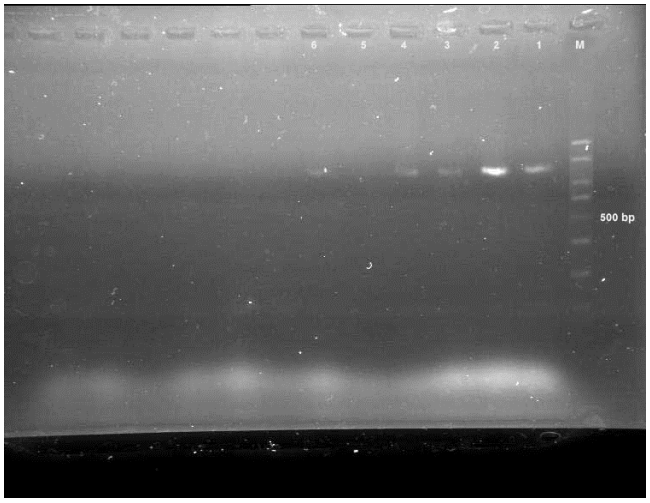

Fig 2: Gel electrophoresis of PCR products of minicircle kDNA of *L major* stained with Ethidium bromide: 1 ladder; 2; Reference strain of *L. major* (MHOM/IR/54/LV39); 2-4 and 6 amplified products of infected samples; 5 negative control.

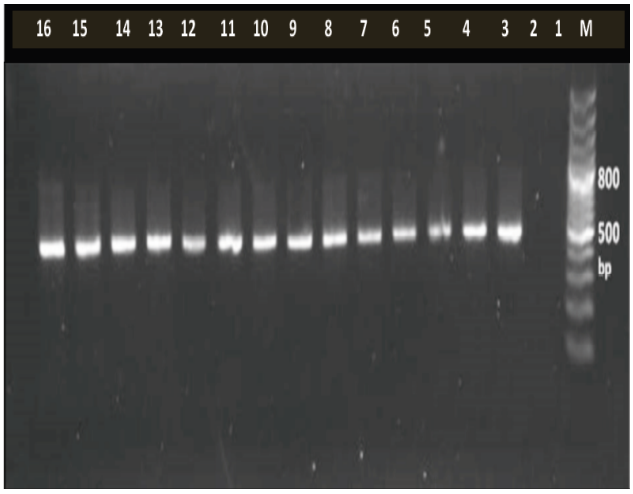

Fig 3: Gel electrophoresis of PCR products of the *Cytb* mitochondrial region of *P. papatasi* stained with Ethidium bromide: 1 ladder; 2 negative control; 3-16 amplified products

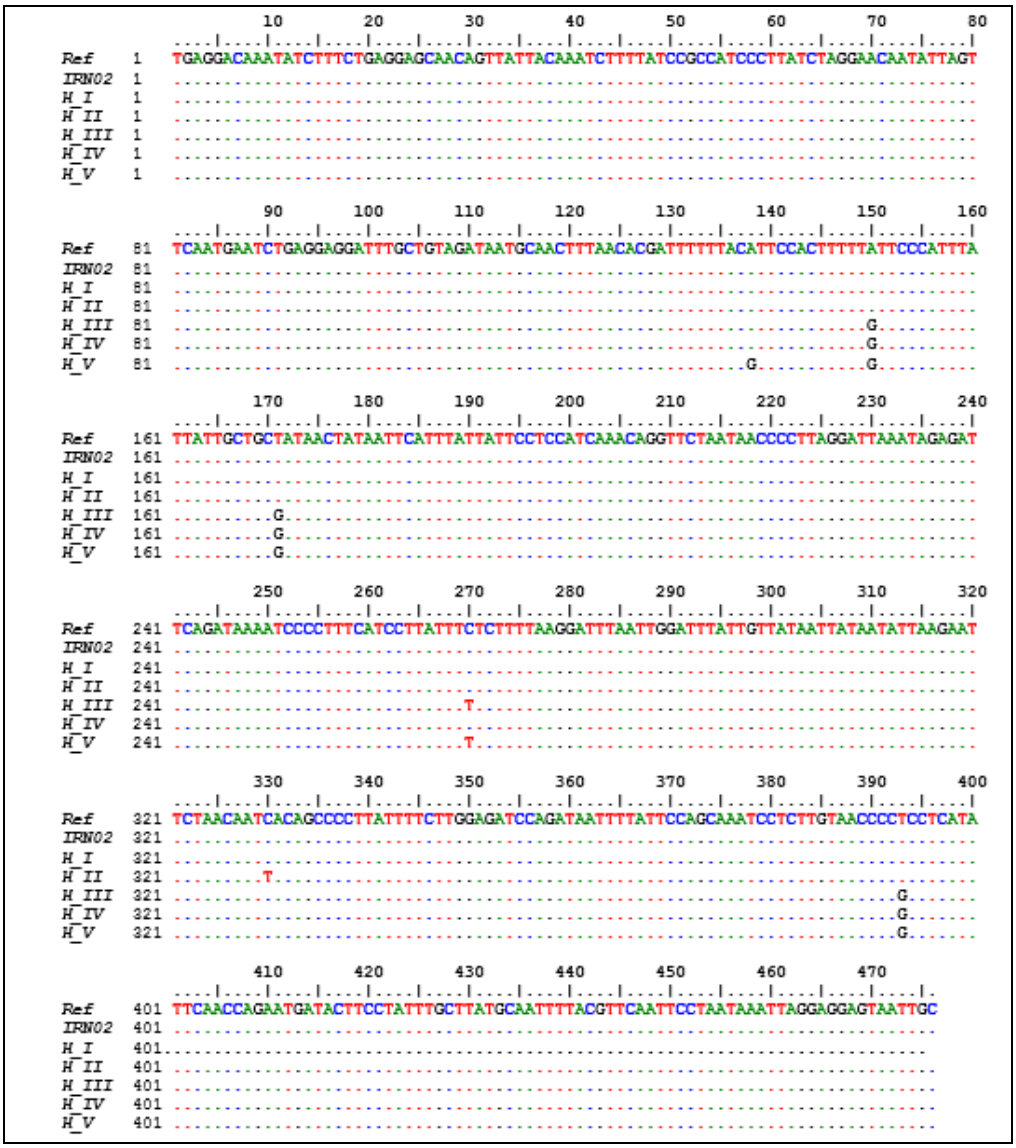

Fig 4: Alignment of the partial sequence of the *Cytb* of the mtDNA of *P. papatasi* haplotypes. Sequences of reference haplotype *P. papatasi* with accessing number (Ref); and Haplotypes!-V from this study were identical in sequence. A dot indicates identity with the reference sequence and a dash indicates a deletion

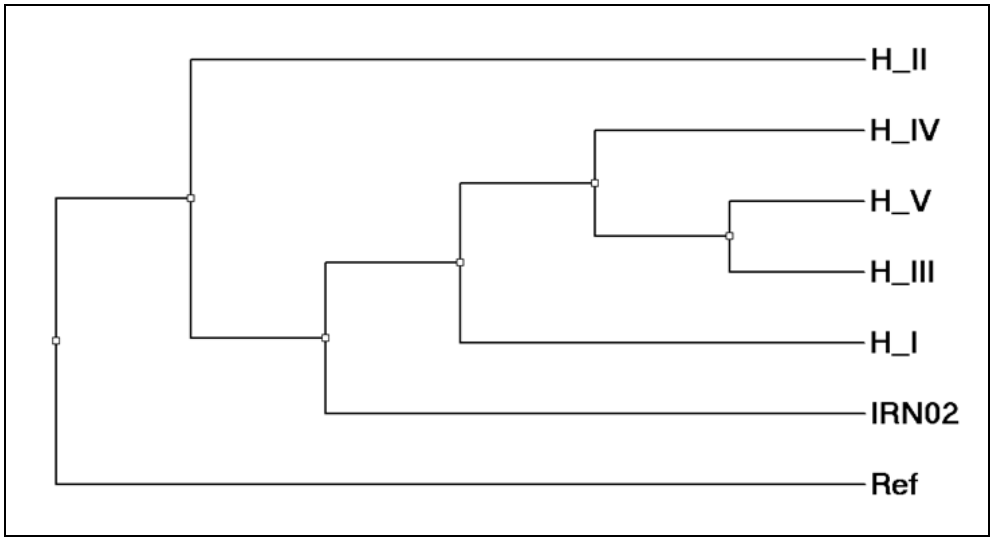

Fig 5: Intuitive phylogeny of different haplotypes of studied population of *P. papaiasi* and haplotypes attributed to IRNO2 and Ref as presented in Parvizi 2006 and Hamarsheh *et al*. 2007. Note the haplotype II as sister group to the remaining haplotypes.

4. Discussion

Phlebotomus papatasi is the main vector of cutaneous leishmaniasis in the Middle East. The present study indicated that *P. papatasi* was the dominant species in the study areas with 71.01% of the indoor, and 28.71% of the rodent burrow sandflies. The high relative frequency of this species from indoors and rodent burrows also were reported from different areas of Iran in previous studies [2]. Regarding the high frequency of this species in study areas and its ability in transmission of zoonotic cutaneous leishmaniasis and papatasi fever, attention of health workers is needed to implement due prevention and control measures.

Based on the results of the present study about 1% of the tested samples of *P. papatasi* were infected with *Leishmania* parasite, with 0.35% infection indoors, and 2.4% infection from rodent burrows. Leptomonad infection of this species has been reported from endemic zoonotic cutaneous leishmaniasis foci and different values of l infectivity have been reported for this species in previous studies [11-14] reported the infection of 2.4% and 5.5% respectively which coincided with the increased case of cutaneous leishmaniasis in Fars province. Findings of Parvizi and Baghban showed the infection rate of *P. papatasi* 4% in central provinces of Iran [15]. In the study of South and South East of Iran the infection rate of this species was also reported 2.1%-5% [16]. Infection rate of *P. papatasi* to *Leishmania* parasite in the present research is in accordance with these studies. However, Bakhshi *et al*., reported the high infection rate (10.7%) of *L. major* and *L. turanica* from Turkaman Sahra and Gonabad, Golestan province [17].

The present study findings indicated that the infection rate in the samples collected from rodent burrows is higher than the infection rate in indoor areas. High infection rate of the samples is associated with the high frequency of infected rodents, the natural reservoirs of cutaneous leishmaniasis in these areas.

Regarding the high frequency of *P. papatasi* in various areas and its infection to *Leishmania* parasite, control measures including indoor residual spraying, using impregnated bed nets and personal protection as well as rodent control are required.

The present study is the first record of infection to *Leishmania* in sandfly samples from Zanjan province and the infection was seen in samples of both indoor and rodent burrows. Cutaneous leishmaniasis is sporadic in this province and indigenous cases have not been reported from it yet. Since one of the infected samples was collected from indoors, and the area is inappropriate for rodent activity, the infection might have been due to other *Leishmania* species. However, further studies could be clarifying in this regard.

Five haplotypes of *Cytb* oxidase group have been observed within the *P. papatasi* populations in the studied areas, among which haplotype I had the highest frequency. In Essingher *et al*., studies on *P. papatasi* populations collected from twelve Middle Eastern countries, 16 haplotypes were reported using the *Cytb* molecular marker [10]. In addition, findings of Hamarasheh *et al*. using the same marker from 20 Middle Eastern countries including 131 geographic areas, 21 haplotypes were observed in 33 polymorphic sites of the amplified fragment [6]. In a similar study, Parvizi *et al*.,(2003, 2006) investigated the partial of *Cytb* in *P. papatasi* among which IRNO2 haplotype had the highest frequency within the collected samples [15,18]. Moreover, in a study of Iran-Iraq border line and Khozestan province haplotype I has a high frequency among haplotypes [19]. They showed that haplotype is similar to IRNO2 haplotype.

Multi-alignment of haplotype sequence data in the present study revealed that haplotype I is completely identical to haplotype IRNO2 presented in previous studies. This haplotype might be more sensitive to infection compared to other haplotypes and all infected samples in our study were in this haplotype. Furthermore, haplotype I was predominant and had the highest frequency among the sandfly populations both indoors and in rodent burrows. Thus, population dynamism within the populations of this sandfly, haplotype I population mobility and the host preference could be of great value in planning and evaluation of control measures in the future studies.

5. Conclusions

Due to high frequency and predominance of *P. papatasi* in the study areas and its capability in transmission of various infectious diseases, health policy makers need to pay attention to the prevention and control measures; planning for indoor residual spraying, using impregnated bed nets and personal protection methods and, rodent control. Observation of infection to *Leishmania* parasite in the collected samples from Zanjan province, and vagueness of epidemiological face of infection, necessitate future studies in this area. Regarding the dominance of haplotype I among the different types of *P. papatasi*, and high capability of this haplotype, in infection to *Leishmania*, investigation of population dynamics, monitoring the population dispersal, and host preference in the future studies could be of great value in the planning of control measures and evaluation of programs.

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