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# Biodiversity, *Leishmania* genetic typing and host identification of phlebotomine species in endemic foci of southeastern Iran

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ABSTRACT

Leishmaniasis is a growing health challenge in many parts of Iran, including Kerman Province. Investigating vector ecology and parasite-harboring capacity is prerequisite to the disease control measures. This study included six provincial sites namely Bam (Bm), Dehbakri (Di), Jiroft (Jt), Mohammad-Abad (Md), Rostam-Abad (Rd) and Darb-e-Behesht (Dt) where sand flies were trapped. The specimens were then identified before being exposed to DNA extraction. PCR-RFLP was used to detect leishmanial infection rates and feeding preference of vectors. Diversity indices indicated that the highest effective numbers of species was in plain sites, whereas, the highest expected numbers of species was in mountainous sites. *P. papatasi* and *P. sergenti* showed similar feeding preferences to both human and animal bloods. *P. papatasi* from indoor catches was found infected with *Leishmania major* at a 2% rate. The ITS1 gene sequences of isolated parasites were >99% similar to related GenBank haplotypes. Bam and Rostam-Abad remain active foci of both types of cutaneous leishmaniasis (CL). Md and Di are prone to visceral leishmaniasis (VL). Jt is not at risk of anthroponotic cutaneous leishmaniasis (ACL) due to absence of *P. sergenti*. Sand flies are absent in Dt, probably because of high elevation and cold climate. In conclusion, patterns of climate and ecosystem changes and vector-host-reservoirs interactions must be carefully scrutinized if leishmaniasis is to be controlled in the stricken sites.

## 1. Introduction

Leishmaniasis remains one of the undermining public health challenges, affecting 98 countries worldwide, where 350 million people live at risk and 12 million are already infected (Akhoundi et al., 2016). This vector-borne disease manifests in two clinical forms in Iran including cutaneous (CL) and visceral leishmaniasis (VL) (Yaghoobi-Ershadi, 2012). It is estimated that about 1.5 million new cases of CL and half a million cases of VL occur globally every year. The CL does not cause death but incurs mental, social and economic damages to patients. The damages include chronic wounds, unsightly skin scars, inflammatory painful ulcers, secondary infection, heavy burden of treatment, prolonged treatment and side effects. VL, however, can be fetal if left untreated (Desjeux, 2004).

The causative agents of the disease are members of unicellular kinetoplastids in the genus *Leishmania*. The zoonotic cutaneous leishmaniasis (ZCL) caused by *Leishmania major* comprises 80 % of cases in many endemic parts of Iran. However, the overwhelming cases of leishmaniasis in Kerman Province (96.5%) are caused by *Leishmania tropica* the agent of anthroponotic cutaneous leishmaniasis (ACL) (Aghasi and Sharifi, 2003; Aghaei et al., 2014). VL being mainly sporadic in Iran, it is endemic in northwestern and southern provinces with 100–300 new cases per year. The causative agent of VL in Iran is the zoonotic *Leishmania infantum*, which infects about 4% of children and 18.4 % of domestic dogs in northwest region (Mohebali, 2013). In Kerman Province, Orzoieh district was reported to be the main endemic focus of VL where both *P. papatasi* and *P. alexandri* were predominant vectors of *L. major* and *L. infantum* respectively (Sharifi et al., 2017).

In Iran, there are 44 confirmed species of sand flies of which 11 have been implicated in the transmission of leishmaniasis, in addition to seven more phlebotomines, which need further documentation (Yaghoobi-Ershadi, 2012). However, some of these doubtful species were previously reported and/or have recently confirmed from Iran such as *P. (Adl.) kabulensis* Artemiev, 1978 (Kassiri et al., 2000; Zahraei-Ramazani et al.,

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2015). There is no common consensus about the number of unidentified sand flies in the country; some put them even up to 10 species (Hazratian et al., 2016). In their recent study, Arzamani et al. (2018) stated the number of Iranian identified phlebotomines to be 47 in addition to 3–5 more uncertain species.

Many authors have emphasized the effects of environmental variables, climate change and niche requirements of sand fly species on their spatial and temporal distribution. However, the impacts of urbanization and environmental degradation on epidemiological patterns and ecological drivers of *Leishmania* transmission may not be ignored. Ebrahimi et al. (2016) specified rainfall patterns during fall and winter seasons, temperature and humidity as main environmental factors, which influence sand fly distribution in Khuzestan Province, southwest of Iran. While they failed to establish any correlation between elevation and distribution pattern, other researchers confirmed such relationships for different environmental settings and sand fly populations (Guernaoui et al., 2006; Simsek et al., 2007; Yared et al., 2017). The distribution of sand flies seems to be species dependent.

This study was meant to investigate the structure of sand fly communities in three endemic counties of Kerman province (Fig. 1) to shed light on biodiversity, distribution and *Leishmania* harboring capacity of present phlebotomines. This seemed necessary in order to synchronize control measures with changing ecological profile of CL in the region.

## 2. Materials and methods

### 2.1. Study areas

The study was carried out in three counties located in the southeast of Kerman Province, of which six sites were selected so that to represent the geographic variations of the study area as follows (Census of the Islamic Republic of Iran, 2006).

#### 2.1.1. Jiroft city (Jt)

It is the capital of Jiroft County located at 180 Km away from the Persian Gulf. The city is known as "Iran's Hindustan" for its tropical climatic and surrounding evergreen forest. The county accommodates the largest forest patch in Kerman Province covering 234,000 hectares. The weather is hot in summer exceeding 50 °C, and cold in winter reaching freezing point. The average rainfall is 200 mm. The county includes mountainous cities such as Darb-e-Behesht (Dt) and Mahammad-Abad (Md) which have temperate climates attracting thousands of tourists as summer resorts each year.

#### 2.1.2. Darb-e-Behesht city (Dt)

Because of its high elevation, the city has cold and snowy weather during winter times and mild temperatures during summer. The elevation of Chahon village in the outskirt of the city exceeds 3000 m above sea level with an average rainfall of 400 mm.

#### 2.1.3. Mohammed-Abad city (Md)

This city is located in the western slopes of Barez Mountain. The climate of the city is temperate and cold with an average rainfall of 350 mm.

#### 2.1.4. Bam city (Bm)

It is the capital of Bam County which has hot and dry weather, sometimes with extreme temperatures due to its proximity to Lut Desert. Local meteorology stations have recorded the highest and lowest temperatures ever at national level during summer and winter seasons respectively. The average annual rainfall is estimated at about 68 mm.

#### 2.1.5. Deh-Bakri city (Di)

Located in Bam County, the city has green landscape and temperate weather due to its mountainous nature. Deh-Bakri provides a refreshing refuge for people escaping the stifling summers of the Lut desert. The average annual rainfall of this city is 400 mm.

#### 2.1.6. Rostam-Abad city (Rd)

It is the capital of Narmashir County, located in the southern part of Kerman province. It has extremely hot weather in summer and very cold temperature in winter, but pleasant weather in springtime. The average annual rainfall of the county is 62 mm (8).

The geographic positions and coordinates of the study sites are presented in Table 1 and Fig. 1.

### 2.2. Sand flies collection

Sand flies were captured using sticky traps. The traps were put in indoor places e.g. inside houses and animal shelters and outdoors in bushes, ruins and close to active rodents nests. On each sampling occasion, six indoor and six outdoor places were selected and ten traps per place were used. The traps were set at dusk and collected at dawn. The stuck adult sand flies were gently removed from the traps using a camel brush and acetone. The sand flies were placed on Whatman filter paper to remove excess castor oil, before being transferred to vials containing 70% alcohol.

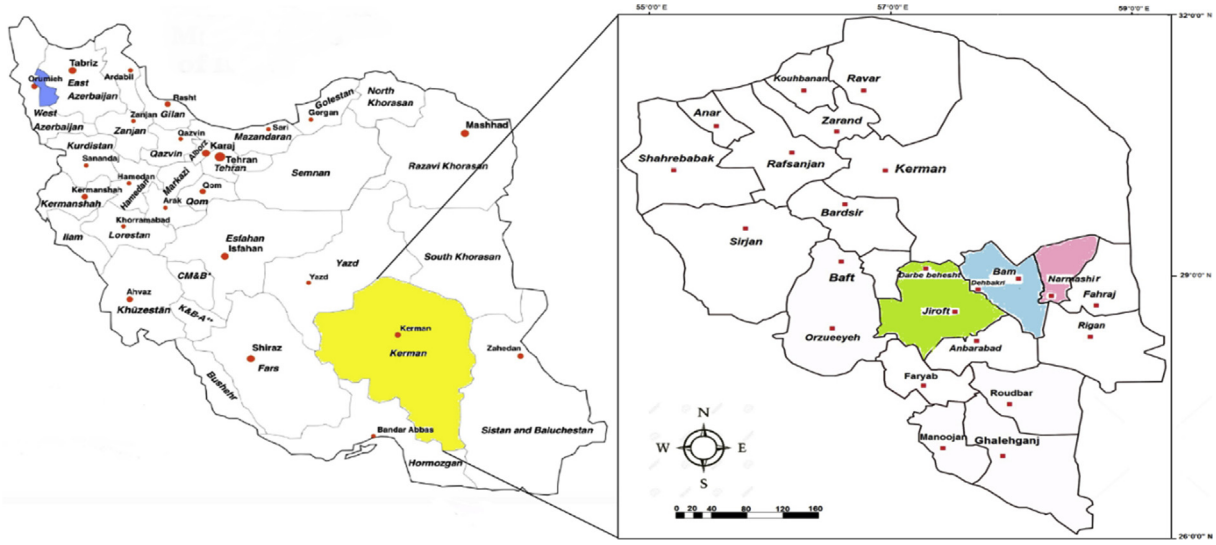



**Fig. 1.** Geographic locations of the study sites in three counties of Kerman Province.

**Table 1**
The details of the study sites in the three counties of Kerman province visited during 2016.

| County | Sites | Date | Altitude | coordinate |
|---|---|---|---|---|
| Bam | Town and outskirt villages | 10th Mar–7th Jun 2016 | 1131m | 29°07′45″N, 58°16′13″E |
| | Deh-Bakri | 18th Apr–22nd Jul 2016 | 2000 m | 29°03′08″N, 57°54′36″E |
| Narmashir | Rostam-Abad | 10th April–5th Jul 2016 | 670 m | 28° 57′ N, 58° 42′ E |
| Jiroft | Town | 3rd Apr–25th May 2016 | 680 m | 28°40′ 13″ N, 57°44′ 13″ E |
| | Mohammad-Abad | 18th Apr–21st May 2016 | 2423 m | 28°47′03″N 57°10′37″E |
| | Darb-e-Behesht | 4th May–23rd Jul 2016 | 2608 m | 29°14′30″N 57°19′31″E |

### 2.3. Sand flies dissection and slide mounting

The specimens were dehydrated by transferring to 80 and 96% alcohol in consecutive steps each for 5 min. Using two dissecting needles, each specimen was picked out and put in a droplet of PBS on a microscopic slide. After removing the specimen's legs, the thorax was pierced with straight needle at the base of the wins and the head was gently removed with bent needle. Spermathecae and genitalia were also pulled out by removing the last 3 abdominal segments. Head, wing, body and posterior abdominal segments were mounted using Canada balsam and covered with a suitable size cover slip. The specimens were dried under 50 °C air cabinet and examined with a light microscope. The specimens were morphologically identified using Iranian phlebotomine morphological keys (Theodor and Mesghali, 1964; Nadim and Javadian, 1976; Seyedi-Rashti and Nadim, 1992).

### 2.4. DNA extraction

The specimens were placed in sterile physiological saline and dissected. Heads and last abdominal segments were mounted on slides using Puri's solution for later morphological identification. The body of the sand flies were put in sterile microtubes, labeled and kept in -20 C for DNA extraction. For DNA extraction, the specimens were first subjected to a digestion step in 400 μL of lysis buffer (50 mM TRIS-HCl, 50 mM NaCl, 20 mM EDTA, 0.5% SDS, pH 8.0) and 10 μL proteinase K (10 mg/mL solution) kept overnight at 60 C. The DNA was then extracted by phenol-chloroform and precipitated by cold absolute ethanol. The DNA was re-suspended in nuclease free water and stored at -20 C until used. The DNA concentration was estimated by absorbance method measuring the ratio of A260/A280. DNA purity of a A260/A280 ratio about 1.8 was considered suitable for further analysis (Tataurov et al., 2008).

### 2.5. PCR-RFLP for parasite identification

Leishmanial contamination of female sand flies was determined using the PCR-RFLP technique. The forward primer (5′-CTGGATCATTTTCCGATG-3′) and the reverse primer (5′-TGATACCACTTATCGCACTT-3′) were used to amplify a 320 bp region of ITS1 of ribosomal RNA gene. The amplification was performed using Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Demark) in 25 $\mu$L of total reaction. The thermocycling conditions included 5 min preheating at 95 °C followed by 30 cycles of 30 Sec. denaturation at 95 °C, 30 Sec. annealing at 51.7 °C, 30 Sec. elongation at 72 °C and 5 min final extension at 72 °C. However, to determine the species of *Leishmania*, the PCR product (10 $\mu$L) was digested with *Hae*III enzyme (2 $\mu$L) in 32 $\mu$L total reaction according to the manufacturer's instructions. The PCR product and its restriction fragments were electrophoresed on 1.5% and 3 % agarose gels repectively at 100 V in TAE buffer (0.04 M Tris acetate and 1 mM EDTA, pH 8) and visualized by UV light upon staining with SafeStain 10,000X SYBR™ (0.12 μg/mL) (Monroy-Ostria et al., 2014).

### 2.6. PCR-RFLP for blood meal identification

The sources of blood meals of captured sand flies were identified with the aid of PCR-RFLP technique. To this end the mitochondrial cytochrome b gene was targeted for molecular diagnostics due to its sufficient genetic variation for vertebrate identification (Kent and Norris, 2005). The DNA amplicon was used to amplify a ~630 bp region of Cyt b gene using F primer (UNFOR403: 5′-TGAGGACAAATATCATTCTGAGG-3′) and the R primer (UNREV1025: 5′-GGTTGTCCTCCAATTCATGTTA-3′). The PCR steps included 5 min preheating at 95 °C followed by 35 cycles of 1 min denaturation at 95 °C, 1 min annealing at 58 °C, 1 min elongation at 72 °C and 7 min final extension at 72 °C. The PCR and restriction reactions and their products were performed and analyzed as described above. The patterns of enzymatic digestion of different hosts are shown in Table 2.

### 2.7. Data analysis

#### 2.7.1. Diversity measurement

The species diversity of phlebotomine sand flies was calculated and compared. The indices used for diversity measurement were as indicated in Table 3.

#### 2.7.2. Sequence typing analysis

The amplicons were extracted from the gel and sequenced in the both directions by the ABI3730XL sequence analyzer (Macrogen, Korea). The sequence chromatograms were edited using Sequencher 4.1 software (Genecodes Corp.).

The nucleotide similarity of PCR amplified ITS1 rDNA gene of *Leishmania* obtained from two infected sand flies, was compared with sequences of *L. major* and *L. tropica* from GenBank using BLAST software (https://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis was performed in MEGA 5 (www.megasoftware.net) using Maximum Likelihood (ML) algorithms with evolutionary distances calculated by Kimura-2 parameter method and 1000 bootstrap value (Kimura, 1980). This research was approved by the Committee of Medical Ethics (no. 52/D/8216 dated 9th Feb. 2016).

## 3. Results

### 3.1. Distribution of sand flies in the collection sites

During a 6-month sampling period, from 10th March to 10th August 2016, samplings were carried out in 27 occasions at weekly intervals during which 1,628 sand flies were captured. Taxonomic identification classified the sand flies under the *Phlebotomus* genus. The phlebotomines included seven species as follows: *P. (Phlebotomus) papatasi*, *P. (Paraphlebotomus) sergenti*, *P. (Paraphlebotomus) kazerouni*, *P. (Paraphlebotomus) alexandri*, *P. (Larroussius) major*, *P. (Larroussius) mascitti* and *P. (Adlerius) longiductus*. The most abundant species were *P. papatasi*, and *P. sergenti* comprising 42% and 57% of total catches respectively. The sex ratios in both species were male-biased so that male to female ratio was 1.97:1. These species were the only collected phlebotomines in plain regions of Narmashir and Bam counties. While *P. papatasi*, was the only species collected from Jt site, two more species namely *P. sergenti* and *P. major* were obtained from the neighboring mountainous site of Md. The most diverse of all sites was Di which

**Table 2**
Digestion of 630 bp fragmnets of Cyt b genes of blood meals retrieved from captured sand flies.

| | Product (bp) | Pattern of digested product (bp) with *Hae*III |
|---|---|---|
| Human | 623 | No digest |
| Dog | 623 | 522/70 |
| Cow | 623 | 310/300 |

**Table 3**
Indices used to measure diversity parameters of study sites.

| Indices | Formula | References |
|---|---|---|
| Expected Species = E(S) | $E(S) = \sum_{i-1}^{s}\left(1 - \frac{\left(\frac{N-N_i}{n}\right)}{\left(\frac{N}{n}\right)}\right)$ | Hurlbert (1971) |
| Shannon's diversity (H′) | H′ = -ΣPiln Pi | Shannon and Wiener, 1949 |
| Shannon Equitability = Shannon Evenness | SE = H′/lnS | Pielou (1969) |
| Number Eq. D (True Diversity) = Simpson's diversity (D1) | N1 = eH′ | Hill (1973) |
| Simpson's Index = True Diversity 2D (Order 2) = Simpson's dominance (D2) | N2 = Reciprocal of Simpson's index = 1/ Σipi2 | Hill (1973) |
| Simpson's Dominance | SD = Σipi2 | Simpson (1949) |
| Gini-Simpson Index = Probability of Interspecific Encounter | PIE = 1-SD | Hurlbert (1971) |
| Berger–Parker dominance (BP) | d = Nmax/N | Berger and Parker (1970) |

Note: Pi = S/N, S = number of individuals of one species, N max = number of individuals in the most dominant species, N = total number of all individuals in the sample, ln = logarithm to base e.

accommodates all seven collected species (Table 4).

### 3.2. Diversity of sand flies in the collection sites

As showed in Table 5, the richness of species in the collection sites increases with altitude. In plain sites of Rd and Bm only two species were trapped, while in mountainous sites of Md and Di three and seven species were collected respectively. The mountainous ecosystems in the study region were rich in flora and fauna, which provide abundant shelters and food resources for sand flies. To account for the difference in sample sizes from study sites rarefaction analysis was done based on minimum sample size in order to compare the richness. The estimated species numbers were again higher in sites with higher elevations and temperate climates. However, calculation of Shannon entropy index indicated that Di site with the highest richness has the lowest effective number of species (N1 = 1.83). This was due to bias of the index to the higher frequency of the most abundant species of the site namely *P. sergenti* with pimax of 87%. Md site as anther elevated site followed with pimax of 75.6 %. Both sites recorded the highest Simpson's dominance indices of 71.9 % and 61.6 % respectively. Simpson's index as a second order diversity indicated even lower values for Di (N2 = 1.39) and Md (N2 = 1.64) diversity than

**Table 5**
Diversity indices of phlebotomine species in study sites.

| Indices | Sites | | | | |
|---|---|---|---|---|---|
| | Rd | Jt | Bm | Di | Md |
| Species Richness R | 2 | 1 | 2 | 7 | 3 |
| Estimated no. of species = E(S) | 2.00 | 1 | 2.00 | 3.94 | 2.97 |
| Berger Parker Index pimax | 51.9% | 100.0% | 55.0% | 84.0% | 75.6% |
| R2 = Shannon Entropy1) H′ | 0.6924 | 0.0000 | 0.6880 | 0.6065 | 0.6614 |
| N1 = eH′ Number Eq. D (True Diversity) | 2.00 | 1.00 | 1.99 | 1.83 | 1.94 |
| N2 = Simpson's Index True Diversity 2D | 2.00 | 1.00 | 1.98 | 1.39 | 1.64 |
| N2/N1 | 1 | 1 | 0.95 | 0.76 | 0.85 |
| R1 = Simpson Dominance SD | 50.1% | 100.0% | 50.5% | 71.9% | 61.6% |
| Shannon Equitability H/lnS | 99.9% | 0.0% | 99.3% | 31.2% | 60.2% |
| Gini-Simpson Index 1-SD | 49.9% | 0.0% | 49.5% | 28.1% | 38.4% |

Shannon entropy index. This is mathematically true as Simpson' index is more biased to species frequencies than Shannon one (Shannon, 1948). Shannon evenness indices showed that Di and Md sites were the least even of all visited sites. The Gini Simpson index also revealed that probabilities of choosing two specimens of the same species in De and Md sites were very low (28.1 and 38.4 %) compared with Rd and Bm sites (49.9 and 49.5 %). This means that the formers are the least diverse site despite their inclusion of a number of rare species.

### 3.3. Blood meal preference of sand fly species

A total of 35 engorged sand flies including 12 *P papatasi* and 23 *P sergenti* females from Bm and Di sites were analyzed for their blood meal preference using RFLP products of their Cytb gene fragments visualized by gel electrophoresis. Figs. 2a and b represents a series of electrophoresis tests undertaken to elucidate the nature of bloods imbibed by collected sand flies particular from sites already known as foci of CL in Kerman Province. Table 6 summarizes the results of blood preference of the abovementioned species. As demonstrated, both tested species have high tendencies to feed on man (43%), although their feeding preference seems to be equally zoophilic (46%). In contrast to *P. sergenti*, the ZCL vector, *P. papatasi*, may occasionally feed on canine (17%) but not on bovine hosts.

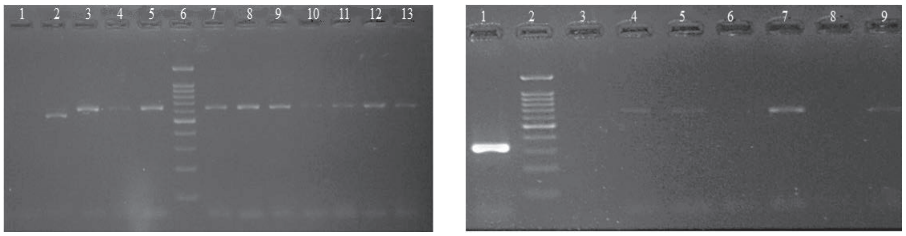
### 3.4. Detection and identification of Leishmania DNA from collected sand flies

Out of 547 captured phlebotomine females belonging either to *P. sergenti* or *P. papatasi* species, 200 were found unengorged (unfed parous and nulliparous) and used for DNA extraction and amplification.

**Table 4**
Distribution of phlebotomine species and their diversity indices in the study sites.

| Elevation (m above sea) | 670 → 2608 | | | | | | N | ♂ (%) | ♀ (%) |
|---|---|---|---|---|---|---|---|---|---|
| | Sites | | | | | | | | |
| Species | Rd Ni (%) | Jt Ni (%) | Bm Ni (%) | Di Ni (%) | Md Ni (%) | Dt | | | |
| *P. (Phlebotomus) papatasi* | 27 (51.9) | 202 (100) | 392 (45.0) | 50 (11.4) | 16 (20.5) | 0 | 687 | 1081 (66) | 547 (34) |
| *P. (Paraphleb.) sergenti* | 25 (49.1) | 0 | 480 (55.0) | 367 (84.0) | 59 (75.6) | 0 | 931 | | |
| *P. (Paraphleb.) kazeruni* | 0 | 0 | 0 | 4 (0.9) | 0 | 0 | 4 | 4 (100) | - |
| *P. (Paraphleb.) alexandri* | 0 | 0 | 0 | 2 (0.5) | 0 | 0 | 2 | 2 (100) | - |
| *P. (Larroussius) major* | 0 | 0 | 0 | 5 (1.1) | 3 (3.8) | 0 | 8 | 8 (100) | - |
| *P. (Larroussius) mascitti* | 0 | 0 | 0 | 3 (0.7) | 0 | 0 | 3 | 3 (100) | - |
| *P. (Adlerius) longiductus* | 0 | 0 | 0 | 6 (1.4) | 0 | 0 | 6 | 6 (100) | - |
| Total samples (N) | 52 | 202 | 872 | 437 | 78 | 0 | 1641 | | |
| No. of species(S) | 2 | 1 | 2 | 7 | 3 | - | | | |
| R1 Simpson Dominance | 0.501 | 1.00 | 0.505 | 0.719 | 0.616 | | | | |
| R2 Shannon Entropy | 0.692 | 0.00 | 0.688 | 0.607 | 0.661 | | | | |

(N = Total Number, Ni = number of individuals of each species).




lane 1: negative control, lane 2: 522 bp fragment indicative of canine blood, lanes 3, 4, 5, 7, 8, 9, 10, 11, 12 and 13: 623 bp fragments indicative of human blood, lane 6: 100 bp ladder

lane 1: 331 bp fragment indicative of cow blood, lane 2: 100 bp ladder, lane 3: negative control, lanes 4, 5, 7 and 9: 623 bp fragments indicative of human blood

**Fig. 2.** Electrophoresis of PCR-RFLP product of Cyt b gene of blood imbibed by sand flies digested by *Hae*III.

**Table 6**
Blood feeding preference of main vector of CL in the study sites.

| Species | Hosts | | | | |
|---|---|---|---|---|---|
| | Human (%) | Bovine (%) | Canine (%) | Others (%) | Total |
| *P.(Ph.) papatasi* | 5 (41.6) | - (0) | 2 (16.8) | 5 (41.6) | 12 |
| *P.(Para.) sergenti* | 10 (43.5) | 2 (8.7) | - (0) | 11 (47.8) | 23 |
| Total | 15 (42.8)* | 2 | 2 | 16 (45.7) ** | 35 |

*Human Blood Index (HBI), ** Animal Blood Index (ABI).

A sample of 100 specimens of *P. papatasi* individually processed resulted in two positive cases for ITS-1 PCR as revealed by 320 bp leishmanial fragments in gel electrophoresis (Fig. 3a). The RFLP reactions using *Hae*III restriction enzyme showed separation of two fragments of about 203 and 134 bp by gel electrophoresis representing *L. major* restriction pattern (Fig. 3b). The contaminated sand flies were collected from indoor traps in Bam site on 7$^{th}$ June, 2016. No leishmanial infection was detected in *P. sergenti* specimens collected from the same site or elsewhere around the same timing.

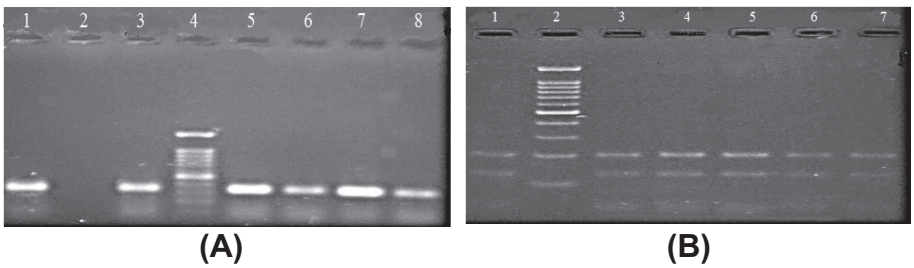
### 3.5. Phylogenetic analysis of sequences of Leishmania ITS1

Comparison through alignment of the ITS1 gene sequences obtained for 2 *Leishmania* infected sand flies with two sequences of *L. major* and one sequence of *L. tropica* retrieved from Genbank revealed the homogeneity of isolated *Leishmania* sequences with *L. major* confirming RFLP results (Fig. 4). In fact, the isolated *Leishmania* differs in only one character with one of retrieved *L. major* sequences showing about 99% similarity. The phylogenetic analysis of the sequences segregated all 11 *L major* haplotypes including 2 from *P. papatasi* females and 9 from Genbank (Fig. 5). The isolated sequences were registered in Genbank with accession numbers MH029154.1 and MH029155.1.

## 4. Discussion

Despite great control efforts undertaken at national and provincial levels for the last decades, leishmaniasis remains a prevailing vector-borne disease in many parts of Iran particularly in southern regions including Kerman province,. This study aimed to contribute to the understanding of leishmaniasis dynamics influenced by diversity and ecological distribution of phlebotomine vectors under different environmental settings. We found that the highest number of phlebotomine sand flies occurs in mountainous sites with temperate and mild climates. Di site has the greatest richness throughout the sampling period with all seven species present, though at uneven frequencies. Md site is the second mountainous site which accommodates three species, namely *P. papatasi*, *P. sergenti* and *P. major*, the principal vectors of ZCL, ACL and VL respectively (Killick-kendrick, 1990; Poursmaelian et al., 2011; Hanafi-Bojd et al., 2015). Given the presence of *P. major*, as a potent vector of VL, in Di and Md sites, these may witness the spread of the disease in near future due to increasing movement of people from neighboring endemic VL foci such as Baft city. The emergence of CL in recent years in the aforesaid sites has followed the same epidemiologic pattern upon increased displacement of infected people in the aftermath of Bam earthquake in 2003 (Aflatoonian et al., 2016).

During our six month survey in subtropical sites of Bm and Rd sites, only two CL vectors were encountered namely *P. sergenti* and *P. papatasi*. These species were evenly distributed in all sampled locations regardless of sample sizes. Although, there seemed to be a setback in the frequency of *P. sergenti* in favor of *P. papatasi* in Bm, given the former reportedly constituted up to 80.3–94% of phlebotomines in the same location (Sharifi et al., 2015). However, in Jt site, the only species caught was *P. papatasi* with no trace of its counterpart *P. sergenti*. This may be behind




Lanes 1, 3, 5, 6, 7 and 8 are fragments measuring 320 bp indicating of *Leishmania* infection, Lane 2 negative control, lane 4 100 bp ladder

lane 1, 3, 4, 5, 6 and 7 produced 2 fragments of about 203 and 134 bp indicative of *Leishmania major* parasite, Lane 2 100 bp ladder

**Fig. 3.** Electrophoresis of PCR (A) and PCR-RFLP (B) products of ITS1 gene digested with *Hae*III restriction enzyme.

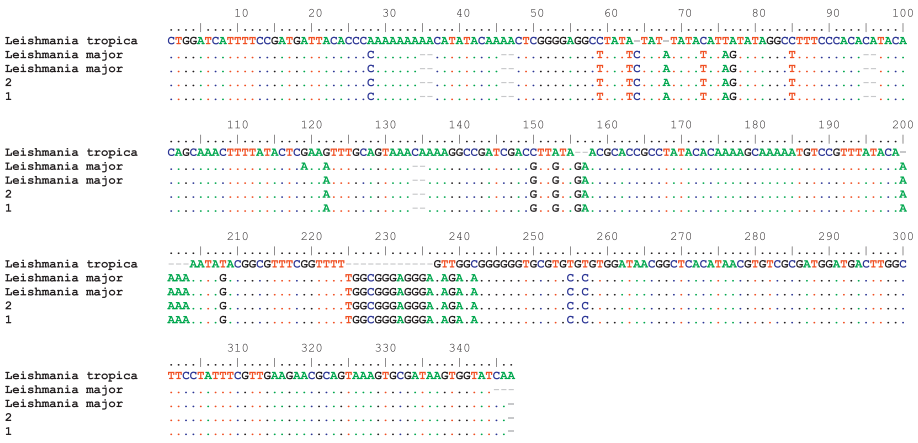



**Fig. 4.** ITS1 sequences alignment from 2 samples of *Leishmania major* (1 and 2) with recorded sequences of *L. major* and *L. tropica* from GenBank using BLAST software. Points and dashes denote identities and gaps respectively.

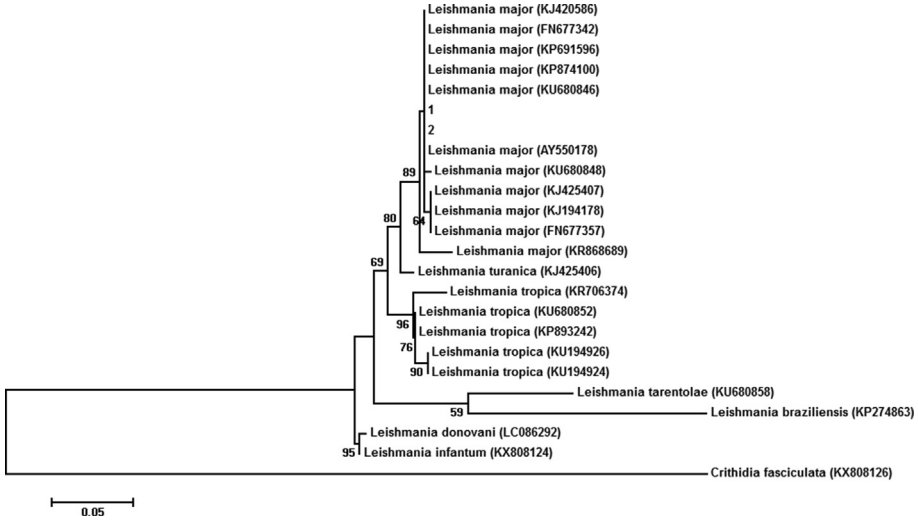



**Fig. 5.** A Maximum Likelihood tree of relationships between haplotypes of ITS1 sequences of *L. major* isolated from *P. papatasi* with its related haplotypes in GenBank using MEGA 5 software. *Crithidia fasciculata* was used as an out-group. Number 1 and 2 indicate understudy haplotypes.

the lack of reports on indigenous transmission of ACL in Jt site so far. All infected cases of ACL in Jt site had a travel history to neighboring foci of the disease. In fact, northern countryside of Jt site was found to be infected with ACL agent, *L. tropica* (Poursmaelian et al., 2011).

The most interesting site was Dt in which no sand fly species was caught at all despite repeated sampling attempts starting from 4th May 2016 onwards. All 880 trapped flies were midges of *Ceratopogonidae* family. Located at close distance from Baft city as an endemic focus of both *L. infantum* and *L. major*, Dt site has no record on any sand fly-borne diseases in its historical background. This may be attributed to cold climate, which does not permit a thriving period of suitable temperatures (11–36 °C) long enough for *Leishmania* agents and their vectors to develop and establish.

Rarefaction analysis showed that diversity of sand flies increases with elevation so that the highest expected number of species was calculated for the mountainous Di site where the highest richness occurred. On the other hand, Shannon entropy and its exponential index measure higher biodiversities for sites located in plain areas such as Rd and Bm, which accommodated two evenly distributed species. This provides another indication on the bias of Shannon index to both evenness and richness of communities. As expected, Simpson' index gave no weight to rare or less abundant species but to the most abundant species in the sample. This is obvious in Table 5 as the highest Simpson's dominance index was calculated for Di site, where the Berger Parker Index was the highest (84%). Adoption of different diversity indices, therefore, may compensate for the shortcoming of commonly used indices in clarifying the complexity.

We observed clear male-biased frequencies (~2:1) for both *P. papatasi* and *P. sergenti* over the whole sampling sites. A significantly greater male to female ratio has been reported for the sand flies of subgenus *Adlerius* in northwest of Iran (Zahraei-Ramazani et al., 2013). In addition, higher male to female ratios of about 5:1 for the same species have been reported by Kamhawi et al. in Jordan who attributed the imbalanced sex ratio to the attraction of *Phlebotomus* males to sticky traps used as the main sampling method in their investigation (Kamhawi et al., 1995). However, Aklilu and co-workers showed that male to female ratios of *P. papatasi* in Ethiopia remain the same (1.87: 1) irrespective of sampling methods including CDC trap, sticky trap and others (Aklilu et al., 2017).

The molecular analysis of imbibed blood by two main vectors of leishmaniasis in the studied sites showed that both *P. papatasi* and *P. sergenti* were equally attracted to human as well as animal hosts for feeding with a bit higher tendency of the latter vector to animal blood. While *P. papatasi* appeared to feed occasionally on canine hosts, *P. sergenti* has a low rate feeding on bovine hosts. Both vectors showed relatively low feeding preferences to human blood (~43 %) similar to the findings of Mozafary and co-workers in Kerman City (Mozafary et al.,

2016). However, much higher human blood index has been reported by studies undertaken in different ecological settings with distinct vector population structures. For example, in Nepal 84.1% of *P. papatasi* was found to feed on human blood but less on dogs (1.1%), bovine (0.6%) or other vertebrates (14.2%) (Burniston et al., 2010). This is because the feeding preference is an ecological niche-dependent parameter across arthropod vectors influenced by habitat suitability (Ayala et al., 2009). La Deau and co-workers postulated that factors such as urban habitat modification and local temperatures strongly affect host-seeking and biting behavior of insect vectors (LaDeau et al., 2015).

The females of *P. papatasi* collected from Bm site were found harboring *L. major* at a low infection rate of 2%. This falls within the range (0.2–10.9%) reported from various ZCL foci in Iran surveyed during 1967–1990 (Yaghoobi-Ershadi, 2012). Kassiri et al. have reported the same rate of infection for *P. papatasi* from neighboring Sistan Va Baluchistan Province (Kassiri et al., 2012). The infection of sand flies with only ZCL agent in already known foci of ACL not only reflects the coexistence of two types of CL, but also emphasizes the occurrence of ecological changes, which favor the spread of ZCL agent and/or its vectors.

## 5. Conclusions

Bam and Narmashir counties remain active foci of both types of CL, although not necessarily with equal endemicity rates. This is because the elements of transmission cycles are influenced by habitat and local ecosystems. In addition to CL, Md and Di sites are prone to VL, given the presence of, *P. major* as a potent vector, and vicinity to Baft City as a VL focus. The vector *P. sergenti* was not discovered in Jt hence the lack of reports on endemic ACL cases. No sand fly vectors has been caught in Dt site despite its proximity to Baft City as a focus for both VL and ZCL. This could be due to its high elevation and cold climate. In general, increasing temperatures, ecosystem changes and prevailing competent vectors, host and reservoirs are risk factors that must be carefully scrutinized should the disease be controlled in this part of the country.

## Declarations

### *Author contribution statement*

Ismail Amiri Ghannat Saman: Conceived and designed the experiments; Performed the experiments.

Mohammad Saaid Dayer: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Majid Pirestani: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### *Competing interest statement*

The authors declare no conflict of interest.

### *Additional information*

No additional information is available for this paper.

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