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ORIGINAL ARTICLE

JPD

Genetic diversity of *Leishmania major* strains isolated from *Phlebotomus papatasi* and *Phlebotomus alexandri* in western Iran based on minicircle kDNA

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Abstract This study aimed to screen the natural infection rate of *Leishmania major* in *Phlebotomus papatasi* and *Phlebotomus alexandri* in two counties (Mehran and Dehloran) of Ilam province as cutaneous leishmaniasis endemic areas in the west of Iran. Furthermore, the genetic diversity of parasite species that are isolated from vectors, was investigated. Sandflies were collected by sticky traps from May 2018 to October 2018. Afterward, specimens were prepared for species identification by morphological features. DNA was extracted from female sandflies, and minicircle kDNA was used to identify *Leishmania* isolates through nested-PCR, followed by genetic diversity between *Leishmania* isolates was investigated by sequence analysis of the amplified minicircle kDNA. Natural infection of the *L. major* was shown in all positive specimens using nested-PCR. Analysis of data from 14 isolates displayed a high level of genetic diversity in *L. major*. In the phylogenetic trees, all of the *L. major* isolates occurred in six clusters. Clusters I, II, III, and VI contained isolated strains from *P. papatasi*. While clusters IV and V contained isolated strains from *P. alexandri*. Genetic diversity of *L. major* isolated from vectors was investigated in western Iran for the first time. According to the results of this study, probably "various clones of *L. major* populations are distributed in the study area.

Keywords *Leishmania* · Minicircle kDNA · Sandflies · Genetic diversity · Iran

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Introduction

Leishmania parasites (Kinetoplastida, Trypanosomatidae) are parasitic protozoans that can infect their hosts through the bite of infected female phlebotomine sandflies (Davami et al. 2011; Moslehi et al. 2020). The parasite can cause unpleasant skin lesions in humans, and it may take a long time to heal and bring severe socio-economic burdens to society (Shirzadi et al. 2015; Ramezankhani et al. 2017; Namdar et al. 2020). Leishmaniasis is a vector–borne disease that is highly prevalent and one of the main public health problems in developing countries, including Iran (Yaghoobi-Ershadi 2012; Salehi et al. 2014). There are about 93 sand fly species reported in the world that transmit 20 *Leishmania* species to humans (Meeting WECot-CotL 2010). Previous studies in Iran have demonstrated that six sand fly species including *P. salehi*, *P. mongolensis*, *P. alexandri*, *P. andrejevi*, *P. caucasicus*, and *P. ansarii* are suspected vectors, and *P. papatasi* serves as the major vector of zoonotic cutaneous leishmaniasis (ZCL)

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due to *L. major* (Afshar et al. 2011; Yaghoobi-Ershadi 2012). *P. alexandri* was also found infected by *L. major* using molecular methods in Khuzestan Province and Kermanshah Province in the neighborhood of the study area (Javadian et al. 1977; Naghian et al. 2020). Iraq-Iran war (from 1980 to 1988) and has recently been involved in the annual passage of pilgrims, mainly from Mehran and Dehloran counties have led to ecological mutability in Ilam Province as the enzootic ZCL foci in western Iran, providing commensurate conditions for blooming heterogeneity and allowing for great diversity among sand fly and *Leishmania* species. The genetic diversity of *Leishmania* spp. may result in different phenotypes and can be related to a variety of clinical advents (Baghaei 2005). This genetic heterogeneity depends on several factors, such as the type of laboratory and molecular marker tools (e.g. mitochondrial or nuclear DNA). Other involving factors include: a source of the *Leishmania* isolates such as vectors, reservoir hosts, and human subjects, and they can be transmitted zoonotically or anthroponotically. Series of DNA biomarkers have been used to analyze genetic diversity, including kDNA (Anders et al. 2002), GP63 (Mauricio et al. 2001), ITS (Mouttaki et al. 2014; Khan et al. 2016), Cytochrome b (Cyt b), and NPT gene (Waki et al. 2007; Kato et al. 2016a, b). Previous studies have shown mitochondrial kinetoplastid DNA (kDNA) is an ideal gene for phylogenetic surveys. The analysis of the amplified minicircle kDNA gene was employed to survey the heterogeneity among *L. major* strains isolated from *P. papatasi* and *P. alexandri* within Ilam province, Iran, in this study.

Materials and methods

Study area

A geographical scale in Ilam Province, located west of Iran selected for the sand fly collection. This area located on the borderline with Iraq country and lies within latitudes of 32° 59′ and 35° 16′ N and longitudes of 46° 25′ and 49° 10′ E. Central areas along with villages and districts of Dehloran and Mehran counties were chosen as the primary sampling region. The sampling regions map was drawn using ArcGIS software v2.18 (Fig. 1).

Sandfly sample collection

40 different zones were chosen for the collection of phlebotomine sand flies, from May 2018 to October 2018 (adult sand flies are absent in other months). To trap sand flies, 44 sticky traps (ST) were located in outdoor and indoor places, and near entries to rodents burrows. All traps were installed at sunset and were collected the next day before sunrise. Collected specimens after detache from the ST were washed to remove the debris and then were kept in 70% ethanol. Dissection of sand flies was done under a loop microscope (Aransay et al. 2000). For taxonomic identification based on standard keys, the head and end of the abdomen of all samples were mounted in a temporary fructose-gum Arabic medium (Seyedi-Rashti and Nadim 1967; Lewis 1982; Seyedi-Rashti et al. 1992), and the residual body sections of female sand flies were transferred to a 1.5 mL Eppendorf tube and stored in absolute ethanol at − 20 °C for molecular surveys.

DNA extraction

Specimens were put in 1.5 mL microtubes and then digested and squelched, in a lysis buffer (Tris–HCl, 10 mM, pH 8.0; proteinase K, 100 µg/mL; SDS, 0.5%; EDTA 10 mM; N-laurilsarcozil, 0.01%). The samples were kept in boiling water until the tissues were completely lysed (1 h). Genomic DNA extraction was carried out using the thorax and abdomen of each sand fly and *Leishmania* promastigotes from medium (positive controls). This process was done using the DNeasy® Blood & Tissue Extraction Kit (Qiagen, Hilden, Germany) based on the directions manufacturer. The concentration of DNA templates was evaluated by the OD ratio of 260 and 280 nm in an ND NanoDrop 1000 (Therm Science). The DNA was kept at − 20 °C until amplified by nested-PCR.

Nested-PCR-based diagnosis

For the proliferation of minicircle kDNA, two steps of nested PCR were used. The combination of primers CSB1XR (5′ATTTTTCGCGATTTTCGCAGAACG3′) and CSB2XF (5′CGAGTAGCAGAAACTCCCGTTCA3′) were used for the first step and the primers LiR (5′TCGCAGAACGCCCCT3′) and 13Z (5′ACTGGGGGTTGGTGTAAAATAG3′) for the second, under the Noyes et al. method (Noyes et al. 1998). The total volume of each PCR round was 25 µL and composition of PCR mix was 1.0 U Taq polymerase (CinnaGen, Tehran, Iran), 50 mM Tris–HCl (pH 7.6), 1.5 mM $MgCl_2$, 200 mM of each dNTP (Roche, Penzberg, Germany), 10 mM CSB1XR, 10 mM CSB2XF and 5 µL template DNA. A Perkin Elmer (PE) thermocycler was used for nested PCR protocols according to this schedule: 5 min at 94 °C followed by 35 cycles of 94 °C, the 30 s, 55 °C, 60 s, 72 °C, 60 s, and a final extension lasting for 5 min at 72 °C. In the following the PCR products were diluted 1:9 with deionized water and then the second step of nested PCR was run using 1 µL of this dilution and primers LiR

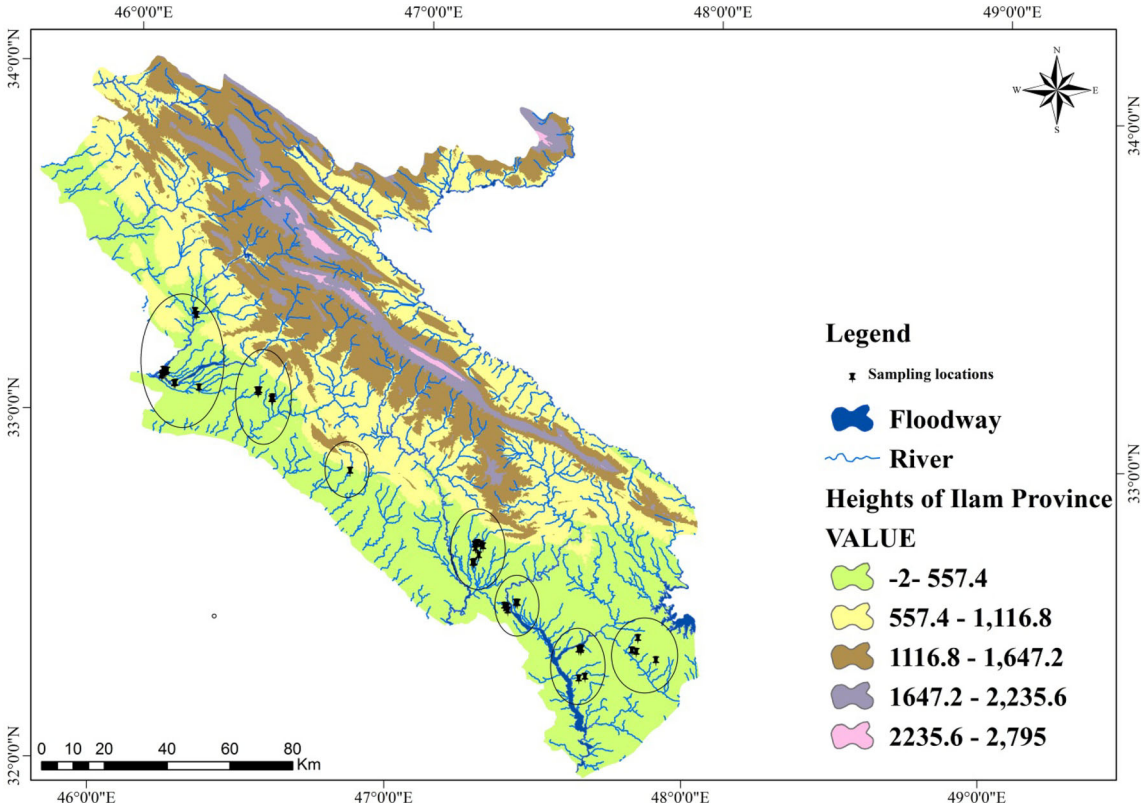

Fig. 1 Ilam Province map, the dark circles show the sampling locations in the counties of Mehran and Dehloran, Qgis v2.18

and 13Z, according to similar thermal profile and materials as the first round.

In each nested PCR run, DNA extracted from *Leishmania* promastigotes, reference strain, MHOM/IR/54/LV39, and DNA samples extracted from male sandflies were used as positive and negative controls respectively.

Sequencing

The amplified DNA fragments were visible on 1.5% agarose gels. A standard DNA marker (Fermentas, Vilnius, Lithuania) was used to measure the size of fragments. All PCR products were subjected to sequencing by MWG (Ebersberg, Germany) for both forward and reversed directions.

Phylogenetic analysis

The Chromas (2.6.6) program was used to see and check the fragments. In the following, the obtained sequences were edited and trimmed using the BioEdit Sequence Alignment Editor and then, were compared with sequences in the NCBI GenBank database using the BLAST (http://blast.ncbi.nlm.nih.gov/) (Camacho et al. 2009). ClustalW options were used for multiple sequence alignments via MEGA-X software. Also, a phylogenetic tree was created based on the Neighbor-Joining (NJ) algoritm (Kumar et al. 2018). Statistical support of the phylogenetic tree was assessed using Bootstrap replications 1000. *Leishmania infantum* was used as an out-group in a phylogenetic tree.

Results

A total of 893 females of *P. papatasi* and 249 *P. alexandri* were collected in trap locations. The physiological condition of both species was blood-fed, semi gravid, gravid, and unfed. 300 females of *P. papatasi* and all hunted females of *P. alexandri* were investigated to isolate *Leishmania* species. Ten females of *P. papatasi* and four females of *P. alexandri* were positive for *L. major* using the nested PCR. In this method bands with a size of 570 bp on 1.5%, agarose gel showed as *L. major* (Fig. 2). The kDNA sequences of 14 positive specimens showed 97–99% similarity to the submitted strain with accession number KY131443.1. The phylogenetic tree according to the sequencing of minicircle kDNA, grouped the 14 isolates into 6 clusters (Fig. 3). Clusters I, II, III, and VI contained isolated strains from *P. papatasi.* While clusters IV and V contained isolated strains from *P. alexandri. Sergentomyia*

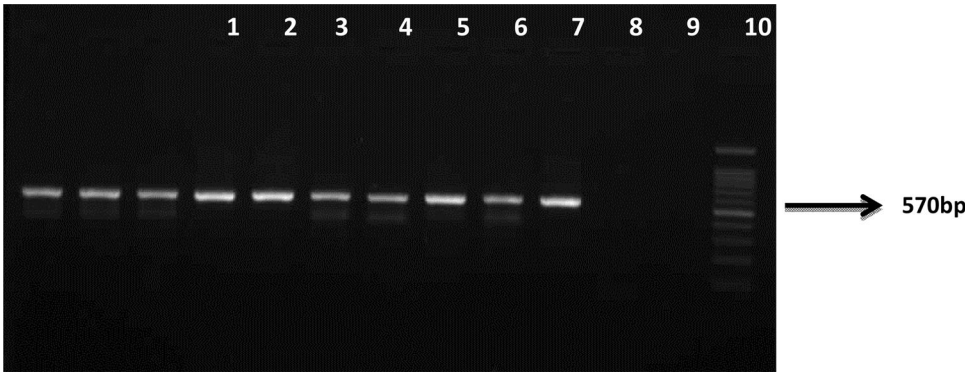

Fig. 2 Agarose gel electrophoresis of amplified minicircle kDNA in nested PCR: Lanes 1–9 *L. major* isolated from sandflies; Lane 10 positive control; Lane 11 and 12 negative control; Lane MM molecular marker

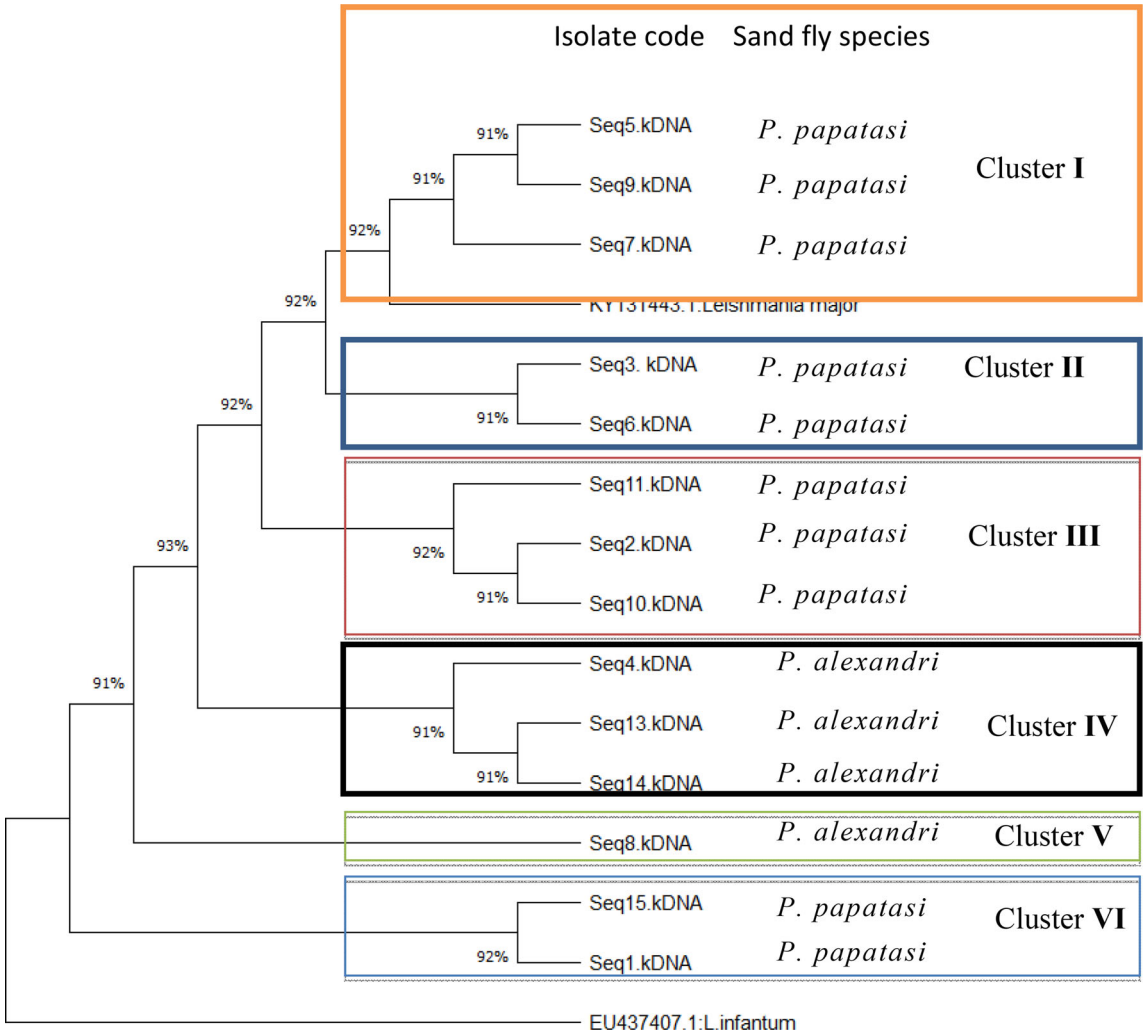

Fig. 3 Clustering algorithms in the phylogenetic tree among *Leishmania* isolates by MEGA-X. *L. infantum* (EU437407.1) and *L. major* (KY131443.1), were used as out-group and standard isolates, respectively

theodori, S. squamipleuris, S. antennata, S. dentata, S. iranica, and S. clydei were other sand fly species that were also found in this study.

Discussion

PCR amplification of kDNA gene was used for the diagnosis and characterization of the genetic diversity of *Leishmania* species in vectors. *L. major* were detected in

10 *P. papatasi* and 4 *P. alexandri* species, respectively. No other species of *Leishmania* were found in this study, and according to the results of this survey, *L. major* is the main species responsible for CL in the study area. Favorable factors such as weather conditions, geographical location, natural disasters such as earthquakes, as well as the presence of reservoirs and carriers of disease in border areas between Iran and Iraq have caused these areas to be part of the hyperemic areas of leishmaniasis. It seems necessary to conduct researches on the agents and carriers of the disease in this region.

To the genetic analysis of *Leishmania* isolates minicircle kDNA gene was used in this study. This fragment contains three highly conserved and variable regions. Conserved blocks of Minicircles kDNA are CSB1, CSB2, and CSB3, which are used to develop PCR and sequence analyses (Noyes et al. 1998; Van der Auwera and Dujardin 2015). In this study, the variable and constant zones of the kDNA with the size of 570 bp were amplified according to the Noyes et al. method (Noyes et al. 1998). This sequence belongs to the CSB2 block and is conserved across many *Leishmania* species. Based on our search, the heterogeneity of *Leishmania* species isolated from vectors based on the kDNA sequencing and to correlate the vectors species with parasite heterogeneity were investigated in this study for the first time. Intrinsic genetic diversity in *Leishmania* spp. occurred due to clonal reproduction and hybridization (Rogers et al. 2014; Ramírez and Llewellyn 2014). The main process that can change genetic composition in biological communities is sexual reproduction (Odiwuor et al. 2011; Kato et al. 2016a, b). Also, sexual recombination that might occur rarely in usually asexual parasites may have a deep effect on their heterogeneity. Ravel et al. and Volf et al. have described enhanced transition possible and appearance new forms of CL in the results of hybrid formation among *L. infantum* and *L. major* (Ravel et al. 2006; Volf et al. 2007). The previous studies concerning the connections between the clinical manifestations of CL and genetic heterogeneity of *L. major* have been carried out in Iran, and a high level of heterogeneity in *Leishmania* parasites, has been reported based on Cyt b, ITS1and kDNA (Baghaei 2005; Shirian et al. 2016; Mohammadpour et al. 2019). These studies have reported a reciprocal link between clinical characteristics of ZCL and the genetic polymorphisms of *L. major*. The polymorphisms of *L. major* causing CL according to ITS PCR-RFLP were evaluated by Baghaei et al. they found a high level of genetic polymorphisms in *L. major* and connections between the clinical outcomes of the CL and the geographical origin (Baghaei 2005). Oryan et al. (2013) and Shirian et al. (2016) studies the mutual connection between the clinical presentations of CL and genetic polymorphisms of parasite according to kDNA, has been investigated. Their studies showed that *L. major* has genetic polymorphisms, and there exists a connection between clinical presentations of the disease in humans and genetic polymorphisms of the parasite. In our study, analyze the minicircle kDNA revealed that this parasite is genetically highly polymorphic. The outcomes of this research displayed polymorphism and heterogeneity in *L. major* isolated from naturally infected sandflies. Genetic factors control *L. major* infection in phlebotomine sand flies so that their ability to support different parasite species or strains is very variable. Some species are highly susceptible, and others refractory to the parasite. The interplay of the promastigote surface adhesin lipophosphoglycan (LPG) and additional factor(s) with sand fly midgut receptors may determine whether a sandfly is a suitable host or not (Wu and Tesh 1990; Dobson et al. 2010). In our study, strains isolated from *P. papatasi* were different genetically from those isolated from *P. alexandri*. This issue that, which strains can be transmitted to humans and cause disease, is a matter that needs further investigation.

Conclusions

The analysis of the *L. major* kDNA gene sequence showed genetic diversity among this parasite that spread in the study area. According to these results, maybe various clones of *L. major* populations are circulating among vectors and reservoir animals in endemic regions of Ilam province. Furthermore, the nested PCR is a sensitive test for the diagnosis of *Leishmania* DNA in naturally infected sandflies.

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Authors' contribution All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials All data generated or analyzed during this study are included in this article.

Declarations

Conflict of interest All authors declare that there are no conflict of interest.

Ethics approval and consent to participate All applicable international and institutional guidelines for conducting the study in 'the houses and domestic animal shelters' were followed. The study protocol was approved by the ethical committee of the Isfahan University

of Medical Sciences, (IR.MUI.MED.REC.1398.523). The guideline of the Institutional Animal and Ethics Committee, Isfahan University of Medical Sciences was used to work in this study.

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