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Molecular detection of *Leishmania* DNA in wild-caught sand flies, *Phlebotomus* and *Sergentomyia* spp. in northern Iran

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ABSTRACT

Leishmaniasis is currently considered a major health problem in Iran, posing an increasing threat to society's development in various dimensions. This study aimed to detect *Leishmania* infection in wild-caught sand flies in Sari City, northern Iran. Sand flies were collected using sticky traps, and *Leishmania* DNA was identified using polymerase chain reaction (PCR) targeting the ITS2-rDNA region, followed by restriction fragment length polymorphism (RFLP) analysis. A total of 138 female sand flies were tested, among which, only 1 specimen of *Ph. papatasi* (11.11 %) and *Ph. major* (14.28 %), 4 specimens of *Ph. kandelakii* (7.27 %) and *Se. dentata* (8.33 %), and 2 specimens of *Se. sintoni* (50 %) were naturally infected with *L.* (*L.*) *major*. This was observed in the ITS2 nested-PCR amplification assays where a ~ 245 bp PCR band was produced. Also, RFLP analysis by *Mnl1* revealed the fragments of 55 and 70 and 120 bp for infected sand flies which are characteristic of *L.* (*L.*) *major*. Most of the sand flies were unfed, collected during warm season, found indoor. This study reperesents the first molecular detection of *L.* (*L.*) *major* in wild-caught sand flies, specifically in *Ph. papatasi* in this region, as well as *Ph. kandelakii* and *Ph. major* in Iran and even the world.

1. Introduction

Leishmaniasis is a significant disease caused by intracellular parasites of the genus *Leishmania*, transmitted through the bite of sand flies (Tzani et al., 2021). It ranks as the third most important vector-borne parasitic disease following malaria and lymphatic filariasis and is among the top seven tropical diseases. This disease poses a serious global health threat due to its diverse clinical manifestations, some of which can be fatal (Barrett and Stanberry, 2009; Tzani et al., 2021).

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There are four types of leishmaniasis in the world, including cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL) and visceral leishmaniasis (VL) (also known as kala-azar and the most serious form of the disease) (Georgiadou et al., 2015). These diseases are spread in the Old and New World with very high epidemiological diversity and are transmitted to humans through the bite of the female sandfly, i.e., *Phlebotomus* in the Old World and *Lutzomyia* in the New World (Georgiadou et al., 2015; Vahabi et al., 2016). Leishmaniasis has spread to numerous tropical, subtropical, and Mediterranean regions, affecting around 102 countries globally and putting an estimated 350 million people at risk, with approximately 12 million infections recorded (Fatemi et al., 2017; Mohammadbeigi et al., 2021). In Iran, Leishmaniasis is caused by three main species: *L.* (*L.*) *tropica*, *L.* (*L.*) *infantum*, and *L.* (*L.*) *major*, with the latter being the most prominent species. (Absavaran et al., 2019; Alexander, 2000).

CL and VL are ancient indigenous diseases in Iran, posing a growing health threat to society and the environment (Yaghoobi-Ershadi, 2012). Approximately 20,000 new cases of leishmaniasis (CL and ZV) are reported in the country each year, with the actual number potentially four to five times higher (Shirmohammadi et al., 2017).

CL, the most common form in Iran, manifests as skin lesions, particularly ulcers, on exposed body parts. It is the second most prevalent parasitic disease transmitted by arthropods after malaria (Mohammadi Azni et al., 2010). The majority of new cases (over 85 %) are reported in 10 countries: Afghanistan, Algeria, Brazil, Colombia, Syria, Libya, Tunisia, Pakistan, Iraq and Iran (WHO, 2023), where two major types of cutaneous leishmaniasis exist: zoonotic cutaneous leishmaniasis (ZCL) caused by *L.* (*L.*) *major* and anthroponotic cutaneous leishmaniasis (ACL) caused by *L.* (*L.*) *tropica* (Yaghoobi-Ershadi et al., 2021).

Over 1000 species of phlebotomine sand flies are known worldwide, with 78 confirmed as human vectors (Galati and Rodrigues, 2023; Maroli and Khoury, 2004). In Iran, 53 phlebotomines (34 *Phlebotomus* and 19 *Sergentomyia*) were reported as confirmed vectors (Jalali et al., 2022). *Phlebotomus papatasi* and *P. sergenti* were identified as proven vectors of ZCL and ACL in various areas of Iran, respectively (Sabzevari et al., 2021). However, other species belonging to the subgenus *Phlebotomus* or females belonging to the group *Caucasicus* have also been reported as vectors of *L. major*, the latter especially as secondary vectors in areas where *Ph. papatasi* is not recorded, or is in low densities (Akhoundi et al., 2013). Various rodents serve as reservoir hosts for ZCL in different endemic foci of Iran (Oshaghi et al., 2009c; Sabzevari et al., 2021), while humans are recognized as reservoirs for ACL in different regions of the country (Yaghoobi-Ershadi, 2012).

VL, also known as kala-azar, is a potentially fatal disease if untreated. Brazil, East Africa, and India are the most affected regions. Globally, an estimated 500,000 to 900,000 cases occur annually, but only a fraction (25–45 %) are reported (WHO, 2023). Caused by *L.* (*L.*) *infantum*, VL affects around half a million of individuals annually in the Mediterranean region and Latin America. In Iran, *Ph. major* and *Ph. kandelakii* play a role as vectors for *L.* (*L.*) *infantum* (Rassi et al., 2012).

It should be noted that traditional and classical methods were reported in the detection of *Leishmania* in sand flies. These methods require the dissection of freshly caught sand flies and the culture of revealed parasites from the sand fly's gut (Rossi et al., 2008). In addition, these techniques are time-consuming, require specialized individuals for dissection, and a large number of samples, as the rate of *Leishmania* infection in sand flies is usually very low, even in highly endemic areas (Sharma and Singh, 2008). Therefore, the use

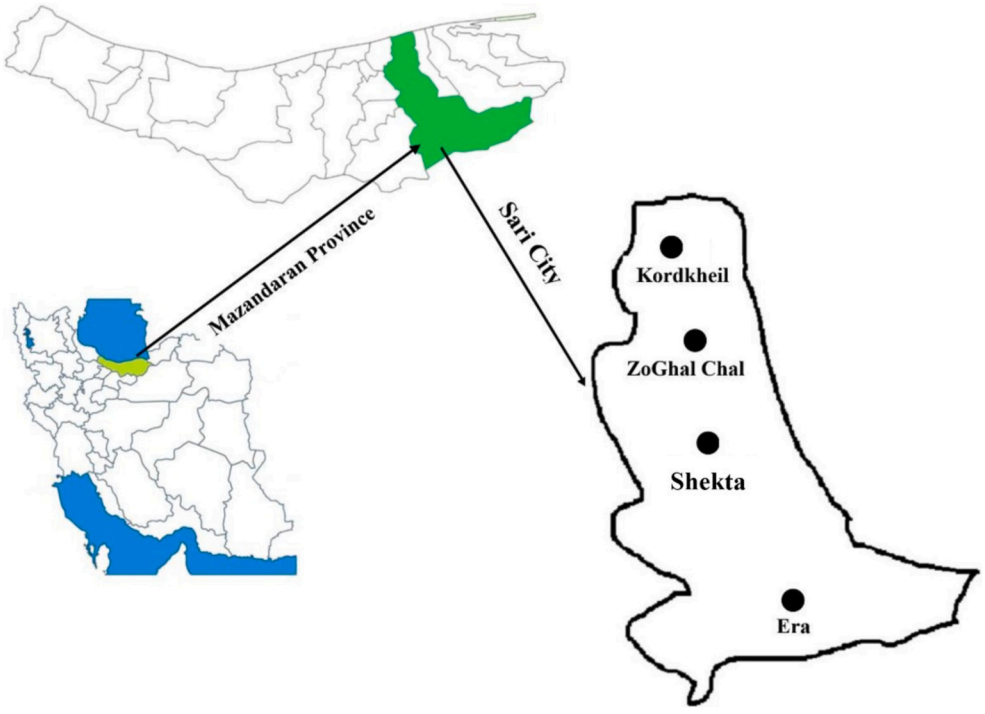

Fig. 1. Study area in Sari City, Mazandaran Province, northern Iran.

of alternative methods to identify *Leishmania* species in sand flies can be very useful. In recent years, molecular methods have been increasingly utilized in epidemiological studies to detect infections and identify *Leishmania* parasites in hosts and vectors (Aransay et al., 2000). The aim of the present study was to identify *Leishmania* infection in wild-caught sand flies by molecular methods in Sari City, Mazandaran Province, northern Iran.

2. Materials and methods

2.1. Study area

The study was carried out in Sari City is located in the Central part of Mazandaran Province, northern Iran, with coordinates between 53°5 longitudes and 36°4 latitudes. It has a population of approximately 309,820 people and an area of 54 km^2, with a moderate and humid climate in summer and a relatively cold climate in winter. Average daily temperature and annual rainfall are reported as 17.7 °C and 824.4 mm, respectively. Sari is one of the oldest and most populated cities in northern Iran. It is an attractive tourist place with several historical and ancient places, mountain, forest and sea. The main occupation of the people is animal husbandry, agriculture, and horticulture. According to the report of positive cases of the disease (local report of provincial health centers) and taking into account geographical and topographical diversity, four villages from each mountain (Era), forest (Shekta), plain (Kordkheil) and urban outskirts (Zoghal Chal) were selected for sampling effort in this study (Fig. 1).

2.2. Sand flies collection

Sand flies were caught monthly from indoors (e.g. corners of rooms, storage, bathrooms and toilets, bedroom, toilet, Warehouse and stable) as well as outdoors (cracks and crevices in the wall, yards, around rodents' nests or animal shelters wall and animal burrows) by using sticky traps (30 papers for indoors and 30 papers for outdoors per station) from April to December 2018. All traps were installed at sunset and collected near sunrise. The sand fly specimens were separated from sticky papers by needle or brush, washed in 96 % ethanol alcohol to remove sticky materials and preserve them. Then, each sample was dissected in a drop of fresh sterile normal saline by cutting the head and end of the abdomen with sterilized forceps and disposable needles. The terminal segments of the abdomen comprising the spermatheca and the heads of females were removed and mounted on glass slides using a drop of Puri's medium and then identified to species level using a morphological identification key (Seyedi and Nadim, 1992). The rest of the body was individually kept in the sterile micro tubes (Eppendorf) with 96 % alcohol and stored at −20 °C for molecular assay.

2.3. DNA extraction

DNA Extraction from the remaining parts of the sand flies was performed by homogenizing them individually in a microtube using a glass pestle according to the Collins protocol and stored at 4 °C. The extraction buffer contained the following components: 20 mM sodium EDTA and 100 mM Tris-HCl, with the pH adjusted to 8.0 using HCl. Additionally, 1.4 M NaCl and 2.0 % (*w/v*) CTAB (cetyltrimethylammonium bromide) were added to the buffer. To prepare the CTAB solution, it should be dissolved by heating to 60 °C and then stored at 37 °C. Just before using the solution, add 0.2 % of ß-mercaptoethanol. For quality control, double-distilled water and DNA extracted from L. *major* (MRHO/IR/75/ER) provided by the Pasteur Institute of Iran, were used as negative and positive control, respectively.

2.3.1. Detection of Leishmania by Polymerase Chain Reaction (PCR) amplification

2.3.1.1. DNA amplification and nested- PCR. The initial screening to detect *Leishmania* species infection in sand flies was performed by nested PCR amplification of ITS2 rDNA, following the protocol previously described by Akhavan (Akhavan et al., 2010b). The primers used to amplify the *Leishmania* spp. ITS2 regions include: outer forward primer (5′-AAA CTC CTC TCT GGT GCT TGC-3′), outer reverse primer (5′-AAA CAA AGG TTG TCG GGG G-3′), inner forward primer (5′- AAT TCA ACT TCG CGT TGG CC-3′), inner reverse primer (5′-CCT CTC TTT TTT CTC TGT GC-3′) (Akhavan et al., 2010b; Hosseini-Vasoukolaei et al., 2016).

Restriction Fragment Length Polymorphism (PCR-RFLP) was used to confirm the identity of *Leishmania* species in positive samples using restriction digestion with *Mnl*I. Restriction analysis was performed in a total solution volume of 15 µL, containing 13 µL of ITS2-rDNA PCR products, 0.5 µL of restriction enzyme (*Mnl*I), 1.5 µL of enzyme reaction buffer, and 3 µL of ddH2O using the conditions recommended the manufacturer (Fermentase) and Akhavan et al. (Akhavan et al., 2010a). The mixture was incubated for 3 h at 37 °C. The products were analyzed in 2 % agarose gel using 1TBE buffer, and SYBR green staining for 55 min at 80 V, visualized under ultraviolet light of the transilluminator. Parasites were identified by comparison with positive controls of L. *major* and molecular weight markers.

Sequencing was performed using Macrogen (South Korea). The obtained sequences were processed and aligned using the Clustal X multiple alignment program (Thompson et al., 1997). To correct ambiguities from the resulting sequences, alignments with sequence data available in GenBank were checked using the Basic Local Alignment Analysis Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/BLAST).

Phylogenetic analysis was conducted using MEGA X version 11 software (Tamura et al., 2021), employing the maximum likelihood method with 1000 bootstrap replicates on all *Leishmania* spp. available in GenBank (Table 1) and *L.*(*L.*) *major* species in this study. This

approach enabled us to generate a well-supported phylogenetic tree, with *T. otospermophilus* serving as the outgroup for the analysis.

3. Results

A total of 334 specimens were collected and morphologically identified. Of these, 138 female sand flies were selected for screening of *Leishmania* parasites. They were *Ph. papatasi* (9, 6.53 %), *Ph. kandelakii* (55, 39.86 %), *Ph.major* (7, 5 %), *Se. dentata* (48, 34.8 %) *Se. sintoni* (4, 3 %) *Se. theodori* (7, 5 %) *Se. sumbarica* (1, 0.73 %), and *Se. antennata* (1, 0.73 %), and Six species were not identified at the species level (Table 2).

Table 1
Details of *Leishmania* spp. ITS2-rDNA sequences used for phylogenetic analysis in this study.

Number	Species	Country	GenBank ID	Length (bp)	Reference
1	*L. mexicana*	Ecuador	AB558242	1049	Kato et al. (2011)
2	*L. mexicana*	Venezuela	AB558251	1044	Kato et al. (2011)
3	*L.amazonensis*	Mexico	AF339753	1127	DS***
4	*L. mexicana*	Mexico	AF466383	1136	DS***
5	*L. turanica*	Uzbekistan	AJ272378	1021	DS***
6	*L. turanica*	Turkmenistan	AJ272379	1022	DS***
7	*L. turanica*	Mongolia	AJ272380	1023	DS***
8	*L. turanica*	Turkmenistan	AJ272381	1022	DS***
9	*L. turanica*	Kazakhstan	AJ272382	1022	DS***
10	*L. major*	Turkmenistan	AJ272383	1102	DS***
11	*L. adleri*	Russia	AJ300480	1090	DS***
12	*L. major*	Sudan	AJ300481	1108	DS***
13	*L.braziliensis*	Brazil	AJ300483	926	DS***
14	*L.braziliensis*	Colombia	AJ300484	926	DS***
15	*L. tropica*	Tunisia	AJ300485	940	DS***
16	*L. gerbilli*	Uzbekistan	AJ300486	1061	DS***
17	*L. infantum*	France	AJ634371	1041	Kuhls et al. (2005)
18	*L. major*	Iran	AJ786163	807	Tashakori et al. (2006)
19	*L. donovani*	Morocco	AM901453	1043	DS***
20	*L. major*	Brazil	DQ300195	1054	DS***
21	*L. major*	Georgia	FJ753395	1117	De Almeida et al. (2011)
22	*L. infantum*	Uzbekistan	FN398341	1038	DS***
23	*L. infantum*	ND**	GU045592	1033	DS***
24	*T. otospermophilus**	ND**	AB190228	7823	Sato et al. (2007)
25	*L. turanica*	Iran	HF545838	138	Bakhshi et al. (2013)
26	*L. gerbilli*	Iran	HF545839	141	Bakhshi et al. (2013)
27	*L. adleri*	ND**	HQ289858	842	DS***
28	*L. turanica*	China	HQ830350	1021	DS***
29	*L. gerbilli*	China	HQ830351	1019	DS***
30	*L. infantum*	China	HQ830353	1008	DS***
31	*L. donovani*	China	HQ830358	997	DS***
32	*L. braziliensis*	Brazil	JQ061322	985	DS***
33	*L. donovani*	Bhutan	JQ730002	1012	Yangzom et al. (2012)
34	*L. major*	Iran	JQ889811	234	DS***
35	*L. major*	Iran	JX183383	579	DS***
36	*L. infantum*	Zambia	LC652645	739	DS***
37	*L.major*	Iran	MF614960	237	DS***
38	*L. turanica*	Iran	MK372246	123	DS***
39	*L. major*	Iran	MK372248	228	DS***
40	*L. braziliensis*	Spain	MT497927	537	DS***
41	*L. braziliensis*	Spain	MT497929	535	DS***
42	*L. guyanensis*	Spain	MT497940	535	DS***
43	*L. major*	Spain	MT497950	683	DS***
44	*L. panamensis*	Spain	MT497966	524	DS***
45	*L. guyanensis*	Spain	MT497969	536	DS***
46	*L. braziliensis*	Spain	MT497975	533	DS***
47	*L. major*	83Shekta	OM985929	212	This study
48	*L. major*	115Era	OM985930	213	This study
49	*L. major*	132Era	OM985931	214	This study
50	*L. major*	138Kordkheil	OM985932	216	This study
51	*L. major*	Iran	ON398783	386	DS***
52	*L. tropica*	Iran	ON398791	341	DS***
53	*L. major*	Iran	ON721105	187	DS***
54	*L. infantum*	Algeria	PP182264	381	DS***

* Out group.
** Not defined.
*** Direct Submission.

The results showed *Ph. papatasi*, 1/9 (11.11 %); *Ph. major*, 1/7(14.28 %); *Ph. kandelakii*, 4/55(7.27 %); *Se. dentata* 4/48(8.33 %) and *Se. sintoni*, 2/4 (50 %) tested positive for *Leishmania* using Nested-PCR of ITS2 rDNA region. The PCR bands obtained from the infected samples matched the *L.*(*L.*) *major* standard strain, producing a band size of 245 bp (Table 2, Fig. 2).

Further analyses showed that they were positive for the ITS2 locus and produced a band of ~245 bp in gel electrophoresis. Also, ITS2 PCR–RFLP analysis by *Mn*II detected the fragments of 55 and 70, and 120 bp for infected sand flies which are characteristic of *L.* (*L.*) *major* and were visualized by separate double bands on gel electrophoresis (Fig. 3). All positive samples were unfed except *Ph. major* (Table 2), which was mostly collected from indoors in mountainous and forested areas in July (Figs. 4, 5, 6).

The ITS2 DNA sequences obtained from the infected sand flies were submitted to the Genbank database with accession numbers OM985929-OM985932. Comparison of these sequences with other available data confirmed it as *L.*(*L.*) *major*.

Blast analysis revealed that the specimens (OM985929–32) shared 96–99 % similarity with *L.*(*L.*) *major* sequences from Iran (Accession No. MK372248), 95–97 % similarity with sequences Spain (Accession No. MT497953) and Central Asia (Accession No. AJ272383).Specimens of 95 and 113 were 79–82 % similar to isolates from Iran, Spain and Central Asia (Accession No. MK372248.1, MT497953.1 and AJ272383.1 respectively). The phylogenetic tree inferred using the maximum likelihood method with a log-likelihood value of −2201.64, is shown in Fig. 7.

4. Discussion

Leishmaniasis remains a significant global health issue due to gaps in understanding the ecology of sand flies, disease epidemiology, and ineffective control measures (Jalali et al., 2022). Identifying naturally infected species and gathering epidemiological data are crucial for planning control strategies (Hamarsheh, 2011).

Sari City, located in northern Iran, near the endemic area (ZCL) of Sahara Turkmen, is facing an emergence of the disease (Sharbatkhori et al., 2014). This is supported by Ghavibazou et al. (Ghavibazou et al., 2018) and Asfaram et al. (Asfaram et al., 2017), who reported cases of cutaneous and visceral leishmaniasis linked to Mazandaran Province. Despite its global prevalence and significant disease burden, there is no commercially available vaccine for human leishmaniasis. Detecting infections in wild-caught sand flies is essential for vector control and reducing the disease burden (Desjeux, 2004; Gillespie et al., 2016). To the best of our knowledge, there is little data about the leishmaniasis and its vector in the area (Ghavibazou et al., 2018; Hosseini-Vasoukolaei et al., 2022; Youssefi et al., 2011). The study is considered as the first research on molecular detection of *Leishmania* DNA within wild-caught sand flies in Sari City, northern Iran.

Molecular techniques, particularly PCR, are more sensitive and efficient for detecting *Leishmania* DNA compared to traditional methods like microscopic dissection and parasite culture. (Azizi et al., 2008; Oshaghi et al., 2009a, 2009b; Vaziri et al., 2023). These methods have been widely used in various parts of the world (Arserim et al., 2021; Maia et al., 2013; Rêgo et al., 2015). In the present study, Nested-PCR method of ITS2 rDNA amplification was used to detect *Leishmania* parasite in wild-caught sand flies. Among 138 the female surveyed, 12 specimens were positive for *L.*(*L.*) *major*, including *Ph. papatasi*, 1/9 (11.11 %); *Ph. major*, 1/7(14.28 %); *Ph. kandelakii*, 4/55(7.27 %); *Se. dentata*, 4/48(8.33 %); *Se. sintoni*, 2/4(50 %). Also, RFLP analysis by *Mn*II confirmed the results of our study.

Phlebotomus papatasi plays a crucial role as the primary vector in transmitting the *Leishmania* parasite to human in both endemic and non-endemic foci of ZCL in Iran (Arzamani et al., 2018; Jalali et al., 2022) and neighboring countries (Ghatee et al., 2020). This species is the most dominant sand fly found around great gerbil nests and human habitats in Iran (Rafizadeh et al., 2016b). *Phlebotomus papatasi* is known as a "selective" vector, specifically supports the development of *L. (L.) major*, although it can transmit multiple *Leishmania* species in endemic area (Dobson et al., 2010; Killick-Kendrick et al., 1996). Studies have detected *L. (L.) major, L. (L.) gerbili,* and *L. (L.) turanica* in *Ph. papatasi* in endemic regions of (Hosseini-Vasoukolaei et al., 2016; Rafizadeh et al., 2016a). This study is the first report on the detection of *L.* (*L.*) *major* DNA from *Ph. papatasi* in Sari City, northern Iran, with an infection rate of 11.11 %, posing a potential threat of ZCL establishment in the region. Various rates of infection of *L. (L.) major* in the species have been reported in different regions of Iran by PCR analysis of the ITS2-rDNA gene of the parasite, followed by RFLP. These include infection

Table 2
Abundance and percentage of phlebotomine sand flies tested and infected with *Leishmania* along with abdominal conditions, in Sari City, northern Iran, 2018.

	tested samples		Infected Positive samples *L. major*		Abdominal status of specimens*			
	N	%	N	%	UF	FF	SG	G
Phlebotomus (Larroussius) kandelakii	55	39.86	4	7.27	*	–	–	–
Sergentomyia (Sergentomyia) dentata	48	34.8	4	8.33	*	–	–	–
Phlebotomus (Phlebotomus) papatasi	9	6.53	1	11.11	*	–	–	–
Sergentomyia (Sergentomyia) theodori	7	5	0	0	–			
Phlebotomus (Larroussius) major	7	5	1	14.28	–	*	–	–
Sergentomyia (Sergentomyia) sintoni	4	3	2	50	*	–	–	–
Sergentomyia (Sergentomyia) antennata	1	0.73	0	0	–	–	–	–
Sergentomyia(Parrotomyia) sumbarica	1	0.73	0	0	–	–	–	–
Unknown	6	4.35	0	0	–	–	–	–
total	138	100	12	8.69				

* UF = Unfed, FF = Fresh fed, SG = Semi gravid, G = Gravid.

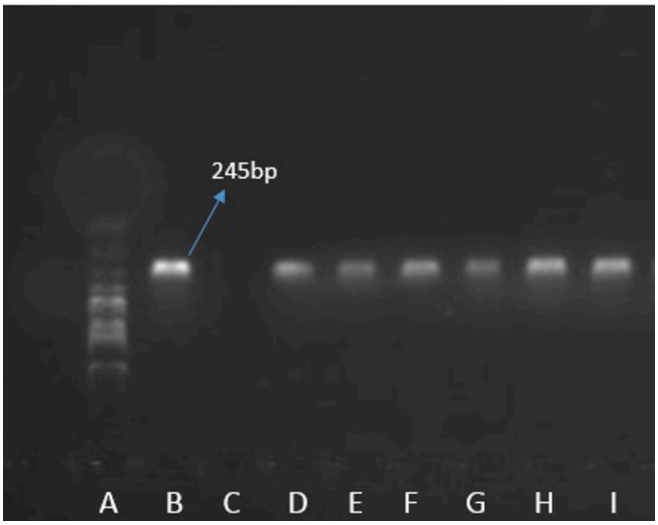

Fig. 2. ITS2 PCR amplification of *leishmania* using Nested- PCR (245 bp).100 bp molecular weight marker (Lane A), Positive control of *L.*(*L.*) *major* (Lane B), Negative control (Lane C), *Ph. papatasi* (Lane D), *Ph. major* (Lane E), *Ph. Kandelakii* (Lane F, G, H, I)

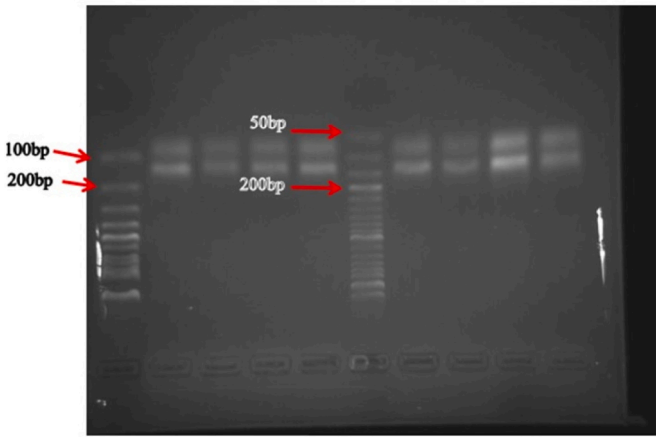

Fig. 3. PCR-RFLP using *Mn*II restriction enzyme. 100bp and 50bp molecular weight marker (Lane A and F, respectively), Positive control of *L.*(*L.*) *major* (Lane B), *Ph. dentata* (Lane C), *Ph. sintoni* (Lane D), *Ph. Kandelakii* (Lane E), *Ph. papatasi* (Lane I) infected sand flies (lane C, D, E and G, H, I, J).

rates of 1.8 % (5 out of 280 *Ph. papatasi* females) in Dehloran City, Ilam Province (Vahabi et al., 2016), 0.3 % (1 out of 372 *Ph. papatasi* females) in Kalaleh City, Golestan Province (Rassi et al., 2008), and 7.80 % in Karun City, Khuzestan Province (Taheri et al., 2024). Moreover, infection rates of 22.08 % and 37 % have been documented in *Ph. papatasi* populations in Varamin City, Tehran Province (Nekouie et al., 2006) and Natanz City, Isfahan Province (Yaghoobi-Ershadi and Akhavan, 1999), respectively. There are some discrepancies between the results of our study and those in the literature, which may be caused by differences in ecological characteristics of the study area, the season, and the abdominal conditions of the collected sand flies (Taheri et al., 2024).

In the present study *L.*(*L.*) *major* DNA was observed in two species, *Ph. kandelakii* and *Ph. major*. As far as we know, this is the first report of natural infection of these species with *L. (L.) major* in Iran and possibly worldwide.

Phlebotomus major is known as a vector of visceral leishmaniasis (VL) in the Mediterranean basin, extending from Morocco to Southeast China (Hoogstraal and Heyneman, 1969; Léger et al., 1979). It a potential vector of *L. infantum* in the western Black Sea region of Turkey (Azizi et al., 2008). In Iran, *Ph. major* has been documented in northern regions, and other areas with reported human VL cases (Azizi et al., 2008; Javadian and Nadim, 1975; Nadim et al., 1978).

Phlebotomus kandelakii has been introduced as the main and proven vector of VL in the main focus of the disease, i.e. North-West (Rasi et al., 2005) and North-East of Iran (Rassi et al., 2012). This species is distributed in Central Asia, Afghanistan, Lebanon, Turkey, Iran and the former Soviet Union (Alvar and Baker, 2002). Previous studies have shown *Leishmania* promastigote infections in *Ph. kandelakii* in Meshkin Shahr, northwest Iran (Nadim et al., 1992). Recent molecular studies confirmed *L.*(*L.*) *infantum* infection in *Ph. kandelaki* in both northwest (Behniafar et al., 2019; Rassi et al., 2005) and northeast Iran, including North Khorasan Province (Rassi et al., 2012).

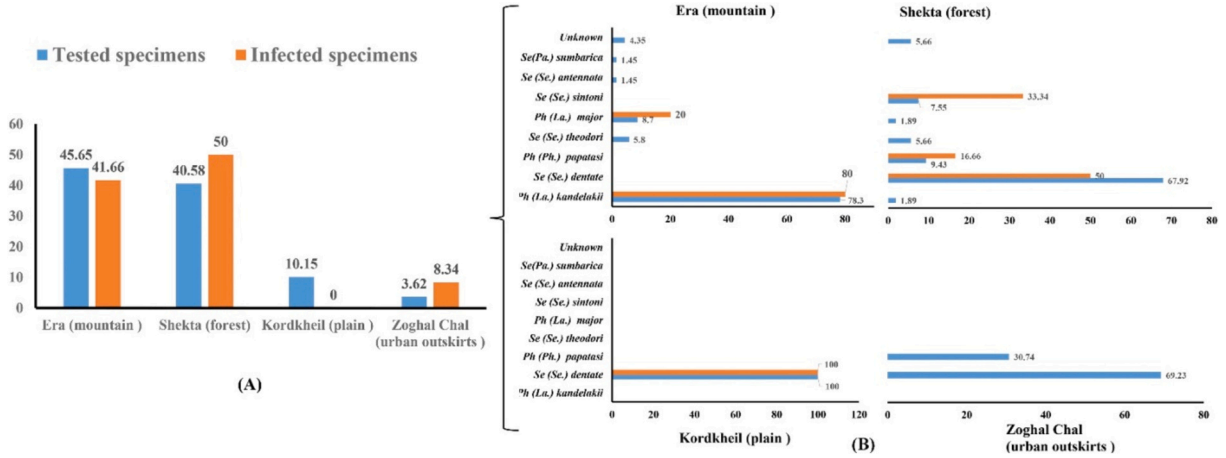

Fig. 4. Percentage of tested and *Leishmania*-infected sand flies by geographic location in Sari City, northern Iran. Panel (A) shows overall data (A), while pannel B categorizes by species.

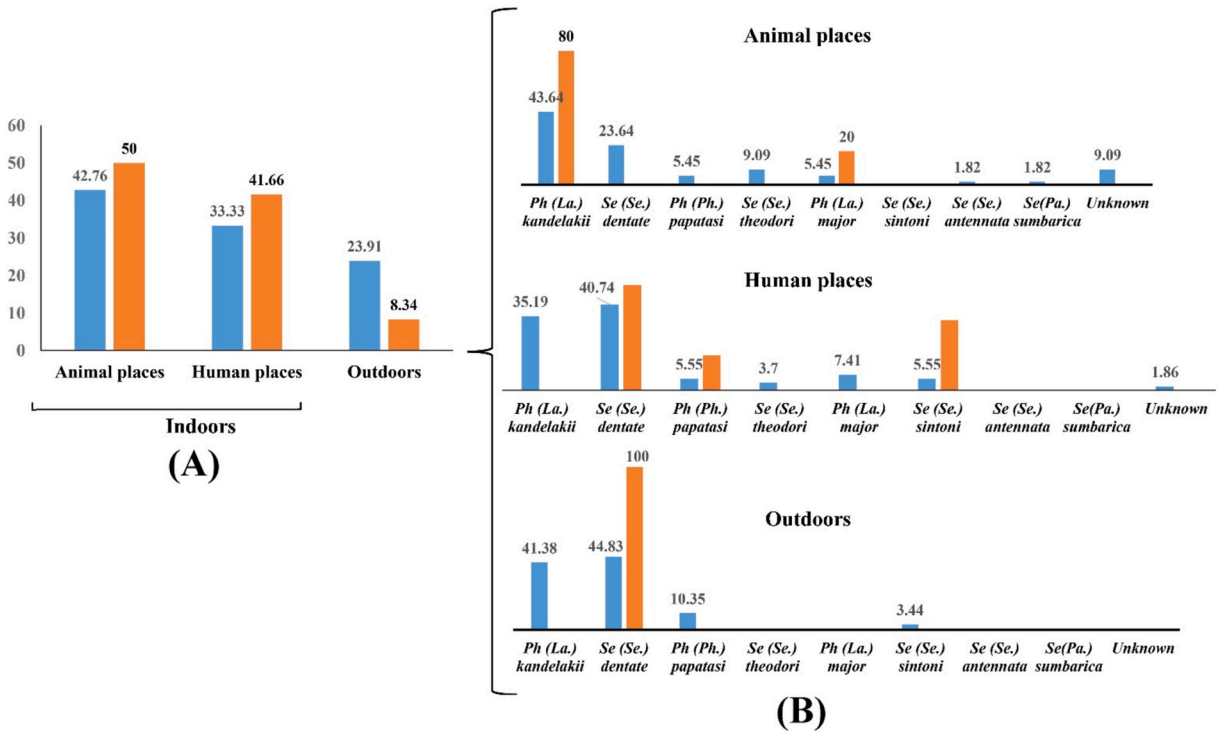

Fig. 5. Percentage of tested and *Leishmania*-infected sand flies from indoor (animal and human dwellings) and outdoor locations in Sari City, northern Iran. Panel (A) presents overall data, while panel (B) categorizes by species.

In our study, *Se. dentata* and *Se. sintoni* were positive for *L.*(*L.*) *major*. As far as we know, this is the first report of natural infection of *Se. sintoni* in the study area and *Se. dentata* in Iran and possibly worldwide. The fact is that *Sergentomyia* species are known as vectors of reptilian *Leishmania* species due to their preference to feed on cold-blooded vertebrates. It is believed that they are not able to transmit *Leishmania* to humans. However, the potential role of *Sergentomyia* as a vector has been documented by the detection of *L. major* DNA in *Se. clydei* and *Se. minuta* in Tunisia (Jaouadi et al., 2015), *Se. minuta* in Portugal (Campino et al., 2013) and *Se. sintoni* in Iran (Parvizi and Amirkhani, 2008). Additionally, promastigote infection of *Se. sintoni* was previously observed in Khorasan Province, northeastern Iran. (Yaghoobi Ershadi et al., 2003). These species exist sympatrically with *Phlebotomus* species, as supported by our findings. Furthermore, they may even display higher abundance levels than *Phlebotomus* (Firouzjaie et al., 2023; Hosseini-Vasoukolaei et al., 2022), contrary to our study's results. This discrepancy may be due to the multifactorial nature of sand fly abundance, which is influenced by geographic location, climate, and the specific ecological niche under investigation.

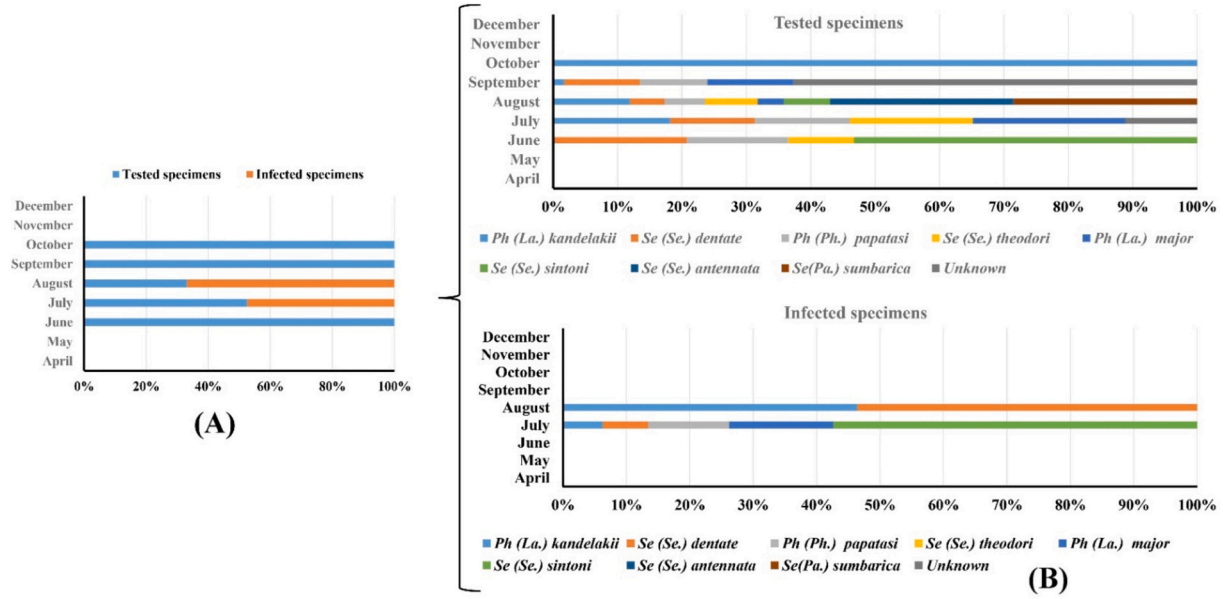

Fig. 6. Percentage of tested and *Leishmania*-infected sand flies by month in Sari City, northern Iran. (A) Overall percentage of tested and infected sand flies across all month. (B) Breakdown of percentages categorized by sand fly species.

In the present study, majority of sand flies were found indoors, with empty abdomen, which indicates the adequate longevity of females to complete the cycle of the parasite in their bodies, and a considerable vectorial capacity in these species. Several studies have highlighted the importance of the abdominal status of sand flies during collection (Rassi et al., 2012; Rassi et al., 2011; Vahabi et al., 2016), noting that vector incrimination is linked to the presence of infective metacyclic promastigotes in the foregut. An empty abdomen suggests successful establishment of the parasite in the midgut and thoracic midgut, where it transforms from a non-infectious to an infectious form (Rassi et al., 2011).

It should be noted that to establish a sand fly as a proven vector in an area, several criteria must be met: (i) frequent natural infection with the same *Leishmania* species found in humans and reservoirs; (ii) presence of the parasite's infective form in the anterior midgut, stomodeal valve, and on naturally or experimentally infected females; (iii) a strong ecological relationship between the vector, humans, and reservoir hosts; and (iv) the vector's ability to support full parasite development post-blood meal and transmit the parasite to a susceptible host during feeding (Killick-Kendrick and Ward, 1981). Therefore, considering the positivity of sand flies to *L.* (*L.*) *major* in the present study, further research is needed to determine which species meet all these criteria to be confirmed as vectors of Old World *Leishmania* species pathogenic to mammals.

The phylogenetic analysis of *Leishmania* spp. ITS2 sequences revealed the relationship between *L.*(*L.*) *major* identified in sand flies from this study and other *Leishmania* species in the GenBank. However, we acknowledge that some nodes exhibit low bootstrap values, likely due to the short sequence length (216 bp) used in our analysis. Based on ITS2 sequences, we observed significant diversity among *L.*(*L.*) *major* isolates, consistent with previous studies that used other molecular markers, such as ribosomal DNA (ITS), cytB, and kDNA, to highlight the heterogeneity of *L. major* isolates (Mahnaz et al., 2011; Mahmoudzadeh-Niknam et al., 2012; Khan et al., 2016; Mohammadiha et al., 2018; Nemati et al., 2024).

It is important to emphasize that comparing *Leishmania* species detected in sand flies with those isolated from humans is crucial for predicting disease transmission risks in endemic areas (Owino et al., 2021). In this study, the *L.*(*L.*) *major* sequences identified in *Phlebotomus* and *Sergentomyia* sand flies was closely related to those isolated from humans in Iran, highlighting their potential role in disease transmission in the target area. Specifically, the *L.*(*L.*) *major* isolate detected in *Se. dentata* (GenBank ID: OM985932) and *Ph. major* (GenBank ID: OM985930) exhibited 98.15 % and 98.12 % similarity, respectively, to *L.*(*L.*) *major* isolated from a patient in Iran (GenBank ID: AJ786163). Furthermore, *L.*(*L.*) *major* detected in *Ph. Kandelakii* (GenBank ID: OM985931) and *Ph. papatasi* (OM985929) showed 97.66 %, and 95.79 % similarity, respectively, to the same human isolate. The phylogenetic analysis confirms the close genetic relationship between *L.*(*L.*) *major* detected in sand flies and isolates from humans, highlighting the potential role of these sand fly species in *L.*(*L.*) *major* transmission within endemic areas.

In conclusion, the results of this study, along with other studies on sand flies in northern Iran, can be useful for epidemiological research and leishmaniasis control programs in the region.

Recommendations

- Further investigation on the assessment and management of *L.* (*L.*) *major* DNA distribution in sand fly populations.
- Implementation of targeted control and prevention programs to reduce the risk of leishmaniasis transmission.

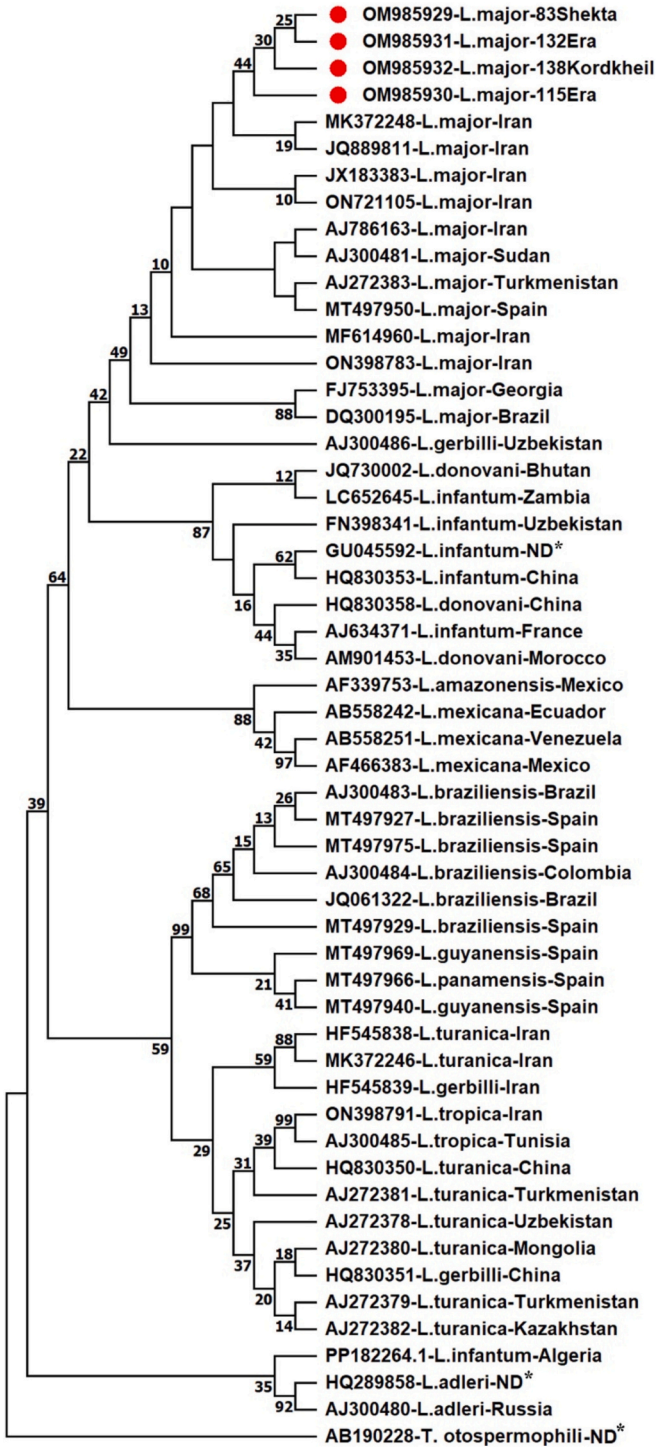

Fig. 7. Maximum likelihood phylogenetic tree based on 216 bp of ITS2-rDNA, illustrating the relations between *L.*(*L.*) *major* sequences from sand flies collected in Sari City, northern Iran (marked in bold with red circles), and 50 reference sequences from GenBank, including 12 *Leishmania* species and *Trypanosoma otospermophili* as the outgroup. The tree was constructed using the HKY + I + G nucleotide substitution model with 1000 bootstrap replicates, displaying bootstrap values >10 % on the branches. *ND = not defined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

- Regular implementation of entomological surveillance to assess sand fly abundance and infection rates in at-risk areas.
- Improvement of diagnostic monitoring and treatment protocols for cutaneous leishmaniasis in humans and animals.
- Prioritization of community education programs on preventive measures against cutaneous leishmaniasis and early symptom identification.
- Promotion of interdisciplinary collaborations between public health officials, entomologists, and researchers to enhance the effectiveness of control efforts.

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CRediT authorship contribution statement

Seyed Hassan Nikookar: Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Mohammad Reza Akbari:** Methodology, Investigation, Data curation. **Mohammad Ali Oshaghi:** Methodology, Investigation, Data curation. **Nasibeh Hosseini-Vasoukolaei:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ahmadali Enayati:** Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Farzad Motevalli-Haghi:** Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mahdi Fakhar:** Visualization, Validation, Resources, Project administration, Methodology, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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