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*Research Article*

# Population Structure of *Leishmania tropica* Causing Anthroponotic Cutaneous Leishmaniasis in Southern Iran by PCR-RFLP of Kinetoplastid DNA

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Iran is one of the six countries with the most cutaneous leishmaniasis (CL) patients. Understanding better the genotypes of the parasite population in relation to geography and climate is critical to achieving better CL control. We aimed to characterise the population structure of *Leishmania tropica*, the cause of anthroponotic cutaneous leishmaniasis (ACL), from important foci in southeast (Bam and Kerman) and southwest (Shiraz) Iran. A total of 39 *L. tropica* isolates from ACL patients from southeast (Bam 14, Kerman 12) and southwest (Shiraz 13) Iran were analysed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of the kinetoplast DNA (kDNA) using restriction enzymes *Msp*I (*HpaII*) and *Cla*I. 37 genotypes were identified among south Iran *L. tropica* isolates. The unweighted pair group method with arithmetic mean (UPGMA) tree obtained from the banding patterns of *Cla*I digested kDNA RFLP distinguished southeast from and southwest *L. tropica* isolates with some subclustering but the *Msp*I derived tree showed greater discrimination with greater subclustering and divergence of the two foci of southeast region but with some overlapping. Although a monophyletic structure has been defined for southeast *L. tropica*, isolates from two foci of southeast Iran were partly discriminated in the current study.

## 1. Introduction

Leishmaniasis is a global vector-borne disease caused by the protozoa belonging to the *Leishmania* genus that is transmitted through the bite of the *phlebotomine* sand fly species [1]. Leishmaniasis is categorised by a spectrum of clinical manifestations from cutaneous and mucocutaneous to visceral forms. Cutaneous leishmaniasis (CL) is the most common form of the disease with 0.7 to 1.2 million new cases per year being reported worldwide [2]. CL causes skin lesions on the exposed sites of the body, leaving life-long scars, notable disability, and psychological distress and has important negative social and economic consequences. Although CL is considered as a significant public health challenge, it remains a neglected disease [3].

Over two-thirds of new CL cases occur in six countries: Afghanistan, Algeria, Brazil, Colombia, Syria, and Iran [4]. Two epidemiological forms of CL are present in Iran, anthroponotic (ACL) and zoonotic cutaneous leishmaniasis (ZCL), and are caused by *L. tropica* and *L. major*, respectively. ZCL

extends mostly in rural regions of the northeast, the central, the west, and the southwest of the country and occurs in some parts of southern and southeast provinces as well [5–14].

ACL is endemic in several large cities, including Tehran, Mashhad, Yazd, Shiraz, and Kerman, and small cities and their surrounding counties such as Bam and Birjand [15–19] (Figure 1).

Population growth and displacement, urbanisation, human behaviour, anthropogenic environmental changes, drug resistance, new agricultural plans, and new parasite-vector relationship have resulted in epidemiological changes and an increased CL incidence rate in different regions [20–23]. Tracking the changing epidemiology of CL can be done with molecular taxonomy studies to determine the *Leishmania* parasite population structure and heterogeneity [24]. Different *Leishmania* genes have been used to study the phylogeny of the *Leishmania* at genus, species and strain levels, including glycoprotein 63 [25], cytochrome b [26], heat shock proteins [27], tubulin [28], N-acetylglucosamine-1-phosphate transferase [29], miniexon [30], kinetoplast minicircle DNA [31], internal transcribed spacer of ribosomal DNA (ITS-rDNA) [32], and microsatellite-containing sequences [33]. The kinetoplast DNA (kDNA) is the mitochondrial DNA of flagellated protozoa of the order kinetoplastida and consists of a network containing two types of duplex DNA circles: (i) a few dozen homogenous maxicircles (~25-50) ranging from 20 to 40 kb and (ii) ~ 5000-10,000 heterogeneous minicircle copies, 800 base pairs (bp) in size, consisting of a 600 bp variable, and a 200 bp conserved region [34, 35]. Although minicircle DNA has a critical role in the function of the trypanosomatid's mitochondrial genes by encoding guide RNAs, which edit the messenger RNA encoded by the maxicircles [36], but can be used to discriminate between strains of the same species based on the heterogeneity of its variable region [37].

Different methods in protein and DNA levels have been used for the study of *Leishmania* diversity in intraspecies level. Multilocus enzyme electrophoresis (MLEE) has been the gold standard for differentiating different species and strains of *Leishmania* based on the electrophoretic pattern of organism's enzymatic systems [38, 39]. However, MLEE is a time consuming procedure that requires the mass cultivation of parasites and has low discriminatory power at the amino acid level. It has been superceded by DNA-based techniques, including polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) of some highly variable genes, sequencing of single or multilocus genes, and multilocus microsatellite sequence typing (MLMT). MLMT and kDNA PCR-RFLP have been found to be the most powerful discriminatory approaches for intraspecies population structure studies [40–42]. There are two considerations regarding these techniques. Variations in the quantity of amplified DNA products and interlaboratory reproducibility have been reported in some studies for kDNA PCR-RFLP and MLMT is very expensive for developing countries (capillary sequencers and fluorescence labelled primers) [15].

We have already reported on the monophyletic population structure of *L. tropica* strains isolated from southeast Iran, by using sequence analysis of the rDNA-ITS locus [15, 43]. There has never been a study in Iran using kDNA PCR-RFLP to examine the intraspecies relationships in *L. tropica* from the principal ACL foci in southeast and southwest Iran. We, therefore, conducted such a study and reported our results herein.

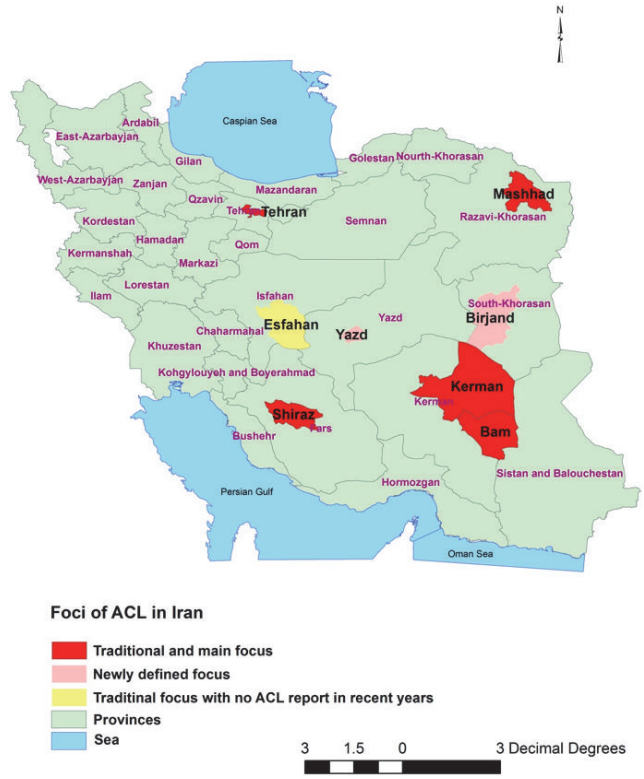


Figure 1: Map (down to county level) showing the main foci of *Leishmania tropica* in Iran. Kerman, Bam, Shiraz, Tehran, and Mashhad are the traditional endemic foci (red polygons). Birjand and Yazd have seen recent introductions of *L. tropica* (pink). *L. tropica* has not been reported from Esfahan in recent years (yellow).

## 2. Material and Methods

*2.1. Study Areas and Samples.* A total of 100 amastigote positive, Giemsa-stained smears were randomly collected (by using records numbers) from patients referred to the Cutaneous Leishmaniasis Control Center in Bam city and health centers in Kerman and Shiraz cities, the main foci of ACL in south Iran in 2013-14 (Figure 1). Kerman city in Kerman county is the capital of Kerman province (southeast Iran). Bam county also belongs to Kerman province and is located in the south border of Kerman county. Shiraz city is the capital of Fars province in southwest Iran. The annual incidence of CL in Kerman county was 369 cases (*L. tropica* mostly). Bam county saw 369 reported CL cases annually; *L. tropica* is reportedly the causative agent of all CL cases [18]. Shiraz city is the traditional focus of *L. tropica* in southwest Iran but recent studies show a changing epidemiology with

ACL confined mostly to the central areas of the city and ZCL caused by *L. major* in the suburbs [16].

*2.2. Microscopic Examination.* Giemsa-stained slides were prepared (as per routine clinic care) from skin lesion scrapings and examined under the microscope. *Leishmania* amastigotes were sought under high power field resolution and the number of amastigotes were reported as negative (0 parasite/1000 HPF), trace (+/- or suspicious), + (1 - 10 parasites/1000 HPF), ++ (1 - 10 parasites/100 HPF), +++ (1 - 10 parasites/10 HPF), and ++++ (1 – 10 parasites/1 HPF). Only slides 2–4+ were selected for molecular evaluation.

*2.3. DNA Extraction.* Tissue scratched from the Giemsa-stained smears were collected in 1.5 ml microtubes containing lysis buffer (Tris 100 mM, EDTA 10 mM, NaCl 100 mM, SDS 1%, and Triton X100 2%). Then, 8-10 $\mu$l proteinase K (10$\mu$g/$\mu$l) was added and the samples were vortexed and incubated at 56°C for one hour. Samples were extracted once with phenol/chloroform (25:24 v/v) and once again with chloroform. Equal volumes of isopropanol and one tenth volume of 3 M NaAc were added to precipitate the DNA. Extraction was followed by washing with 70% ethanol. The DNA precipitant was dried and then suspended in 50 $\mu$l ultrapure water.

*2.4. kDNA PCR.* kDNA PCR was performed to identify the *Leishmania* species and produce enough product for the later kDNA RFLP. kDNA was amplified by using the primers 13Z (5′-ACT GGG GGT TGG GTG TAA AAT AG-3′) and LiR (5′-TCG CAG AAC GCC CCT-3′). The PCR mixture consisted of 12.5$\mu$l of 2x premix (Ampliqon, Denmark), 20 pmol of each primer, 5 $\mu$l of template DNA, and enough water up to 25 $\mu$l. The cycling PCR conditions were 95°C for 5 min followed by 35 cycles of 94°C for 45s, 55°C for 60 s, and 72°C for 90 s and a final extension at 72°C for 7 min in a thermal cycler machine (Applied Biosystems 2700). Distilled water instead of DNA template was used in each run as a negative control. A clinical isolate with a laboratory confirmed species finding as *L. tropica* and one reference strain of *L. major* (MRHO/IR/75/ER) were used for species identification. The species specific band sizes discriminate *L. tropica* and *L. major* [37]. The PCR products were subjected to 1.2% agarose gel electrophoresis and visualized by a transilluminator. A 100 bp DNA marker was used for sizing the bands. A total of 39 smears that were identified as *L. tropica* were selected: (i) 14 from Bam district (ID sample: B18, B25 to B29, B31, B33 to B39), (ii) 12 from Kerman (B7 to B17 and B32), and (iii) 13 from Shiraz (B1 to B6, B19 to B24 and B30).

*2.5. kDNA-Restriction Fragments Length Polymorphism.* kDNA-minicircle amplicons were exposed to the restriction enzymes *Msp*I (*Hpa*II) and *Cla*I (Fermentas, Lithuania) for 2 h, in a final reaction volume of 15$\mu$l containing 1 U of restriction enzyme, 1.5$\mu$l of 10X buffer, 6-10 $\mu$l PCR product (depending on the sharpness of the obtained band), and enough ultrapure distilled water at 37°C. An aliquot of 10-15 $\mu$l of enzyme-treated products was applied in each well in the gel. The kDNA RFLP profiles were electrophoresed on 2% Metaphor agarose (Cambrex, USA) and the obtained bands were visualized by a transilluminator. A 100 bp DNA marker was used as standard to estimate the band sizes.

Table 1: The patient's demographic and lesion data.

| Patient's data | percent |
|---|---|
| Sex | |
| Male | 48.7 |
| Female | 51.3 |
| Total | 100 |
| Age | |
| <10 | 28.2 |
| 10-20 | 23.1 |
| 20-60 | 41 |
| >60 | 7.7 |
| Total | 100 |
| Lesion sites | |
| Face and neck | 35.9 |
| One hand | 38.4 |
| Both hands | 7.7 |
| One leg | 12.8 |
| Both legs | 2.6 |
| Hand and leg | 2.6 |
| Total | 100 |

*2.6. Data Analysis.* The kDNA RFLP banding pattern for each isolate was analysed by NT SYS software version 2 [44]. All bands sizes were recorded in a table and scored as one for band presence and zero for band absence. Finally, the banding pattern for each isolate was recorded and two trees were inferred, using the unweighted pair group method with arithmetic mean (UPGMA), based on the banding pattern obtained using *Msp*I and *Cla*I restriction enzymes. The clusters constituting the trees were named by alphabetic letters for better interpretation. The genotypes (banding patterns) obtained using *ClaI* and *MspI* enzyme-based digestion of kDNA were named GCs and GMs, respectively, and GM-GC was used for the final genotypes from both banding patterns.

## 3. Results

The patient's demographic and lesion data are shown in Table 1. Enzymatic digestion with both *Msp*I and *Cla*I enzymes revealed different patterns for the isolates of *L. tropica* obtained from different foci. The approximate band sizes obtained by digestion of isolates with *Msp*I were 600, 580, 550,520, 500, 480, 450, 400, 360, 330, 310, 300, 280, 270, 250, 240, 220,190, 180, and 160 bp. Likewise, different band sizes were found among PCR products digested by *ClaI*: 600, 550, 480, 450, 400, 380, 340, 280, 250, 200, and 140 bp (Figure 2).

*3.1. Genotypes Obtained by Digestion of kDNA by ClaI.* Digestion of the kDNA products of isolates obtained from Bam

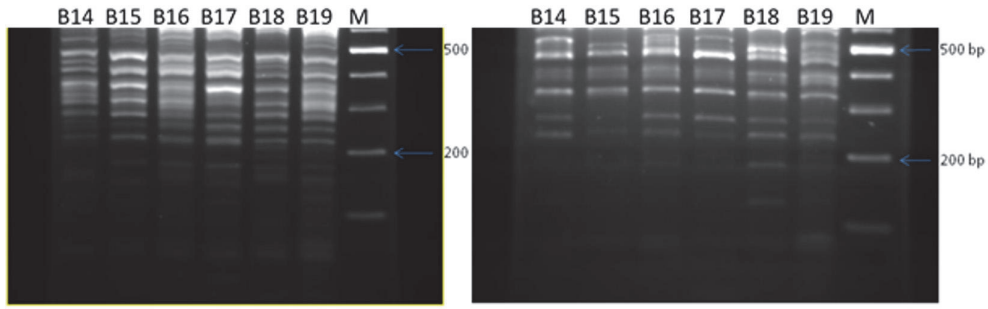


Figure 2: The banding patterns obtained by digestion of kDNA of the same samples by the enzymes *ClaI* (right) and *MspI* (left). The higher number of bands was obviously obtained when *MspI* was used. B14-B17 samples were from Kerman and B18 and b19 were obtained from Bam and Shiraz, respectively.

showed 11 genotypes among 14 isolates: GC1-GC3, GC5, GC6, GC8, GC9, GC14, GC15, GC18, and GC24. Three genotypes were found in more than one isolate: GC3 (B34 and B38), GC5 (B26 and B28), and GC9 (B18 and B39) in this focus. Among 13 isolates from Shiraz, 10 genotypes were found: GC1, GC2, GC7, and GC16-22; three isolates (B2 B4, and B6) belonged to genotype GC17 and two other isolates (B19, B21) to genotype GC19. Genotypes GC1, GC2, GC4, GC8, GC10-13, GC15, and GC23 were found from Kerman county; three (B13, B15, and B16) of 12 Kerman isolates were from one genotype (GC2).

Several genotypes were found in more than one focus: (i) two genotypes GC8 (B10-Kerman and B29-Bam) and GC15 (B17-Kerman and B31-Bam) were found in Kerman and Bam, (ii) GC18 was present in Shiraz and Bam (B5-Shiraz and B25-Bam isolates), and (iii) genotypes GC1 (B1-Shiraz, B7-Kerman and B36-Bam) and GC2 (B3-Shiraz, B33-Bam, B13-Kerman, B15-Kerman and B16-Kerman) were found in all three foci.

*3.2. Genotypes Obtained by Digestion of kDNA with MspI.* GM10, GM14, GM18, GM21-GM29, and GM34 were found among isolates obtained from Bam county. All banding patterns were different except for two isolates, B33 and B34, which were GM25. Therefore, 13 different genotypes were revealed among the 14 isolates of Bam county. The genotypes from the Kerman isolates were GM12, GM13, GM14, GM15, GM16, GM17, GM19, GM20, and GM30-GM32; two isolates (B13 and B15) belonged to the GM17 genotype, so 11 different genotypes were revealed in this focus. Only one genotype, GM14, was found in both Kerman (B9) and Bam (B18). In Shiraz, there were 11 genotypes from 13 isolates, consisting of GM1-GM9, GM11, and GM33; GM1 (B1 and B6 isolates) and GM3 (B2 and B4 isolates) were observed in two isolates.

*3.3. Phenetic Tree Topology Based on the Banding Patterns Obtained from kDNA Digestion with ClaI Enzyme.* Two main clusters were generated on the UPMGA tree (Figure 3). Most cases were found in cluster 1, which consisted of four main subclusters A to D. Subcluster A comprised seven genotypes, GC1-GC7, from 15 isolates. GC1 and GC2 came from all three foci but GC3, GC5, and GC6 only from Bam county. GC4 and GC7 were from Kerman and Shiraz, respectively. Of the 15 isolates, 12 isolates (80%) were from Bam and Kerman. In subcluster B, three genotypes, GC8-10, were found in five isolates from Kerman and Bam counties. Subcluster C consisted of five genotypes, GC11-15, and six isolates from Kerman and Bam counties. One isolate from Shiraz (B30) was GC16 genotype that represented a separate taxon between subclusters C and D. Subcluster D consisted of five genotypes, GC17-21, from nine isolates; eight and one were from Shiraz and Bam district, respectively.

Cluster 2 was comprised of three isolates, each with a different genotype: (i) GC22 (B24) from Shiraz, (ii) GC23 (B14) from Kerman, and (iii) GC24 (B35) from Bam; the Bam and Kerman genotypes were grouped in a subcluster.

*3.4. Phenetic Tree Topology Based on the Banding Patterns Obtained from kDNA Digestion with MspI Enzyme.* In this phenetic tree, there were two obvious clusters (A and B) for the *L. tropica* strains (Figure 4). Cluster A contained 13 isolates; 12 from Shiraz and only one from Bam and had two main subclusters, AI and AII. The AI subcluster contained nine genotypes, including GM1-GM9 genotypes, all from Shiraz. Subcluster AII comprised genotypes GM10 and GM11 from Bam and Shiraz, respectively.

Cluster B subclustered into BI and BII subclusters, containing 25 and one isolate, respectively, with further division of the BI subcluster into subclusters BI-a and BI-b and, in turn, branching of the BI-a into two main groups, BI-a-1 and BI-a-2. The BI-a-1 group contained 10 genotypes, GM12-21 (12 isolates) from Bam and Kerman, whereas the BI-a-2 group contained eight genotypes (GM22-29) only from Bam county. Subcluster BI-b contained four genotypes, GM30-33 (4 isolates), three from Kerman and one from Shiraz county. One isolate from Bam comprised subcluster BII.

Although 24 and 34 banding patterns were obtained from the digestion of kDNA with *Cla*I and *Msp*I enzymes, respectively, isolates B13 and B15 (Kerman) and B2 and B4 (Shiraz) showed the same genotypes in both enzymatic digestions that were identified GM17-GC2 and GM3-GC17. Therefore, a total of 37 different genotypes were identified from 39 *L. tropica* isolates from southern Iran based on the both banding patterns obtained from the digestion by both enzymes for each sample (Table 2).

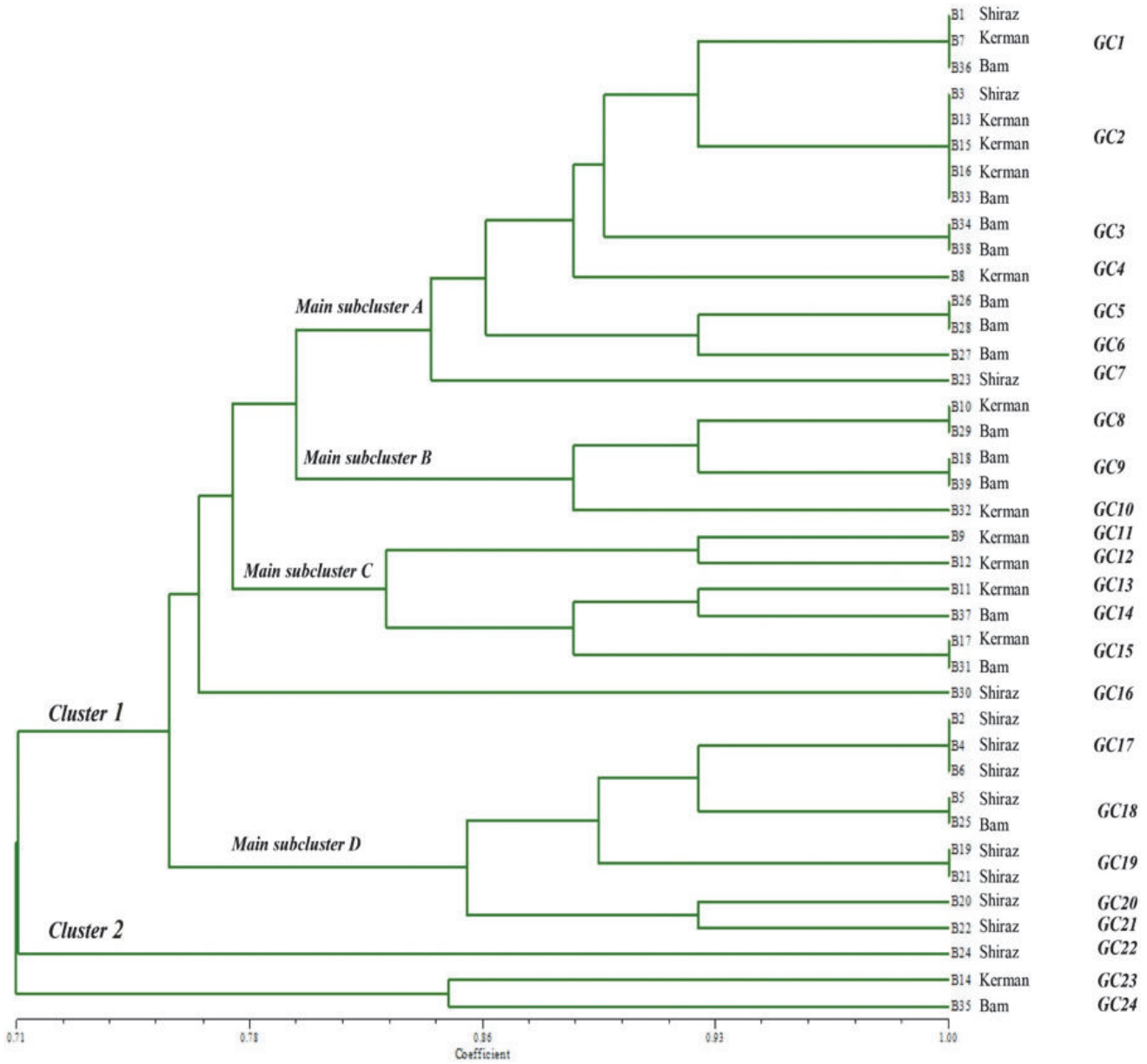


Figure 3: The UPGMA generated tree based on the banding patterns obtained from digestion of kDNA with *ClaI* enzyme showing clusters 1 and 2 and subclusters (A-D) of cluster 1. Subclusters A to C comprise most genotypes from Bam and Kerman and subcluster D most genotypes from Shiraz.

## 4. Discussion

In this study, a total of 37 genotypes were identified from 39 *L. tropica* isolates obtained from three main foci of ACL in southern Iran, Shiraz (southwest) and Bam and Kerman (southeast). The UPGMA tree topology, based on *Msp*I enzyme digested kDNA, showed well-differentiated *L. tropica* strains in two main clusters with a predominance of Shiraz in Cluster A and B group subclustered into mixed Kerman Bam isolates or Bam isolates alone. The tree topology obtained from *Cla*I digested kDNA distinguished southwest from southeast isolates but was unable discriminate between two main foci of southeast Iran. The *Cla*I enzyme has fewer cutting sites in the kDNA and has intrinsically less discriminating power than the *Msp*I enzyme. Nevertheless, both enzymes had broadly consistent results.

There are distinct geographical, environmental, and climatic differences between southeast and southwest Iran. The southeast has a predominantly desert climatic with high temperatures, low rainfall, and low flora and fauna diversity and density; the southwest is a mountainous region that sees greater rainfall and is covered with more green land cover, including pastures and forests [45, 46]. These differences may have led to the evolution of different *L. tropica* populations.

We have already shown that a monophyletic structure of *L. tropica* exists in southeast Iran based on the sequence

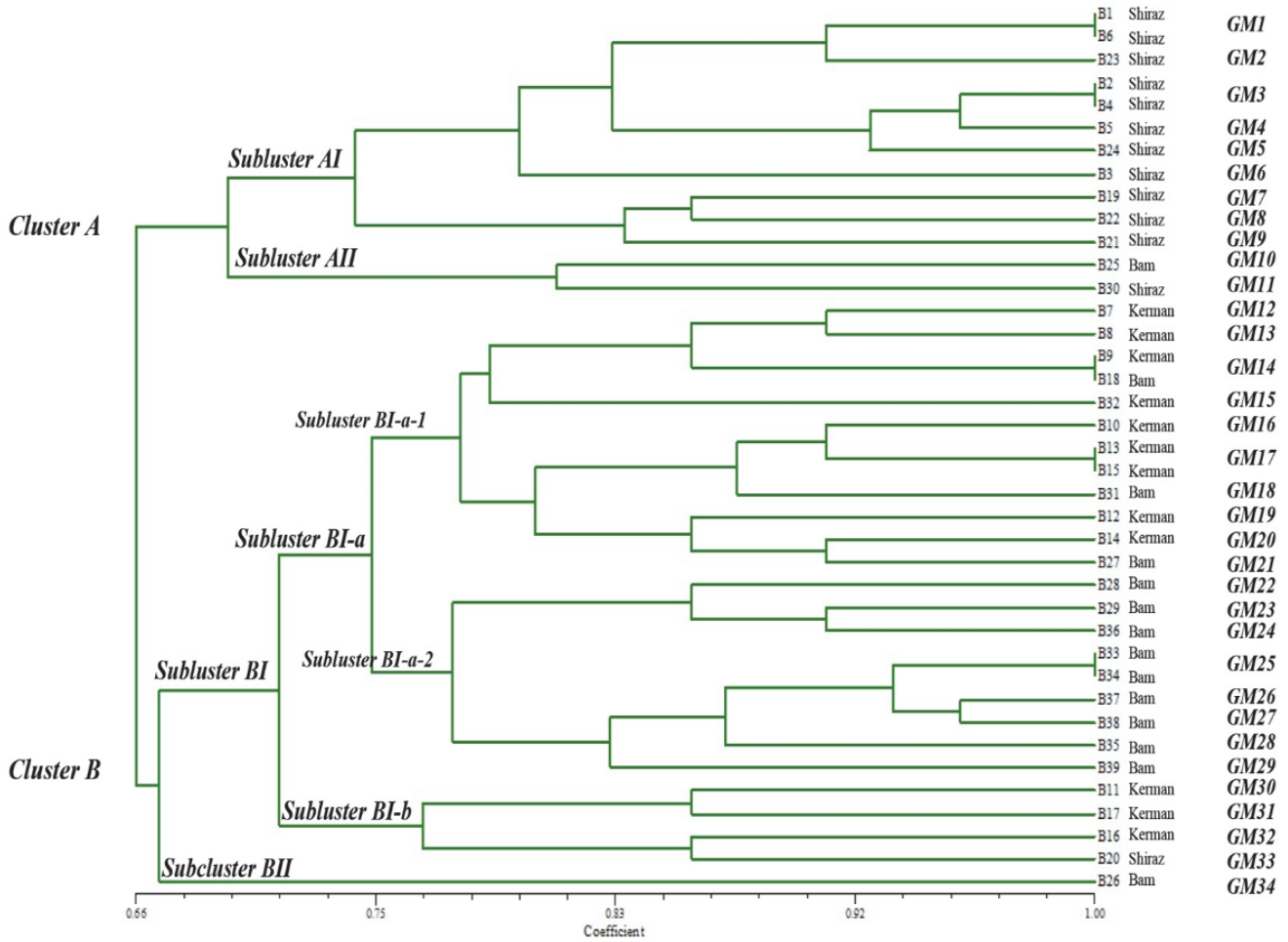


Figure 4: The UPGMA generated tree based on the banding patterns obtained from digestion of kDNA with *MspI* enzyme (n=34 genotypes). Cluster A isolates, except one, were from Shiraz. Cluster B included only isolates from Kerman and Bam.

analysis of ITS-rDNA [15] and that there is close genetic relationship between east and southeast Iranian *L. tropica* [17]. Moreover, two populations of *L. tropica* are present in Herat, west Afghanistan, in which the minor population was found to be more homogenous with southeast Iranian isolates and was assumed to have originated from that population [47]. ITS-rDNA analysis of Shiraz *L. tropica* isolates also distinguished two subgroups; one group was clustered with southeast and east *L. tropica* and another group was distributed in the another main cluster and was found to be more similar to northeast isolates [17]. Herat region in Western Afghanistan and Shiraz city in southwest Iran are mountainous regions in contrast to arid desert regions which surround the southeast-east regions. We hypothesize that climate affects the sandfly life cycle and evolution and that aridity and humidity are important drivers for *L. tropica* diversity in Iran which we believe it explains the *L. tropica* genetic diversity between humid western Afghanistan and arid east-southeast Iran [48]. Aridity along with some other human and climatic factors also affected the distribution of visceral leishmaniasis in southwest Iran as one of the two main foci of VL in Iran where aridity confined the distribution of VL [48–50].

Sequence variation among the kinetoplastid minicircle of *Leishmania* species occurs rapidly in nature [34]. Therefore, in comparison to ITS sequence analysis, kDNA PCR-RFLP is able to reveal more difference between *L. tropica* populations. This explains the diversity and subclustering seen in some *L. tropica* isolates from southeast foci and the divergence between Bam and Kerman *L. tropica* populations in comparison to previous data obtained by ITS sequence analysis in south Iran [15]. Bam and Kerman are adjoining counties in Kerman province that are surrounded by large deserts and the mean annual rainfall showed arid weather in both foci but there are some environmental differences between them. The presence of hundreds hectares of orchards, including palm and citrus trees, in Bam and its rural environs can be seen as green images by satellite data analysis [51]. These vegetation and crop residue and the local humidity resulting from irrigation produce suitable breeding microfoci for *P. sergenti*, the main vector for transmitting *L. tropica* in Iran. In addition, many agricultural workers are exposed to these

Table 2: The samples ID, geographical origin, and related GM, GC, and GM-GC genotypes (banding patterns).

| Isolate ID | Geographical origin | | | Genotypes | | |
|---|---|---|---|---|---|---|
| | Bam (SE) | Kerman (SE) | Shiraz (SW) | GM | GC | GM-GC |
| B1 | | | ✔ | GM1 * | GC1 *** | GM1-GC1 |
| B2 | | | ✔ | GM3 * | GC17 * | GM3-GC17 |
| B3 | | | ✔ | GM6 | GC2 *** | GM6-GC2 |
| B4 | | | ✔ | GM3 * | GC17 * | GM3-GC17 |
| B5 | | | ✔ | GM4 | GC18 ** | GM4-GC18 |
| B6 | | | ✔ | GM1 * | GC17 * | GM1-GC17 |
| B7 | | ✔ | | GM12 | GC1 *** | GM12-GC1 |
| B8 | | ✔ | | GM13 | GC4 | GM13-GC4 |
| B9 | | ✔ | | GM14 | GC11 | GM14-GC11 |
| B10 | | ✔ | | GM16 | GC8 ** | GM16-GC8 |
| B11 | | ✔ | | GM30 | GC13 | GM30-GC13 |
| B12 | | ✔ | | GM19 | GC12 | GM19-GC12 |
| B13 | | ✔ | | GM17 * | GC2 *** | GM17-GC2 |
| B14 | | ✔ | | GM20 | GC23 | GM20-GC23 |
| B15 | | ✔ | | GM17 * | GC2 *** | GM17-GC2 |
| B16 | | ✔ | | GM32 | GC2 *** | GM32-GC2 |
| B17 | | ✔ | | GM31 | GC15 ** | GM31-GC15 |
| B18 | ✔ | | | GM14 | GC9 * | GM14-GC9 |
| B19 | | | ✔ | GM7 | GC19 * | GM7-GC19 |
| B20 | | | ✔ | GM33 | GC20 | GM33-GC20 |
| B21 | | | ✔ | GM9 | GC19 * | GM9-GC19 |
| B22 | | | ✔ | GM8 | GC21 | GM8-GC21 |
| B23 | | | ✔ | GM2 | GC7 | GM2-GC7 |
| B24 | | | ✔ | GM5 | GC22 | GM5-GC22 |
| B25 | ✔ | | | GM10 | GC18 ** | GM10-GC18 |
| B26 | ✔ | | | GM34 | GC5 * | GM34-GC5 |
| B27 | ✔ | | | GM21 | GC6 | GM21-GC6 |
| B28 | ✔ | | | GM22 | GC5 * | GM22-GC5 |
| B29 | ✔ | | | GM23 | GC8 ** | GM23-GC8 |
| B30 | | | ✔ | GM11 | GC16 | GM11-GC16 |
| B31 | ✔ | | | GM18 | GC15 ** | GM18-GC15 |
| B32 | | ✔ | | GM15 | GC10 | GM15-GC10 |
| B33 | ✔ | | | GM25 * | GC2 *** | GM25-GC2 |
| B34 | ✔ | | | GM25 * | GC3 * | GM25-GC3 |
| B35 | ✔ | | | GM28 | GC24 | GM28-GC24 |
| B36 | ✔ | | | GM24 | GC1 *** | GM24-GC1 |
| B37 | ✔ | | | GM26 | GC14 | GM26-GC14 |
| B38 | ✔ | | | GM27 | GC3 * | GM27-GC3 |
| B39 | ✔ | | | GM29 | GC9 * | GM29-GC9 |

∗ indicates that the genotypes were found in more than one isolate in one geographical focus.
∗∗ and ∗ ∗ ∗ indicate that the genotypes were found in more than one isolate in two and all three geographical foci, respectively.
The underlined GM-GC genotypes (GM17-GC2 and GM17-GC2) are found in more than one isolate. 37 GM-GC genotypes were identified from 39 L. tropica isolates.

sandflies while they work in the orchards and this probably explains the fivefold higher CL incidence in Bam county compared to Kerman despite having a lower population. 71% and 14% of all CL cases have occurred in Bam and Kerman counties in Kerman province [18]. The Bam Kerman environmental differences may also have affected the *P. sergenti* strains and help explain the genetic variation in *L. tropica* strains from these foci of southeast Iran.

Previously, there had been movement of people between Bam and Kerman but following the devastating earthquake in Bam in 2003 and massive destruction of buildings (more than 70%), many people fled from Bam to live in neighbouring

cities and villages, particularly Kerman city [52, 53]. The cluster B group analysis found that the subcluster B1-a-2 comprised isolates from Bam county (which included most isolates from Bam county in the tree) but subcluster B1-a-1 consisted of isolates from both Kerman and Bam. This topology supports the notion of an influx of *L. tropica* strains and gene flow by the immigration of people from Bam to Kerman. Other studies have showed heterogeneity of *Leishmania* in limited geographical areas using kDNA as a high-resolution marker. Using a minisatellite probe and kDNA RFLP analysis for genomic fingerprinting, two clusters of *L. tropica* strains were found from Kfar Adunim village in the Judean desert in the Palestinian West Bank. One strain was inconsistent with other strains from the area, suggesting it was imported from another endemic focus [54]. Another study showed that a man made barrier wall has decreased human and Canidae movements (Canidae are reservoirs of *L. infantum*) and resulted in the divergence of two separate clusters of *L. infantum* isolates in two geographical regions either side of the wall [31].

Salvatore *et al.* (2016) studied the heterogeneity of *L. infantum* isolates obtained from dogs in seven regions in Northern Italy and showed five different kDNA RFLP patterns that were specific to geographical origin [55]. Similar findings have been reported from Portugal. Of 13 *L. infantum* genotypes obtained among 120 isolates by kDNA RFLP-RFLP, only three were found country wide while the other 10 genotypes could discriminate *L. infantum* population in northern focus from those in central and southern regions [56]. In Majorca island, *L. infantum* has less variability compared to isolates from mainland Spain and can be explained by its geographical isolation from the mainland resulting in an ecological niche with limited canine and human movement into Majorca [57].

Environmental differences between geographical regions may directly affect vector populations and their parasites due to probable parasite-vector coevolution. Kuhls *et al.* (2008) showed that sandfly vectors might play a role in sustaining genetic diversity of *Leishmania* [58] and other studies suggest sandfly vectors might play a role in selecting specific parasite strains at a regional level and, therefore, contribute to the genetic structure of *Leishmania* [59, 60]. The existence of two genetically divergent populations of *L. tropica* in southeast and southwest Iran may be due to specific strains of *P. sergenti* sandflies that have adapted to the specific climatic and environmental conditions in these two regions. Based on sequence analyses of mitochondrial genes, Moin-Vaziri *et al.* (2007) demonstrated three lineages of *P. sergenti* strains in Iran, which diverged according to geographical region into southwest, northwest-central-southeast, and northeast Iran [61]. Although these findings are consistent with divergence of Iranian southeast and southwest *L. tropica,* they are discordant with our *L. tropica* genetic data that show a divergence of the southeast *L. tropica* genotypes [61].

Compared to other countries and *Leishmania* species studies, the highest number of different *L. tropica* genotypes is found in southern Iran based on kDNA RFLP patterns [31, 34, 40, 55–57, 59, 60, 62] and the current study has confirmed higher *L. tropica* heterogeneity which previously was found in comparison to other species [42]. The number of genotypes obtained with kDNA PCR-RFLP depends on the *Leishmania* species, the type of digesting enzyme, and cutting sites. To the best of our knowledge, the only other *L. tropica* study that used kDNA PCR-RFLP was a study from northern Palestine; in that study, the genetic heterogeneity was less compared to our study [60]. Clonal propagation of an asexually reproducing organism is the main method for the proliferation of *Leishmania* but sexual recombination is also considered important for producing genetic diversity as evidenced by the identification of hybrid species like *L. major* and *L. arabica* in Saudi Arabia and *L. braziliensis* and *L. panamensis* in Nicaragua [63–65] and studies reporting evidences of recombination in *Leishmania* species in the New and Old Worlds [41, 66–70]. Ghatee *et al.* (2013) showed at least two different alleles with a 100 bp gap in one allele for ITS-rDNA in Iranian *L. tropica* isolates and proposed that sexual recombination caused this 100 bp DNA fragment deletion/insertion; this may also partly explain the considerable genetic variations found in our current study [43]. Evidences of existence of at least two alleles for ITS locus of *L. tropica* from some other endemic foci in the World have been reported by Schönian *et al.* (2001) and Mauricio *et al.* (2004) [42, 71].

## 5. Conclusion

Using kDNA PCR-RFLP we found considerable heterogeneity in *L. tropica* from southern Iran, 37 genotypes from 39 isolates, which may be part explained by sexual recombination that previously was hypothesized for Iranian *L. tropica*. A clear distinction between southeast and southwest and divergence with overlapping in the former were found in *L. tropica* populations in south Iran that may have been driven by the different geographical and climatic conditions between these regions. Increased human movement after the 2003 earthquake in Bam probably resulted in mixing of *L. tropica* strains in the southeastern foci. These results will be used for recognition and following up epidemiological changes as well as for control measures and also for further kDNA RFLP studies in other ACL foci in northeast and central regions of Iran to complete the *L. tropica* genetic picture in Iran.

## Data Availability

Access to these data will be considered by the author upon request.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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## References

[1] Z. Assimina, K. Charilaos, and B. Fotoula, "Leishmaniasis: an overlooked public health concern," *Health Science Journal*, vol. 2, no. 4, pp. 196–205, 2008.

[2] J. Alvar, I. D. Vélez, C. Bern et al., "Leishmaniasis worldwide and global estimates of its incidence," *PLoS ONE*, vol. 7, no. 5, Article ID e35671, 2012.

[3] M. Kassi, A. K. Afghan, R. Rehman, P. M. Kasi, and M. Kassi, "Marring leishmaniasis: the stigmatization and the impact of cutaneous leishmaniasis in Pakistan and Afghanistan," *PLOS Neglected Tropical Diseases*, vol. 2, no. 10, article e259, 2008.

[4] World Health Organization, "Leishmaniasis," 2016, http://www.who.int/mediacentre/factsheets/fs375/en/.

[5] A. Nadim and M. Faghih, "The epidemiology of cutaneous leishmaniasis in the Isfahan province of Iran: I. The reservoir II. The human disease," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 62, no. 4, pp. 534–542, 1968.

[6] A. Nadim and M. A. S. Rashti, *A brief review of the epidemiology of various types of leishmaniasis in Iran*, 1971.

[7] E. Javadian and A. Mesghali, "Studies on cutaneous leishmaniasis in Khuzestan, Iran. Part I. The leptomonad infection of sandflies," *Bulletin de la Societe de Pathologie Exotique*, vol. 67, no. 5, pp. 513–516, 1974.

[8] E. Javadian, A. Nadim, G. H. Tahvildare-Bidruni, and V. Assefi, "Epidemiology of cutaneous leishmaniasis in Iran: B. Khorassan. Part V: report on a focus of zoonotic cutaneous leishmaniasis in Esferayen," *Bulletin de la Societe de Pathologie Exotique et de ses Filiales*, vol. 69, no. 2, pp. 140–143, 1976.

[9] E. Javadian, M. Dehestani, and N. Nadim, "Confirmation of Tatera indica (Rodentia: Gerbilldae) as the main reservoir host of zoonotic cutaneous leishmaniasis in the west of Iran," *Iranian Journal of Public Health*, vol. 27, no. 1-2, pp. 55–60, 1998.

[10] M. Tashakori, S. Ajdary, A. Kariminia, F. Mahboudi, and M. H. Alimohammadian, "Characterization of Leishmania Species and L. major Strains in Different Endemic Areas of Cutaneous Leishmaniasis in Iran," *Iranian Biomedical Journal*, vol. 7, no. 2, pp. 43–50, 2003.

[11] Y. Rassi, E. Javadian, M. Jalali, M. Motazedian, and H. Vatndoost, "Investigation on Zoonotic Cutaneous Leishm-aniasis, Southern Iran," *Iranian Journal of Public Health*, vol. 33, no. 1, pp. 31–35, 2004.

[12] S. Alimoradi, H. Hajjaran, M. Mohebali, and F. Mansouri, "Molecular identification of Leishmania species isolated from human cutaneous leishmaniasis by RAPD-PCR," *Iranian Journal of Public Health*, vol. 38, no. 2, pp. 44–50, 2009.

[13] K. Azizi, F. Abedi, and M. D. Moemenbellah-Fard, "Identification and frequency distribution of Leishmania (L.) major infections in sand flies from a new endemic ZCL focus in southeast Iran," *Parasitology Research*, vol. 111, no. 4, pp. 1821–1826, 2012.

[14] Y. Rassi, A. Saghafipour, M. R. Abai, M. A. Oshaghi, M. Mohebali, and R. Mostafavi, "Determination of Leishmania parasite species of cutaneous leishmaniasis using PCR method in Central County, Qom Province," *Zahedan Journal of Research in Medical Sciences*, vol. 15, no. 12, pp. 13–16, 2013.

[15] M. A. Ghatee, I. Sharifi, K. Kuhls et al., "Heterogeneity of the internal transcribed spacer region in Leishmania tropica isolates from southern Iran," *Experimental Parasitology*, vol. 144, no. 1, pp. 44–51, 2014.

[16] S. Izadi, H. Mirhendi, N. Jalalizand et al., "Molecular epidemiological survey of cutaneous leishmaniasis in two highly endemic metropolises of iran, application of fta cards for dna extraction from giemsa-stained slides," *Jundishapur Journal of Microbiology*, vol. 9, no. 2, 2016.

[17] M. Karamian, K. Kuhls, M. Hemmati, and M. A. Ghatee, "Phylogenetic structure of Leishmania tropica in the new endemic focus Birjand in East Iran in comparison to other Iranian endemic regions," *Acta Tropica*, vol. 158, pp. 68–76, 2016.

[18] F. Sharifi, I. Sharifi, M. Zarean et al., "Spatial distribution and molecular identification of *Leishmania* species from endemic foci of South-Eastern Iran," *Iranian Journal of Parasitology*, vol. 7, no. 1, pp. 45–52, 2012.

[19] H. Hajjaran, M. Mohebali, S. Mamishi et al., "Molecular identification and polymorphism determination of cutaneous and visceral leishmaniasis agents isolated from human and animal Hosts in Iran," *BioMed Research International*, vol. 2013, Article ID 789326, 7 pages, 2013.

[20] I. Sharifi, M. R. Aflatoonian, A. R. Fekri et al., "A comprehensive review of cutaneous leishmaniasis in kerman province, southeastern iran- narrative review article," *Iranian Journal of Public Health*, vol. 44, no. 3, pp. 299–307, 2015.

[21] N. L. Sharma, V. K. Mahajan, N. Ranjan, G. K. Verma, A. K. Negi, and K. I. S. Mehta, "The sandflies of the Satluj river valley, Himachal Pradesh (India): Some possible vectors of the parasite causing human cutaneous and visceral leishmaniases in this endemic focus," *Journal of Vector Borne Diseases*, vol. 46, no. 2, pp. 136–140, 2009.

[22] M. Svobodova, J. Votypka, J. Peckova et al., "Distinct transmission cycles of Leishmania tropica in 2 adjacent foci, northern Israel," *Emerging Infectious Diseases*, vol. 12, no. 12, pp. 1860–1868, 2006.

[23] M. Svobodová, B. Alten, L. Zídková et al., "Cutaneous leishmaniasis caused by Leishmania infantum transmitted by Phlebotomus tobbi," *International Journal for Parasitology*, vol. 39, no. 2, pp. 251–256, 2009.

[24] A. L. Banuls, M. Hide, and M. Tibayrenc, "Molecular epidemiology and evolutionary genetics of Leishmania parasites," *International Journal for Parasitology*, vol. 29, no. 8, pp. 1137–1147, 1999.

[25] I. L. Mauricio, M. W. Gaunt, J. R. Stothard, and M. A. Miles, "Genetic typing and phylogeny of the Leishmania donovani complex by restriction analysis of PCR amplified gp63 intergenic regions," *Parasitology*, vol. 122, no. 4, pp. 393–403, 2001.

[26] G. E. Luyo-Acero, H. Uezato, M. Oshiro et al., "Sequence variation of the Cytochrome b gene of various human infecting members of the genus *Leishmania* and their phylogeny," *Parasitology*, vol. 128, no. 5, pp. 483–491, 2004.

[27] J. Fraga, A. M. Montalvo, S. De Doncker, J.-C. Dujardin, and G. Van der Auwera, "Phylogeny of Leishmania species based on the heat-shock protein 70 gene," *Infection, Genetics and Evolution*, vol. 10, no. 2, pp. 238–245, 2010.

[28] A. Mendoza-Leon, J. C. Havercroft, and D. C. Barker, "The RFLP analysis of the $\beta$-tubulin gene region in New World Leishmania," *Parasitology*, vol. 111, no. 1, pp. 1–9, 1995.

[29] K. Waki, S. Dutta, D. Ray et al., "Transmembrane molecules for phylogenetic analyses of pathogenic protists: Leishmania-specific informative sites in hydrophilic loops of trans-endoplasmic reticulum N-acetylglucosamine-1-phosphate transferase," *Eukaryotic Cell*, vol. 6, no. 2, pp. 198–210, 2007.

[30] J. Marfurt, A. Nasereddin, I. Niederwieser, C. L. Jaffe, H.-P. Beck, and I. Felger, "Identification and differentiation of *Leishmania* species in clinical samples by PCR amplification of the miniexon sequence and subsequent restriction fragment length polymorphism analysis," *Journal of Clinical Microbiology*, vol. 41, no. 7, pp. 3147–3153, 2003.

[31] A. Nasereddin, K. Azmi, C. L. Jaffe et al., "Kinetoplast DNA heterogeneity among *Leishmania infantum* strains in central Israel and Palestine," *Veterinary Parasitology*, vol. 161, no. 1-2, pp. 126–130, 2009.

[32] K. Kuhls, I. L. Mauricio, F. Pratlong, W. Presber, and G. Schönian, "Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex," *Microbes and Infection*, vol. 7, no. 11-12, pp. 1224–1234, 2005.

[33] S. Ochsenreither, K. Kuhls, M. Schaar, W. Presber, and G. Schönian, "Multilocus microsatellite typing as a new tool for discrimination of Leishmania infantum MON-1 strains," *Journal of Clinical Microbiology*, vol. 44, no. 2, pp. 495–503, 2006.

[34] D. P. Alonso, D. L. Costa, I. L. De Mendonça, C. H. N. Costa, and P. E. M. Ribolla, "Short report: heterogeneity of *Leishmania infantum chagasi* kinetoplast DNA in Teresina (Brazil)," *The American Journal of Tropical Medicine and Hygiene*, vol. 82, no. 5, pp. 819–821, 2010.

[35] J. Shlomai, "The structure and replication of kinetoplast DNA," *Current Molecular Medicine*, vol. 4, no. 6, pp. 623–647, 2004.

[36] S. Brewster and D. C. Barker, "Analysis of minicircle classes in Leishmania (Viannia) species," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 96, no. 1, pp. 55–63, 2002.

[37] H. A. Noyes, H. Reyburn, J. W. Bailey, and D. Smith, "A nested-PCR-based schizodeme method for identifying *Leishmania* kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of *Leishmania tropica* in Pakistan," *Journal of Clinical Microbiology*, vol. 36, no. 10, pp. 2877–2881, 1998.

[38] F. Pratlong, J. A. Rioux, J. Dereure et al., "Leishmania tropica in Morocco. IV—Intrafocal enzyme diversity," *Annales de Parasitologie Humaine Et Comparee*, vol. 66, no. 3, pp. 100–104, 1991.

[39] F. Pratlong, J. Dereure, C. Ravel et al., "Geographical distribution and epidemiological features of Old World cutaneous leishmaniasis foci, based on the isoenzyme analysis of 1048 strains," *Tropical Medicine & International Health*, vol. 14, no. 9, pp. 1071–1085, 2009.

[40] Y. Botilde, T. Laurent, W. Q. Tintaya et al., "Comparison of molecular markers for strain typing of *Leishmania infantum*," *Infection, Genetics and Evolution*, vol. 6, no. 6, pp. 440–446, 2006.

[41] N. Chargui, A. Amro, N. Haouas et al., "Population structure of Tunisian Leishmania infantum and evidence for the existence of hybrids and gene flow between genetically different populations," *International Journal for Parasitology*, vol. 39, no. 7, pp. 801–811, 2009.

[42] G. Schönian, L. Schnur, M. El Fari et al., "Genetic heterogeneity in the species Leishmania tropica revealed by different PCR-based methods," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 95, no. 2, pp. 217–224, 2001.

[43] M. A. Ghatee, I. Sharifi, A. A. Haghdoost et al., "Spatial correlations of population and ecological factors with distribution of visceral leishmaniasis cases in southwestern Iran," *Journal of Vector Borne Diseases*, vol. 50, no. 3, pp. 179–187, 2013.

[44] F. Rohlf, *Numerical taxonomy and analysis system (NTSYSpc) version 2.0*, Department of Ecology and Evolution Sate University of New York, New York, NY, USA, 1998.

[45] G. A. Heshmati, "Vegetation characteristics of four ecological zones of Iran," *International Journal of plant production*, vol. 1, no. 2, pp. 215–224, 2012.

[46] M. A. Ghatee, A. A. Haghdoost, F. Kooreshnia et al., "Role of environmental, climatic risk factors and livestock animals on the occurrence of cutaneous leishmaniasis in newly emerging focus in Iran," *Journal of Infection and Public Health*, vol. 18, pp. 425–433, 2018.

[47] M. Fakhar, H. Pazoki Ghohe, S. A. Rasooli et al., "Genetic diversity of Leishmania tropica strains isolated from clinical forms of cutaneous leishmaniasis in rural districts of Herat province, Western Afghanistan, based on ITS1-rDNA," *Infection, Genetics and Evolution*, vol. 41, pp. 120–127, 2016.

[48] M. Fakhar, M. Karamian, M. A. Ghatee, W. R. Taylor, H. Pazoki Ghohe, and S. A. Rasooli, "Distribution pattern of anthroponotic cutaneous leishmaniasis caused by Leishmania tropica in Western Afghanistan during 2013-2014," *Acta Tropica*, vol. 176, pp. 22–28, 2017.

[49] B. Sarkari, T. Naraki, M. A. Ghatee, S. A. Khabisi, and M. H. Davami, "Visceral leishmaniasis in Southwestern Iran: a retrospective clinico-hematological analysis of 380 consecutive hospitalized cases (1999-2014)," *PLoS ONE*, vol. 11, no. 3, Article ID e0150406, 2016.

[50] M. A. Ghatee, A. A. Haghdoost, F. Kooreshnia et al., "Role of environmental, climatic risk factors and livestock animals on the occurrence of cutaneous leishmaniasis in newly emerging focus in Iran," *Journal of Infection and Public Health*, 2017.

[51] K. Tierney, B. Khazai, L. T. Tobin, and F. Krimgold, "Social and public policy issues following the 2003 Bam, Iran, earthquake," *Earthquake Spectra*, vol. 21, no. 1, pp. S513–S534, 2005.

[52] F. Nadim, M. Moghtaderi-Zadeh, C. Lindholm et al., "The Bam earthquake of 26 December 2003," *Bulletin of Earthquake Engineering*, vol. 2, no. 2, pp. 119–153, 2004.

[53] I. Sharifi, N. Nakhaei, M. R. Aflatoonian et al., "Cutaneous leishmaniasis in bam: A comparative evaluation of pre- and post- earthquake years (1999-2008)," *Iranian Journal of Public Health*, vol. 40, no. 2, pp. 49–56, 2011.

[54] L. F. Schnur, A. Nasereddin, C. L. Eisenberger et al., "Multifarious characterization of Leishmania tropica from a Judean desert focus, exposing intraspecific diversity and incriminating Phlebotomus sergenti as its vector," *The American Journal of Tropical Medicine and Hygiene*, vol. 70, no. 4, pp. 364–372, 2004.

[55] D. Salvatore, A. Di Francesco, G. Poglayen et al., "Molecular characterization of leishmania infantum strains by kinetoplast DNA RFLP-PCR," *Veterinaria Italiana*, vol. 52, no. 1, pp. 71–75, 2016.

[56] S. Cortes, I. Mauricio, A. Almeida et al., "Application of kDNA as a molecular marker to analyse Leishmania infantum diversity in Portugal," *Parasitology International*, vol. 55, no. 4, pp. 277–283, 2006.

[57] C. Chicharro, M. A. Morales, T. Serra, M. Ares, A. Salas, and J. Alvar, "Molecular epidemiology of Leishmania infantum on the island of Majorca: A comparison of phenotypic and genotypic tools," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 96, no. 1, pp. 93–99, 2002.

[58] K. Kuhls, C. Chicharro, C. Canavate et al., "Differentiation and gene flow among European populations of Leishmania infantum MON-1," *PLOS Neglected Tropical Diseases*, vol. 2, no. 7, article no. e261, 2008.

[59] P. E. M. Ribolla, L. T. Gushi, M. D. S. Pires e Cruz et al., "Leishmania infantum Genetic Diversity and Lutzomyia longipalpis Mitochondrial Haplotypes in Brazil," *BioMed Research International*, vol. 2016, Article ID 9249217, 11 pages, 2016.

[60] K. Azmi, L. Schnur, G. Schonian et al., "Genetic, serological and biochemical characterization of Leishmania tropica from foci in northern Palestine and discovery of zymodeme MON-307," *Parasites & Vectors*, vol. 5, no. 1, p. 1, 2012.

[61] V. Moin-Vaziri, J. Depaquit, M.-R. Yaghoobi-Ershadi et al., "Intraspecific variation within Phlebotomus sergenti (Diptera: Psychodidae) based on mtDNA sequences in Islamic Republic of Iran," *Acta Tropica*, vol. 102, no. 1, pp. 29–37, 2007.

[62] S. Cortes, C. Chicharro, I. Cruz, J. M. Cristovão, C. Cañavate, and L. Campino, "Genetic diversity of human zoonotic leishmaniasis in Iberian Peninsula," *Zoonoses and Public Health*, vol. 58, no. 4, pp. 234–237, 2011.

[63] D. A. Evans, W. P. Kennedy, S. Elbihari, C. J. Chapman, V. Smith, and W. Peters, "Hybrid formation within the genus Leishmania?" *Parassitologia*, vol. 29, no. 2-3, pp. 165–173, 1986.

[64] J. M. Kelly, J. M. Law, C. J. Chapman, V. E. Guillaume J.J.M, and D. A. Evans, "Evidence of genetic recombination in Leishmania," *Molecular and Biochemical Parasitology*, vol. 46, no. 2, pp. 253–263, 1991.

[65] A. A. Belli, M. A. Miles, and J. M. Kelly, "A putative Leishmania panamensis/Leishmania braziliensis hybrid is a causative agent of human cutaneous leishmaniasis in Nicaragua," *Parasitology*, vol. 109, no. 4, pp. 435–442, 1994.

[66] M. B. Rogers, T. Downing, B. A. Smith et al., "Genomic Confirmation of Hybridisation and Recent Inbreeding in a Vector-Isolated Leishmania Population," *PLoS Genetics*, vol. 10, no. 1, Article ID e1004092, 2014.

[67] C. Ravel, S. Cortes, F. Pratlong, F. Morio, J.-P. Dedet, and L. Campino, "First report of genetic hybrids between two very divergent Leishmania species: Leishmania infantum and Leishmania major," *International Journal for Parasitology*, vol. 36, no. 13, pp. 1383–1388, 2006.

[68] D. Nolder, N. Roncal, C. R. Davies, A. Llanos-Cuentas, and M. A. Miles, "Multiple hybrid genotypes of Leishmania (Viannia) in a focus of mucocutaneous leishmaniasis," *The American Journal of Tropical Medicine and Hygiene*, vol. 76, no. 3, pp. 573–578, 2007.

[69] S. Odiwuor, S. De Doncker, I. Maes, J.-C. Dujardin, and G. Van der Auwera, "Natural Leishmania donovani/Leishmania aethiopica hybrids identified from Ethiopia," *Infection, Genetics and Evolution*, vol. 11, no. 8, pp. 2113–2118, 2011.

[70] N. S. Akopyants, N. Kimblin, N. Secundino et al., "Demonstration of genetic exchange during cyclical development of Leishmania in the sand fly vector," *Science*, vol. 324, no. 5924, pp. 265–268, 2009.

[71] I. L. Mauricio, J. R. Stothard, and M. A. Miles, "Leishmania donovani complex: Genotyping with the ribosomal internal transcribed spacer and the mini-exon," *Parasitology*, vol. 128, no. 3, pp. 263–267, 2004.