Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Molecular characterization of sandflies and *Leishmania* detection in main vector of zoonotic cutaneous leishmaniasis in Abarkouh district of Yazd province, Iran

Jafari R[1], Najafzadeh N[3,4], Sedaghat MM[2], Parvizi P[3*]

[1]*National Institute Of Health Research, Isfahan Health Research Station, Iran*
[2]*Department of Medical Entomology and Vector Control, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
[3]*Molecular Systematic Laboratory, Parasitology Department, Pasteur Institute of Iran, Tehran, Iran*
[4]*Department of Microbiology, Islamic Azad University, Science and Research Branch of Fars, Iran*

ARTICLE INFO

Article history:
Received 10 July 2013
Received in revised form 15 August 2013
Accepted 15 September 2013
Available online 20 October 2013

Keywords:
Zoonotic cutaneous leishmaniasis
Phlebotomus papatasi
Leishmania major
Sandfly
Central Iran

ABSTRACT

Objective: To assess molecular characterization, distribution, seasonal activities of sandfly species and *Leishmania* parasites infecting them for this zoonotic cutaneous leishmaniasis focus. **Methods:** The collections were carried out in 2009-2011 using CDC traps, Sticky Papers and manual aspirator in and around the villages in Abarkouh district. Individual sandflies were characterized by PCR amplification and sequencing of fragments of their mitochondrial cytochrome b gene. *Leishmania* parasite infections within sandflies were performed by targeting Cyt b, ITS-rDNA, k-DNA and microsatellite genes. **Results:** The PCR assays detected only *Leishmania major* (*L. major*). All infections (30) were found in the abundant and widespread vector *Phlebotomus papatasi* (*P. papatasi*). Small numbers of other sandfly species were also screened for infections, but none was found. *Sergentomyia sintoni* and *P. papatasi* were the predominant members in all locations of this district and in all habitats throughout the trapping season. Only five other sandfly species were found, namely *Phlebotomus ansari*, *Phlebotomus caucasicus*, *Phlebotomus sergenti*, *Sergentomyia dentata* and *Sergentomyia merviney*. **Conclusions:** In the current survey, the only infections detected are of *L. major* in females of *P. papatasi* (30 out of 190). The rates of infection of *P. papatasi* by *L. major* are not significantly different in compare with other locations in Iran with no diversity of parasite strains. Zoonotic cutaneous leishmaniasis may have emerged only recently in Abarkouh district, and the reason may well be the instability of the transmission cycles there.

1. Introduction

Zoonotic cutaneous leishmaniasis (ZCL) is a group of arthropod borne parasitic disease of human and other mammals caused by infections with protozoan haemoflagellates of the genus *Leishmania* Ross in the family Trypanosomatidae, order Kinetoplastida. *Leishmania* parasites transmit by sandflies that live and breed in gerbil burrows[1,2]. All sandflies incriminated as vectors of Leishmaniasis cannot be found all over the world, and its limitation in each region, depends on geographic situations, altitude and climate of the area, the ecology and biology of the sandflies itself, and the reservoir hosts[3]. More than 50 species of sandflies have been reported in Iran[4,5], but only minorities of them are common in ZCL foci and bite gerbils and/or people. So the accurate identification of the sandflies is essential and important to find the only proven or potential vector of ZCL for control program considerations. In males of some sandfly species, external genitalia provide

*Corresponding authors: Parvizi P, Molecular Systematic Laboratory, Parasitology Department, Pasteur Institute of Iran, Tehran, Iran.
Tel: 00 98216649 6414
Fax: 00982166968855
E-mail: parp@Pasteur.ac.ir

the only diagnostic morphological characters; the females of these species could not be separated morphologically based on the structure of the spermathecae or the weakly developed pharyngeal armature[6,7]. Since these female species of sandflies are impossible to identify from each other, molecular methods are needed[7,8].

Understanding of distribution, fauna, sandfly's species, and seasonal activities in each region, holds a great importance when facing ZCL[4,9].

Abarkouh district in Yazd province is located at an altitude of 1 510 meters above sea level (a.s.l.) (4 954 feet). And its coordinates are: 31 ° 07′44″N 53 ° 16′57″E 31.13 ° N 53.28 ° E. Abarkouh is a part of an important focus of ZCL in centre of Iran (Figure 1). Regarding to records received from health care authorities in the area, ZCL has noticeably been increased over the past few years, or the cases of the disease had an increase or decrease from one region to another and the necessity of a study on distribution, fauna, sandfly's species, seasonal activities, ecology, biology and detection of *Leishmania* parasite within sandflies in the area has been requested by the authorities and that is the main reason of the following research.

Figure 1. Location of villages, Abarkouh district in Yazd province of Iran, where sandflies were sampled and screened for *Leishmania* infections.

The mitochondrial genome has been shown to be a good source of accessible genetic variation, and analysis of mitochondrial DNA (mt DNA) variation has been used to understand evolutionary biology at both the intra specific and inter specific levels[8,10]. Among the insects, the first mitochondrial genomes were obtained by cloning and sequencing, *eg.* for *Drosophila yakuba*[11] and then by using the polymerase chain reaction (PCR), *eg.* for *Anopheles quadrimaculatus*[12]. Cytochrome b (Cyt b), which is usually useful for the molecular systematic of insect species in related genera but not always for the phylogenetics of higher taxa[13].

Over the last decade, molecular methods as an accurate identification of *Leishmania* species, have been successfully improved[14-16]. The high sensitivity and specificity are the main advantages of the method. The number, stage and localization of the *Leishmania* and field studies in vectors applied even in areas with low rates of infection in the last few years[17,18].

Since there has been no record on molecular characterization, distribution, seasonal activities and *Leishmania* infection in sandfly species in Abarkouh district of Yazd province, this study was designed to lighten these aspects of ZCL in the area.

2. Materials and methods

2.1. Collection, morphological and identification of adult sandflies

The collections were carried out in 2009-2011, during the seasons of activity of adult sandflies, all the study sites were situated at the edge of rural villages on the cultivated plain, mostly at the edges of 3 regular sampling villages and 3 random ones, near Abarkouh city (Yazd province) (Figure 1).

Three types of traps were used for sandfly collection sticky papers which were placed over night in bedrooms, storerooms and toilets from indoors, ruined outhouses and at the entrances to gerbil burrows, and CDC miniature light traps which were set overnight to sample sandflies in domestic animal shelters, and a manual aspirator was sometimes used by a single collector to capture sandflies resting inside houses in the morning[19].

Samples were stored in −70 ℃ or narcotized with cigarette smoke and those caught on sticky papers were removed with needles or fine brushes dipped in 70% ethanol. All specimens were then stored in analytical grade 80% ethanol, firstly at 4 ℃ (in Abarkouh) and later at −20 ℃ (in Systematic molecular laboratory Pasteur Institute Iran, Tehran).

All sandflies were identified based on external and internal morphological characters of the head and abdominal terminalia[20,21], which were slide-mounted in Berlese fluid following dissection with sterilized forceps and micro-needles[19]. This was carried out in a room away from the molecular biology laboratory, to reduce the risk of PCR carryover, i.e. the "contamination" of a genomic DNA

sample with a DNA fragment that had already been amplified by PCR. The extraction of DNA was performed in molecular systematic laboratory of Pasteur using ISH-Horovize method with minor modifications[19].

2.2. DNA amplification and sequence analysis

For molecular characterization of sandflies, DNA extracted from the thorax and attached anterior abdomen of each sandfly was used to amplify a fragment of Cyt b with the primer pair CB1-SE/CB-R06 from single-fly by PCR[19].

For *Leishmania* detection PCR was performed on each female *Phlebotomus papatasi* (*P. papatasi*) using three genes: ITS-rDNA gene, using ITS1F and ITS2R4 primers; Kinetoplast DNA gene, using LINR4, LIN17 and LIN19 primers; and microsatellite DNA using ITSmF1 and ITSmR2 primers[2,22].

The fragments were directly sequenced, and the sequences edited and aligned using Sequencher™ v. 4.4 to permit the identification of haplotypes (= unique sequences) and their phylogenetic analysis using MEGA4 and PAUP* softwares[23,24].

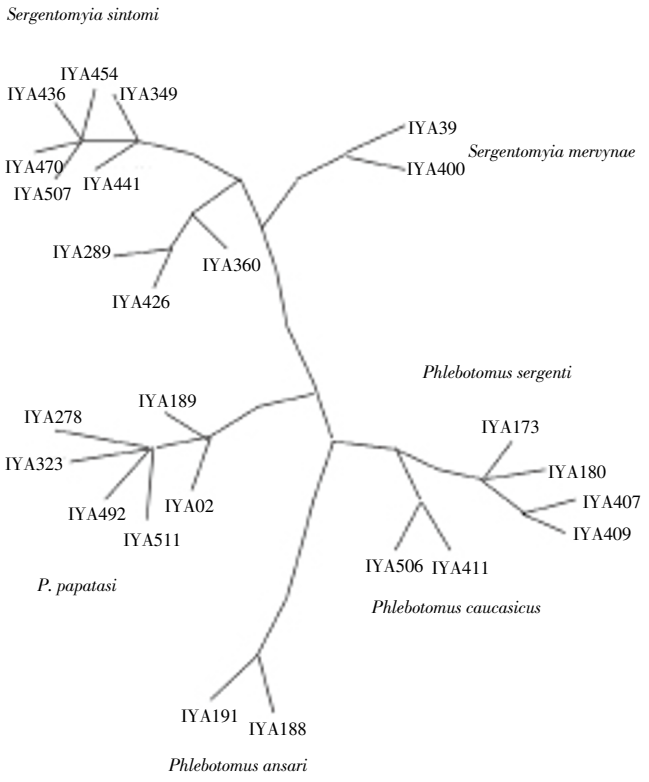

Figure 2. Unrooted consensus phylogenetic tree for DNA sequences of Cyt b (715 nucleotides) of sandfly species were collected from Abarkouh district, produced by heuristic parsimony search using PAUP*.

3. Results

Total of 2 476 sandflies were collected and identified from different villages of Abarkouh focus, Yazd province. A total of 2 033 sandflies were captured from outdoors including gerbil borrowers, yards, ruins, cave craves and etc; and 433 sandflies were captured from indoor places including animal shelters, bedrooms, bathrooms and *etc.* (Table 1).

A total of 876 males and 1 880 female sandflies were identified by morphological and molecular characters. Cyt b fragment sequences were obtained for all seven sandfly species from Abarkouh district. A total of 25 haplotypes were identified (Figure 2).

Unrooted phylogram showed two primary branches, and each of these had subgroups with relatively long branches. One of the primary branches had 3 branches belonged to 3 *Sergentomyia* species. *Sergentomyia sintoni* had 6 haplotypes, *Sergentomyia dentata* 3 haplotypes and *Sergentomyia merviney* 2 haplotypes. The second primary branch had 3 main branches which separated 3 subgenus of *Phlebotomus*. *P. papatasi* had 6 haplotypes and *Phlebotomus* (*Synphlebotomus*) *ansari* 2 haplotypes and also *Phlebotomus caucasicus*, *Paraphlebotomus sergenti* species together 6 haplotypes (*Phlebotomus caucasicus* 2 and *Phlebotomus sergenti* 4). The pairwise genetic distances between

Table 1
Distribution of collected samples of sandfy on different villages of Abarkouh focus, Yazd Province, Iran.

Location		*S. mervynae*		*S. dentata*		*S. sintoni*		*P. ansari*		*P. sergenti*		*P. caucasicus*		*P. papatasi*		Total
		F	M	F	M	F	M	F	M	F	M	F	M	F	M	
Regular villages	Abarkouh	9	0	56	13	443	219	0	0	2	1	1	8	106	91	949
	Esfand abad	7	4	34	0	198	38	74	36	0	0	12	3	62	74	542
	Harooni	17	9	59	32	396	124	28	2	3	0	0	4	168	143	985
Random villages	Khoramabad	0	0	10	0	0	1	0	3	2	1	1	3	7	0	28
	chahgir	0	0	8	0	0	0	0	0	0	0	0	0	9	3	20
	Bahman	0	0	73	12	0	11	5	0	0	0	0	8	85	32	226
	Harook	0	0	0	0	0	0	0	0	0	0	0	0	5	1	6
Total		33	13	240	57	1 037	393	107	41	7	2	14	26	442	344	2 756
Indoor places		0.60%		8.60%		33.50%		7.20%		0.20%		2.00%		47.90%		100.00%
Rodent burrows		1.60%		8.00%		62.70%		5.20%		0.20%		1.00%		21.30%		100.00%

S. mervynae: Sergentomyia mervynae; S. dentata: Sergentomyia dentata; S. sintoni: Sergentomyia sitoni; P. ansari: Phlebotomus ansari; P. sergenti: Phlebotomus sergerti; P. caucasicus: Phlebotomus caucasicus.

Table 2

Leishmania detection in female sandflies by targeting three genes in different locations in Abrakouh focus, Yazd province, Iran.

Sandflies´taxonomy/ location		*P. papatasi*				*P. sergenti*			*P. caucasicus*			*P. ansari*			*S. dentata*			Total
		ITS-rDNA	k-DNA	Micro	Total	ITS-rDNA	k-DNA	Micro	ITS-rDNA	k-DNA	Micro	ITS-rDNA	k-DNA	Micro	ITS-rDNA	k-DNA	Micro	
Bahman	Esfand Abad	10(4)	7(3)	12(3)	13(4)*	0	0	0	5(0)	2(0)	5(0)	10(0)	0	10(0)	1(0)	0	7(0)	53(4)
	Khoram Abad	15(4)	2(0)	8(6)	25(6)	2(0)	0	0	1(0)	0	0	0	0	0	0	0	0	28(6)
	Gonbad Ali	10(0)	0	6(2)	16(2)	0	0	0	0	0	0	0	3(0)	2(0)	0	0	0	21(2)
Abarkouh	Abarkouh	3(1)	2(1)	4(1)	9(1)	2(0)	0	0	0	1(0)	0	0	0	0	5(0)	0	0	17(1)
	Harook	0	0	5(1)	5(1)	0	0	0	0	0	0	0	0	0	0	0	0	5(1)
Harooni	Harooni	83(8)	7(2)	7(1)	97(8)	0	0	3(0)	0	0	0	0	0	5(0)	3(0)	0	3(0)	111(8)
	Chahgir	10(2)	0	15 (8)	25(8)	0	0	0	0	0	0	0	0	0	0	0	0	25(8)
Total		[131(19), 18 (6), 57(22)]=190(30)				4(0)	0	3(0)	6(0)	3(0)	5(0)	10(0)	3(0)	17(0)	9(0)	0	10(0)	260(30)

**Leishmania* positive, Micro: Microcatellite, *P. sergenti: Phlebotomus sergenti; P. caucasilus: Phlebotomus caucasicus: P. ansari: Phlebotomus onsari; S. dentata: Sergentomyia dentata.*

haplotypes of within species were low but high between subgenus of species and even higher considering different genus (Figure 2).

P. papatasi is the dominant species in Abarkouh district of Yazd province. *Phlebotomus ansari*, *Phlebotomus caucasicus*, *Phlebotomus sergenti* species together contain 8.6% of captured samples and do not have a considerable population or seasonal activity in the area. *Sergentomyia sintoni* is the dominant species in outdoor places in Abarkouh district of Yazd province, and can be found in all months of the year with a respectively high seasonal activity. *Sergentomyia dentata*, *Sergentomyia merviney* species together contains 18% of conducted samples and they do not have a considerable population or seasonal activity. Adult sandfly's seasonal activity for *P. papatasi* and *Sergentomyia sintoni* were shown in Figure 3. A total of 50.05% *P. papatasi* were male and 49.95% female. About *Sergentomyia sintoni*'s seasonal activity and sex ratio; 23.61% of samples collected were male and the rest (76.39%) were female (Table 1); 96.6% of *P. papatasi* was identified as parous and 3.4% noliparus.

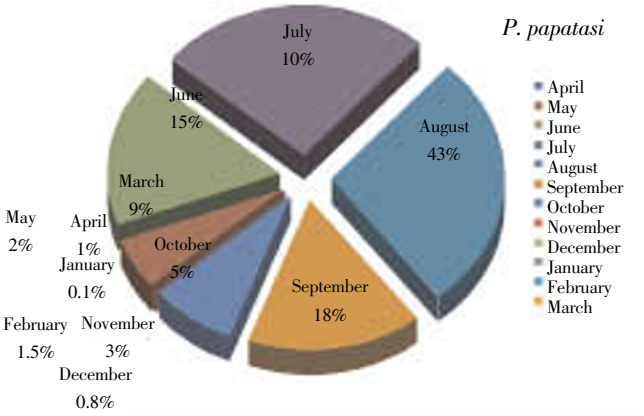

Figure 3. Seasonal activity of *P. papatasi* sandfly in the study area in 2011.

As for *Leishmania* parasite infection, a total of 260 female sandflies were studied and after performing PCR targeting ITS-rDNA, k-DNA and microsatellite DNA in over 190 female *P. papatasi* 30 of them were found to be infected with *Leishmania* parasites. No *Leishmania* infection was found in other sandfly species. The detail results are shown in Table 2. Sixteen out of 190 *P. papatasi* were examined at least with two of ITS-rDNA, k-DNA and/or microsatellite DNA genes and 17 out of 30 *Leishmania* positive samples were identified with ITS-rDNA, k-DNA and/or microsatellite DNA genes (Table 2). All the infected females of *P. papatasi* contained developing or fully developed eggs (Table 2). For accurate identification of *Leishmania* species and announcing the main causative agent of ZCL, RFLP and sequencing was performed on positive PCR products of ITS-rDNA gene with high amount of parasite existence; Based on molecular analysis, 15 out of 30 positive samples had enough DNA or readable sequences and firmly identified as *Leishmania major* (*L. major*) (Table 2).

Haplotype of ITS-rDNA found in *P. papatasi* were identified by aligning the sequences with those of *Leishmania* species in GenBank. Eighteen haplotypes of *Leishmania* were analyzed using PAUP* software (Figure 4).

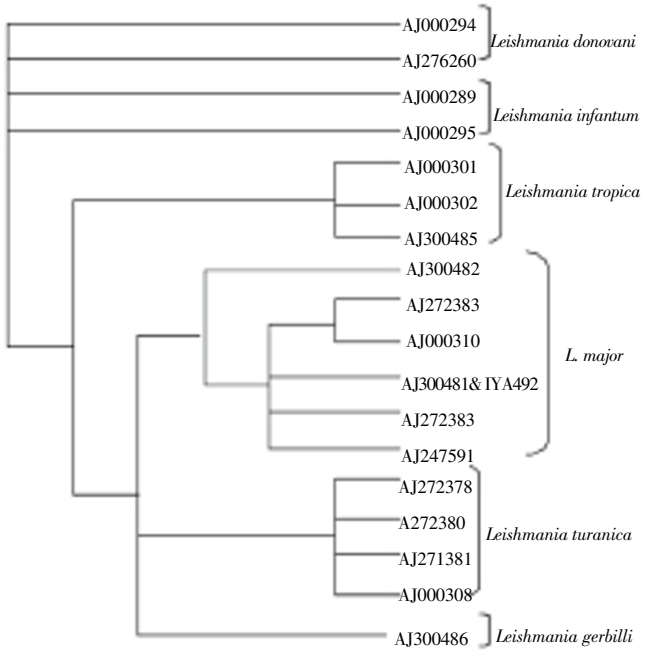

Figure 4. Relationship of ITS-rDNA sequences (ITS1F/ITS2R4 fragment) of different strains of *Leishmania*.
The consensus most parsimonious cladogram (of 234 shortest trees of length = 62) was found by a branch and bound search using PAUP*.

4. Discussion

We have previously sequenced the ITS-rDNA gene of *Leishmania*, in order to compare the diversity of parasite species and strains found in the sandfly vectors (Diptera, Psychodidae) of *L. major* in foci of ZCL in Golestan province, northeast Iran, and in Isfahan province, central Iran[2,25,26]. We now report the use of the same standardized techniques to assess the *Leishmania* parasites found in sandflies in another one of the Iran foci of ZCL, in Yazd province to the east of Isfahan. In the current investigation of *Leishmania* in the sandflies of Abarkouh district, Yazd province, the only infections detected were of *L. major* in females of *P. papatasi* (30 out of 190; 15.79%). There was not any diversity of *L. major* in *P. papatasi* with only one haplotype sequences. The overall infection rates in *P. papatasi* to compare with our previous finding are not significantly different (*Chi*-squared test: $P > 0.05$)[26,27].

Eighteen haplotype sequences of the 435 bp ITS1F/ITS2R4 fragment were aligned for phylogenetic analysis in PAUP*, representing the *L. major* found in the *P. papatasi* in Abarkouh district and some *Leishmania* from GenBank. The tree (the consensus most parsimonious cladogram (of 234 shortest trees of length = 62) constructed by the branch and bound search using PAUP* method is presented as a cladogram.

The rates of infection of *P. papatasi* by *L. major* were of the same in Isfahan, Golestan and Fars provinces, where this sandfly is considered to be the principal vector[2,25-27]. However, there is an important difference between these provinces. No other gerbil parasites were detected in *P. papatasi* from Abarkouh district, Yazd province. In contrast, both *Leishmania turanica* and *Leishmania near gerbilli* were detected in *P. papatasi* in Isfahan and Golastan provinces[2].

No *L. major* or other gerbil parasites were detected in other six sandfly species maybe because of low densities of these sandfly species were tried to incriminate as vectors[28].

We conclude that our failure to find *Leishmania turanica* and *Leishmania near gerbilli* in sandflies from Abarkouh district, Yazd province can explain by the absence or low densities of the incriminated or suspected sandfly vectors, which include *Phlebotomus* (*Paraphlebotomus*) *caucasicus* and the very similar *Phlebotomus* (Pa.) *mongolensis*, in addition to the more frequently infected *P. papatasi*.

From 2 476 sandflies, seven species were identified using on their morphological characters of head and abdomen terminal. Based on the Cyt b fragment examined, phylogenetic analyses were performed to help investigate the relationship between haplotypes, species, subgenera and genera of sandflies were caught from Abarkouh and the geographical variation among haplotypes of each sandfly species. Most sandfly species showed only recent divergence in this location of Iran, because the genetic distances between haplotypes of each sandfly species were small. But evidences for isolation by species were found. Firstly, all the haplotypes of different sandfly species from Abarkouh, Iran could not be shown to belong to a single network, whereas most from single sandfly species did belong to a single network. Secondly, there were more haplotypes that were found from some sandfly species. The sandfly fauna of Abarkouh district, Yazd province was dominated by *Sergentomyia sintoni* (1430 out of 2 756; 51.8 %) and *P. papatasi* (786 out of 2 756; 28.52%). The second one is the incriminated vector of *L. major* throughout southwest Asia and North Africa, and first one is believed to prefer feeding on lizards[29]. The abundance of S. sintoni in our recent collections is because most of this sandfly species were caught from outdoors (gerbil burrows). Seasonal Activity of adult *P. papatasi* stars from June and finishes in November in Abarkouh, Yazd with one pick in July and August in 2011[30].

In Iran, only *P. papatasi* was judged to be a proven vector of *L. major*, although *Phlebotomus caucasicus*/*Phlebotomus mongolensis* and *Phlebotomus ansarii* were only acknowledged as possible vectors[2,31,32].

It is important to understand the distributions of sandflies and the *Leishmania* species infecting them to each ZCL focus in Iran and to identify which *Leishmania* species are frequently infecting potential sandfly vectors.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The work was supported by the Pasteur Institute of Iran; grant 501awarded to Dr. Parviz Parvizi, Another part of this wok was funded by National Institute Of Health Research (NIHR) project No 63-9024. The authors would like to thank Abarkouh Health Care authorities and the personnel of NIHR in Yazd and Isfahan Health Research Stations for their assistance and support during this study in collections of sandflies in the field. We thank Mehdi Baghban, Hassan Soleimani, Mohammad Hosein Arandeian, Mohammad Hadi Farah Zadi, Hosein Dehghan Mangabadi for helping with the field working and Elnaz AlaeeNovin for helping in Molecular Systematic Laboratory. A part of this research through MSc studentships to Narmin Najafzadeh based at the Pasteur Institute of Iran, Tehran, and registered for a Islamic Azad University, Science and Research Branch of Fars, Iran.

References

[1] WHO. *Control of the leishmaniasis. Technical report series No.793.* Geneva: World Health Organization; 1990.

[2] Parvizi P, Ready P. Nested PCRs and sequencing of nuclear ITS-

rDNA fragments detect three *Leishmania* species of gerbils in sandflies from Iranian foci of zoonotic cutaneous leishmaniasis. *Trop Med Int Health* 2008; **13**: 1159–1171.

[3] Ready PD. Biology of *Phlebotomine* sandflies as vectors of disease agents. *Annu Rev Entomol* 2013; **58**: 227–250.

[4] Yaghobi Ershadi MR. *Phlebotomine* sand flies (Diptera: Psychodidae) in Iran and their role on *Leishmania* transmission. *J Arthropod-Borne Dis* 2012; **6**(1): 1–17.

[5] Kasiri H, Javadian E, Seyedi-Rashti MA. Liste des *Phlebotominae* (Diptera: Psychodidae) d'Iran. *Bull Sco Path Exot* 2000; **93**(2):129–130.

[6] Theodor O, Mesghali A. On the phlebotomine of Iran. *J Med Entomol* 1964; 285–300.

[7] Parvizi P, Tahehrkhani H, Ready PD. *Phlebotomus caucasicus* and *Phlebotomus mongolensis* (Diptera: Psychodidae): indistinguishable by the mitochondrial cytochrome b gene in Iran. *Bull Entomol Res* 2010; **100**: 415–420.

[8] Esseghir S, Ready PD, Killick-Kendrick R, Ben-Ismail R. Mitochondrial haplotypes and phylogeography of *Phlebotomus* vectors of *Leishmania major*. *Insect Mol Biol* 1997; **6**: 211–225.

[9] Mahmadallie SS, Pesson B, Ready PD. Multiple genetic divergence and population expansions of a Mediterranean sandfly *Phlebotomus ariasis* in Europe during the Pleistocene glacial cycle. *Heredity* 2010; **1**: 1–13.

[10]Pesson B, Ready JS, Benabdennbi I, Martin-Sanchez J, Esseghir S, Cadi-Soussi M, et al. Sandflies of the *Phlebotomus perniciosus* complex: mitochondrial introgression and a new sibling species of *P. longicuspis* in the Moroccan Rif. *Med Vet Entomol* 2004; **18**: 25–37.

[11]Clary DO, Wolstenholme DR. The mitochondrial DNA molecular of *Drosophila yakuba*: nucleotide sequence, gene organization, and genetic code. *J Mol Evol* 1985; **22**: 252–271.

[12]Mitchell SE, Cockburn Andrew F, Seawright JA. The mitochondrial genome of *Anopheles quadrimaculatus* species A: complete nucleotide sequence and gene organization. *Genome* 1993; **36**: 1058.

[13]Simmons RB, Weller SJ. Utility and evolution of Cytochrome b in insects. *Mol Phylogenet Evol* 2001; **20**: 196–210.

[14]Hajjaran H, Mohebali M, Alimoradi S, Abaei MR, Edrissian GhH. Isolation and characterization of pathogenic *Leishmania turanica* from *Nesokia indica* (Rodentia, Muridae) by PCR-RFLP and ITS1 sequencing in Iran. *Trans R Soc Trop Med Hyg* 2009; **103**: 1177–1179.

[15]Mahmoudzadeh-Niknam H, Ajdary S, Riazi-Rad F, Mirzadegan E, Rezaeian A, Khaze V, et al. Molecular epidemiology of cutaneous leishmaniasis and heterogeneity of *Leishmania major* strains in Iran. *Trop Med Int Health* 2012; [Epub ahead of print].

[16]Schönian G, Kuhls K, Mauricio I. Molecular approaches for a better understanding of the epidemiology and population genetics of *Leishmania*. *Parasitology* 2011; **138**: 405–425.

[17]Bensoussan E, Nasereddin A, Jonas F, Schnur LF, Jaffe CL. Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. *J Clin Microbiol* 2006; **44**: 1435–1439.

[18]Al-Jawabreh A, Diezmann S, Müller M, Wirth T, Schnur LF, Strelkova MV, et al. Identification of geographically distributed sub-populations of *Leishmania* (*Leishmania*) *major* by microsatellite analysis. *BMC Evol Biol* 2008; **8**: 183.

[19]Parvizi P, Benlarbi M, Ready PD. Mitochondrial and Wolbachia markers for the sandfly *Phlebotomus papatasi*: little population differentiation between peridomestic sites and gerbil burrows in Isfahan province. *Iran Med Vet Entomol* 2003; **17**: 351–362.

[20]Nadim A, Javadian E. Key for species identification of sandflies (Phlebotominae; Diptera) of Iran. *Iran J Public Health* 1976; **5**: 35–44.

[21]Lewis DJ. A taxonomic review of the genus *Phlebotomus* (Diptera: Psychodidae). *Bull Br Mus nat Hist Entomol* 1982; **45**: 121–209.

[22]Aransay AM, Scopulica E, Tselentis Y. Detection and identification of *Leishmania* DNA within naturally infected sand flies by semi-nested PCR on minicircle kinetoplastic DNA. *Appl Environ Microbiol* 2000; **66**: 1933–1938.

[23]Tamura K, Dudley J, Nei M, Kumar S. Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007; **24**: 1596–1599.

[24]Swofford DL. *PAUP*: phylogenetic analysis using parsimony (* and other methods) version 4.0*. Sunderlard: Sinauer Associates; 2002.

[25]Parvizi P, Mauricio I, Aransay AM, Miles MA, Ready PD. First detection of *Leishmania major* in peridomestic *Phlebotomus papatasi* from Isfahan province, Iran: comparison of nested PCR of nuclear ITS ribosomal DNA and semi-nested PCR of minicircle kinetoplast DNA. *Acta Trop* 2005; **93**: 75–83.

[26]Parvizi P, Baghban N, AlaeeNovin E, Absavaran A. Detection, identification and molecular typing of *Leishmania major* in *Phlebotomus papatasi* from a focus of zoonotic cutaneous leishmaniasis in central of Iran. *Exp Parasitol* 2010; **124**: 232–237.

[27]Parvizi P, Akhoundi M, Mirzaei H. Distribution, fauna and seasonal variation of sandflies, simultaneous detection of nuclear internal transcribed spacer ribosomal DNA gene of *Leishmania major* in *Rhombomys opimus* and *Phlebotomus papatasi*, in Natanz District in Central Part of Iran. *IBJ* 2012; 16 (2): 113–120.

[28]Rassi Y, Oshaghi MA, MohammadiAzani S, Abaei MR, Rafizadeh S, Mohebali M, et al. Molecular detection of *Leishmania* infection due to *Leishmania major* and *Leishmania turanica* in the vectors and reservoir host in Iran. *Vector-Borne and Zoonotic Dis* 2011; **11**: 145–150.

[29]Nadim A, Seyedi-Rashti MA, Mesgali A. On the nature of leptomonads found in Sergentomyia sintoni in Khorassan, Iran and their relation to Lizard leishmaniasis. *J Trop Med Hyg* 1968; **71**: 240.

[30]Müller GC, Kravchenko VD, Schlein Y. Seasonal and spatial changes of sand fly species in a canyon in the Carmel Mountains. *J Vec Ecol* 2011; **36**(Suppl 1): S118–S127.

[31]Killick-Kendrick R. Phlebotomine vectors of the leishmaniasis: A review. *Med Vet Entomol* 1990; **4**: 1–24.

[32]Yaghoobi-Ershadi MR, Javadian E, Tahvildare-Bidruni GH. The isolation of *Leishmania major* from *Phlebotomus* (*Paraphlebotomus*) *caucasicus* in Isfahan Province, Islamic Republic of Iran. *Trans R Soc Trop Med Hyg* 1994; **88**: 518–519.