

## Original Article

# Genetic Polymorphism of *Leishmania* major in Two Hyper Endemic Regions of Iran Revealed by PPIP-PCR and ITS- RFLP

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## Abstract

**Background:** Zoonotic cutaneous leishmaniasis (ZCL) is a polymorphic disease that may show various clinical manifestations. Although it is suggested that the genetic variability of the parasite is one of the factors influencing clinical manifestations of leishmaniasis, no data exists regarding genetic polymorphism of *Leishmania major* (*L.major*). This study investigates the determination of genetic variations within the species of *L.major* isolates from different cases of ZCL in two hyper-endemic areas of Iran.

**Methods:** A variety of nucleic acid detection methods that target both DNA and RNA have been developed. Among these, the polymerase chain reaction (PCR) method proved to be a highly sensitive and specific technique. Species identification was based on permissively primed intergenic polymorphic-polymerase chain reaction (PPIP-PCR) and restriction fragment length polymorphism analysis of amplified internal transcribed spacer (ITS-RFLP) in the ribosomal operon of *L.major* from clinically different forms of ZCL. The DNA products were amplified by PCR, followed by digestion of the PCR product with restriction enzymes. The profiles were visualized in agarose gel under ultraviolet (UV) light.

**Results:** The PCR product obtained for all isolates was about 1060 bp in size. Different patterns of PPIP-PCR and ITS-RFLP in the ribosomal operon were classified as I, II, III, IV, and V. This classification was according to the number and localization of bands. Results of this research detected the genetic and clinical polymorphism of *L. major*, and showed that strain A was more frequent than other strains.

**Conclusion:** The *L.major* causing ZCL in Isfahan, Iran is genetically a highly polymorphic species and PPIP-PCR exposed more genetic polymorphism among clinical samples in Isfahan, Iran.

**Keywords:** Internal transcribed spacer, *Leishmania major*, PPIP-PCR, RFLP

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## Introduction

**L**eishmaniasis is endemic in 88 countries, with an estimated annual rate of 1 – 1.5 million cases of cutaneous leishmaniasis (CL).<sup>1</sup> About 90% of CL cases occur in seven countries, including Iran.<sup>2</sup> In Iran there are at least two species of old world *leishmania*, which are responsible for self-healing ulcerative disease and occasionally metastatic CL. Zoonotic CL (ZCL) is endemic throughout Iran.<sup>2,3</sup>

A variety of nucleic acid detection methods targeting both DNA and RNA have been developed. Polymerase chain reaction (PCR) has been proven to be a highly sensitive and specific technique. In the present study, we used restriction fragment length polymorphism analysis of amplified internal transcribed spacer (ITS-RFLP) in the ribosomal operon and permissively primed intergenic polymorphic-polymerase chain reaction (PPIP-PCR) to investigate the genetic variations among *Leishmania major* (*L.major*) isolates from Isfahan, Iran. Then, findings were correlated with the clinical manifestations of ZCL in Isfahan, Iran, where ZCL is a major public health problem.

## Materials and Methods

### Study area and population

Of 600 patients with suspected CL who referred to the Research Center for Skin Disease and Leishmaniasis in Isfahan, Iran (SEDIGHE-TAHERE), 440 were positive. Of 67 patients from Ahwaz (H EFDEH-SHERIVER Health Center) 44 were positive (Table1). Study approved by Ethics Committee, patients signed informed consents.

This study selected 120 cases at random, aged between 1 to 50 years (average age: 9 years) with clinically suspected ZCL lesions and a disease history of 2 to 4 weeks, unless otherwise stated. They were referred for diagnosis to the Research Center for Skin Disease and Leishmaniasis in Isfahan, Iran during a period of 6 months from September 2005 to March 2006, the period when ZCL cases are more common in this area. All patients were residents of Isfahan, Iran and Ahwaz, Iran. They had neither previous records of infection nor medication, except for lupoid forms of CL, and had no apparent differences in nutritional status or in their general states of health. We excluded patients who did not meet these criteria from the study.

### Parasite samples

Diagnosis of leishmaniasis was confirmed by the demonstration of amastigotes in slit smears and/or the presence of flagellated promastigotes in a Novy-McNeal-Nicolle (NNN) medium from the skin lesions of 120 patients with typical and atypical lesions. For parasitological investigation, three skin samples

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**Table 1.**The clinical features of different forms of Zoonotic Cutaneous Leishmaniasis present in Isfahan & Ahwaz, Iran.

| Type of ZCL  | Main characteristics   | Location                             | Number of patients | %    | Duration (weeks) | Size (cm <sup>2</sup> ) | Genotype |
|--|--|--------------------------------------|--------------------|------|------------------|-------------------------|----------|
| <b>Volcano-shape</b>                                   | Raised turgid margins central serous crust, resembles a flattened volcano.                                 | Hands, face, feet, neck, trunk, etc. | 448                | 92.6 | 3–5              | 1–4                     | A        |
| <b>Lupoid form</b>                                     | Acute lesion with persistent red, occasionally pale appearance with brown color.                           | Face                                 | 3                  | 0.7  | 1–3 years        | 2–10                    | D        |
| <b>Wheal form</b>                                      | Unulcerated wheal, smooth and elevated above the surrounding skin, Resembles a wheal.                      | Hands                                | 2                  | 0.4  | 2–4              | 4–12                    | B        |
| <b>Psoriasis form</b>                                  | Superficial with raised patches of skin covered with silvery scales.                                       | Hands, face                          | 3                  | 0.6  | 3–4              | 3–20                    | B1       |
| <b>Pian-bois-like (Plain, boil-like) leishmaniasis</b> | Widely scattered multiple ulcers   | Hands, face, feet, neck, trunk       | 2                  | 0.4  | 2–4              | 1–7                     | B2       |
| <b>Hyperkeratotic form</b>                             | Erythematous papulonodules and plaque, resembles psoriasis, covered with dry, horny build dead skin cells. | Shoulders, limbs                     | 4                  | 0.8  | 5–7              | 1–2                     | A2, A, G |
| <b>Pyoderma gangrenosum</b>                            | Margin always flat, may start with a blister. Develops rapidly after a few days.                           | Hands, feet                          | 6                  | 1.3  | 4–5              | 5–15                    | A1       |
| <b>Ecthyma form</b>                                    | Hard serous crust, which exudes pus on pressure or removing crust. Margins not firm on palpation.          | Limbs                                | 8                  | 1.6  | 3–4              | 5–25                    | A2       |
| <b>Erysipelas form</b>                                 | An erythematous, indurated, slightly squamous, plaque with sharply defined borders                         | Elbows, hands                        | 2                  | 0.4  | 4–7              | 3–6                     | G        |
| <b>Patch tiny papularform</b>                          | A cutaneous ulcer and nodules extended proximally from the ulcer. Some crusting and slight oozing.         | Face                                 | 2                  | 0.4  | 3–4              | 2–3                     | A, B2    |
| <b>Nodular form</b>                                    | Firm-elevated erythematous papules (with exudative surface).   | Limbs                                | 4                  | 0.8  | 21–32            | 1–2                     | B2       |

**Table 2.** Result of PPIP-PCR band Profiles of samples isolated from Two-hyperendemic area of cutaneous leishmaniasis.

| Profile banding | A <sub>1</sub> | A <sub>2</sub> | B    | B <sub>1</sub> | B <sub>2</sub> | C    | D   | G    |
|-----------------|----------------|----------------|------|----------------|----------------|------|-----|------|
| 515             | 515            | 515            | 515  | 515            | 515            | 515  | 515 | 515  |
| 400             | 400            | 400            | 400  | 400            | 400            | 400  | —   | —    |
| 310             | ----           | ----           | 310  | 310            | ----           | ---- | 310 | ---- |
| 220             | 220            | 220            | ---- | ----           | ----           | 260  | 220 | ---- |
| 180             | 180            | 180            | ---- | ----           | ----           | 220  | 180 | 470  |
| —               | —              | —              | —    | —              | —              | 180  | —   | 220  |
| —               | —              | —              | —    | —              | —              | —    | —   | 180  |

With heavy band; A1 and B2 with three heavy bands and the others with 4 heavy bands. : — ; No band : ----

were collected from the edge of the lesions using sterile surgery blades. Two were used for direct smear and stained with giemsa and the third sample was inoculated into sterile screw top tubes that contained blood agar slanted NNN medium and incubated at 25 ± 1°C.

The isolated promastigotes were subcultured in RPMI-1640 medium supplemented with 15% fetal bovine serum. They were washed twice by re-suspension in buffered saline and centrifuged. Pellets were stored frozen at -70°C until further processing.

In summary, parasites from a 15 mL mid-logarithmic phase of bulk culture were harvested by centrifugation (700 × g for 20 min at 4°C) and washed three times in ice-cold sterile PBS (pH 7.2).

#### DNA preparation

For species identification, the DNA isolated from promastigotes in culture was extracted using a commercial extraction kit (High

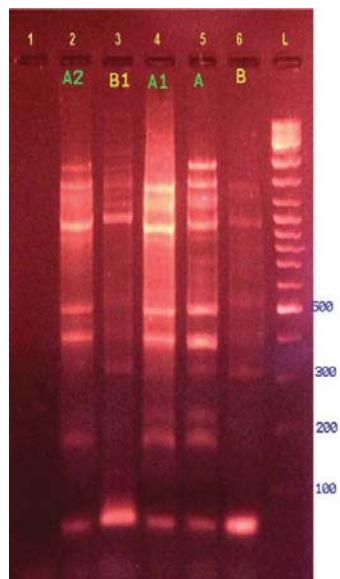
Pure Template DNA Preparation Kit, Roche, Germany) according to the instructions of the manufacturer. Identification of species was based on PPIP-PCR.

#### PPIP-PCR procedure

PCR amplification was performed in a 50 μL volume with specific leishmania primer (2B) and nonspecific primer (2A).<sup>4</sup> The DNA was amplified in 50 mL of PCR that consisted of 1.25 units taq DNA polymerase, 2.5 mL of PCR buffer, 200 mL of each deoxyribonucleotide, and 10 – 20 ng of DNA. Amplification products were separated in a 1% agar gel and visualized under ultraviolet light after staining with ethidium bromide. *L.major* (MHOM/IR/15/ER) and *L.tropica* (MHOM/SU/79/K27)<sup>3</sup> were used as positive controls. Distilled water instead of the DNA template was used as the negative control.

**Table 3.** Genetic variability with restriction enzymes in ITS-rDNA

| Enzyme        | Profile banding I | Profile banding II | Profile banding III | Profile banding VI | Profile banding V | consideration   |
|---------------|-------------------|--------------------|---------------------|--------------------|-------------------|---|
| <b>Alu1</b>   | 500               | 700                | 500                 | ---                | ---               | Profile banding II is seen in mix infection                       |
|               | 300               | 500                | 300                 | ---                | ---               |   |
|               | 200               | 400                | 200                 | ---                | ---               |   |
|               | 60                | 200                | 100                 | ---                | ---               |   |
|               | ---               | 60                 | ---                 | ---                | ---               |   |
| <b>Dde1</b>   | 480               | 480                | 600                 | ---                | ---               | Profile banding of group in c band profile II Coordinate PCR-PPIP |
|               | 340               | 340                | 480                 | 480                | ---               |   |
|               | 210               | 210                | 340                 | 340                | ---               |   |
|               | 80                | 150                | 220                 | 210                | ---               |   |
|               | ---               | 80                 | ----                | 60                 | ---               |   |
| <b>Taq1</b>   | 440               | 440                | 440                 | 440                | 700               | ---   |
|               | 300               | 300                | 300                 | 300                | 440               |   |
|               | 280               | 280                | 280                 | 280                | 300               |   |
|               | 100               | ---                | 100                 | 180                | 280               |   |
|               | 50                | 50                 | ---                 | 50                 | 180               |   |
| <b>HaeIII</b> | 500               | 500                | ---                 | ---                | ---               | ---   |
|               | 320               | 320                | ---                 | ---                | ---               |   |
|               | 280               | 280                | ---                 | ---                | ---               |   |
|               | 70                | ---                | ---                 | ---                | ---               |   |
|               | ---               | ---                | ---                 | ---                | 50                |   |
| <b>Hinf1</b>  | 500               | 500                | ---                 | ---                | ---               | ---   |
|               | 80                | ---                | ---                 | ---                | ---               |   |
| <b>Rsa1</b>   | 650               | 1300               | ---                 | ---                | ---               | ---   |
|               | 180               | 700                | ---                 | ---                | ---               |   |
|               | 80                | ---                | ---                 | ---                | ---               |   |
| <b>Scrf1</b>  | 1060              | 900                | 900                 | 1200               | ---               | Profile banding VI is seen in mix infection                       |
|               | ---               | 100                | 150                 | 900                | ---               |   |
|               | ---               | ---                | 100                 | 80                 | ---               |   |

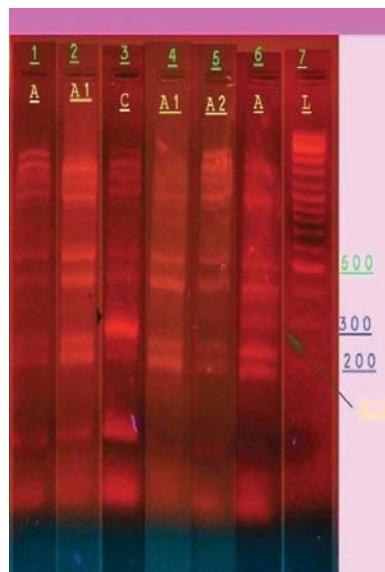


**Figure 1.** PCR profiles of different strains of *Leishmania major* from human cases of cutaneous leishmaniasis obtained with primer LITSV and LITSR.

Profile bandings of permissively primed intergenic polymorphic of *leishmania major* directly from clinical samples.

Lane 1 negative control and 5 standard profile of *L. major*. Lane 2-4 ,6 *L. major* from clinical samples of patients.

Lane 7. 1 kb ladder are indicated, Isfahan, Iran.L3&6 without 220 and 180bp bands.L2,4, and 5 show 180 and 220 bp molecular weight bands. B and B1 show another profile bandings (table 1).



**Figure 2.**PCR profiles of different strains of *Leishmania major* from human cases of cutaneous leishmaniasis obtained with primer LITSV and LITSR.

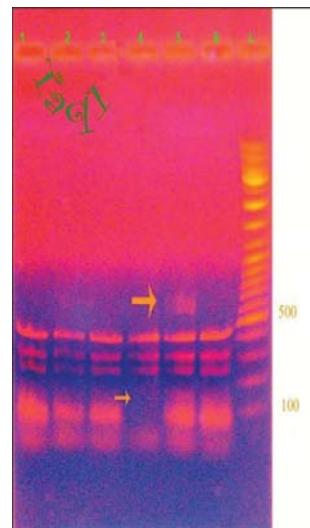
Profile bandings of permissively primed intergenic polymorphic of *leishmania major* directly from clinical samples.

Lane 1- 6 *L.major* from clinical samples. Lane 7. 1 kb ladder are indicated , Isfahan, Iran.L1 &L6 show profile band A,with 310bp band. L2 with three high molecular weight bands. , profile band of C was like profile band of A but it has 261bp band instead of 310bp.

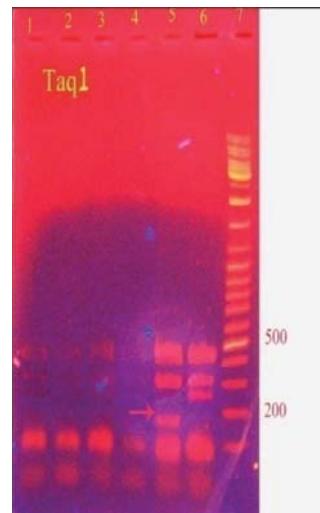


**Figure 3.** Restriction of amplified ITS regions of different of *L.major* isolated from human cases of cutaneous leishmaniasis.

Profile bandings of ITS-rDNA digested with restriction enzyme taq1. ITS-PCR amplification products of *L major* isolated from different forms of ZCL. Lane 7: 1 kb ladder DNA size marker. Lane 1 reference strain (positive control). Lanes 2-6 *L.major* isolated from different forms of ZCL with the enzyme taq1. Restricted with this enzyme show that *L.major* has special site for restricted enzyme taq1 but some are different.



**Figure 4.** Restriction of amplified ITS regions of different of *L.major* isolated from human cases of cutaneous leishmaniasis. (Profile bandings of ITS-rDNA digested with restriction enzyme taq1. ITS-PCR amplification products of *L major* isolated from different forms of ZCL. Lane 7: 1 kb ladder DNA size marker. Lane 1 reference strain (positive control). Lanes 2 - 6 *L.major* isolated from different forms of ZCL with the enzyme taq1. L4 and L5 have deletion and addition band).



**Figure 5.** Restriction of amplified ITS regions of different of *L.major* isolated from human cases of cutaneous leishmaniasis.

Profile bandings of ITS-rDNA digested with restriction enzyme taq1. ITS-PCR amplification products of *L major* isolated from different forms of ZCL. Lane 7: 1 kb ladder DNA size marker. Lane 1 reference strain (positive control). Lanes 2 to 6 *L.major* isolated from different forms of ZCL with the enzyme taq1. L5 show that is different , with addition band.

#### Internal analysis and RFLP-PCR procedure

PCR was used to amplify the ITS-rDNA region with the primers LITSV (5-ACACTCAGGTCTGTAAAC-3) and LITSR (5-CTGGATCATTTCCGATG-3).<sup>5,6</sup>

The PCR products that contained the amplified regions were digested with seven different restriction enzymes (Taq1, Hae III, Rsa1, Scrf1, Alu1, Dde1, and Hinf1). The ITS sequence was amplified from all isolates and reference strains were digested separately with restriction enzymes for 2 hr, according to the manufacturer's instructions.

Amplification and restriction products were separated in a 1% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. Fragment sizes were estimated in comparison with bands of DNA molecular weight markers (Hyper ladder I; 1 kb DNA ladder; Bioline, Biology Laboratory Supplies and Reagents, UK). Throughout the study, genomic DNA from reference strain *L.major* was used as the positive control and distilled water as the negative control.

Digestion of the internal transcribed spacer from ribosomal DNA with seven enzymes enables identification of five different patterns. PPIP-PCR showed nine distinct banding profiles (Figure 1) and ITS-rDNA showed five different schizodemes (Figure 2).

To identify *L. major* by PPIP-PCR, major bands were 510 bp (500 – 553 bp) molecular weight as essential band and in majority 310 bp molecular weight, *L.tropica* with two major bands 470 and 340 bp (310 – 370 bp) molecular weight, and some minor bands.

#### Results

Restriction length polymorphism analysis was performed on the entire ITS region in the ribosomal operon of 120 isolates of *L.major*. One reference strain was amplified using PCR primers, which specifically amplify *Leishmania* ITS, located between the small subunit RNA gene and the large subunit RNA gene and included the 5.8 RNA gene sequences.

The PCR products obtained for all isolates and the reference strain were approximately 1060 bp in size for the entire ITS amplicon, which was indicative of *Leishmania* (with the exception of two isolates). Amplification products were not observed when distilled water was used instead of template DNA as the negative controls.

The resulting pattern of bands depended on both the restriction enzyme chosen and the type of isolates. Using each enzyme, the patterns of the bands was classified into five groups according to the number and the localization of the bands. This classification also corresponded to the shape of skin lesions from which promastigotes were isolated (data not shown).

Comparing the patterns with those of the reference DNAs, the PPIP-PCR assay also identified all analyzed samples as *L. major*. All had 515 bp, and the majority contained 310 bp polymorphic bands (except for two cases that probably had mixed infections). PPIP-PCR showed that *L.tropica* separated from *L.major* with 470 and 340 bp molecular weight bands. However, the PCR product profiles separated into nine distinct types and subtypes according to the number and the localization of band. *Leishmania major* banding profiles classifies as II, III, IV, V groups and I.

Table 2 shows different profiles of PPIP-PCR.

Each of these groups separated into one, two, or three subtypes. Profile *Lm* I, separated into three subtypes: A, A1, and A2. Profile *Lm* II had three subtypes: B, B1, and B2. Profile *Lm* III (C), IV (D), and V (G) each had one subtype.

Profile A consisted of 515 bp, 400 bp, and 310 bp major bands (strong), with 220 bp and 180 bp as light molecular weight bands, and four heavily molecular weight bands. The frequency of this profile band was more than the others (Figures 1 and 2).

Digestion of the PCR products of ITS-rDNA from 120 samples with seven restriction enzymes produced different patterns of banding profiles which were dependent on the endonuclease restriction enzyme.

Digestion of ITS from ribosomal DNA with seven enzymes (*Alu*1, *Hae*III, *Hinf*1, *Dde*1, *Taq*1, *Rsa*1, and *Scrf*1) enabled us to identify five different patterns. However, using each enzyme, the patterns of the bands were classified into five groups according to the number and localization of the bands. Table 3 shows genetic variation with the restriction enzymes.

By *Alu*1, *Taq*1, *Hae*III, *Rsa*1, and *Scrf*1 restriction enzymes, we can digest ITS-rDNA, but *Scrf*1 in mix infection of *L.major* and *L.tropica*, as ordinary revealed high molecule weight. *Scrf*1 could not digest ITS-rDNA, except two cases, that cut *L.major* ITS-rDNA which revealed light molecular weight. Restriction analysis of the amplified ITS ribosomal DNA revealed intra-specific variations (Figures 3, 4, and 5), but not as many as the PPIP-PCR method.

Thus, PPIP-PCR showed nine distinct banding profiles and ITS-rDNA showed five different schizodemes. Thus, PPIP-PCR Showed 9 distinct banding profiles and ITS-rDNA-RLFP showed five different schizodeme. In some cases, two procedures confirmed similarity.

In this study, profile banding A separated from the reference strain in reaction to the restriction enzyme. Strain A is the predominant strain in Isfahan, Iran. DNA of strain A was extracted with a High Pure PCR Template Preparation Kit. After DNA colonization in the ptz57R vector plasmid, the ligation reaction was transformed in *E.coli* XL1blue and Hb101 as competent cells. After extraction of the plasmid and confirmation of DNA, it was then sent for sequencing. It was confirmed that strain A differed from the reference strain.

## Discussion

Since ZCL is the most important endemic disease in two hyper-endemic areas of Iran, determination of *L.major* genetic variations seems necessary for designing appropriate control programs.

The PPIP-PCR technique and the ITS-RFLP method have revealed species-specific fragment patterns that can be used to identify *Leishmania* strains at the species level.<sup>5,6</sup> The PCR approaches used in this study detected a comparably high degree of genetic variability among the different strains of the species *L.major*.

Thus, PPIP-PCR Showed 9 distinct banding profiles and ITS-rDNA-RLFP showed five different schizodeme (table 3). The results of this research detected the genetic polymorphism of *L.major*, and showed that strain A was more frequent than the other strains.

In accordance with the present findings, considerable

heterogeneity has been reported within the ITS-RFLP of strains of *L.tropica* and *L.aethiopica*,<sup>5,6</sup> and other new world species.<sup>7</sup>

In another study in Iran, five genotypes of ITS1-ITS2 were identified from Kashan, Tehran, Desful, Dehloran, and Damghan.<sup>8</sup> In accordance with the present study, identical results were obtained using the *taq*1 enzyme.

PPIP-PCR showed more strains than ITS-rDNA-RFLP. This may be explained by the fact that sequence variation may go undetected by RFLP analysis, since the restriction enzymes survey only a subset of the total variable sites.<sup>8</sup> However, little attention has been paid to their capacity to accurately resolve sequence variation. For example, analysis of DNA fragments produced by PCR-RFLP and random amplified polymorphic DNA (RAPD) relies on the separation of fragments by agarose gel or polyacrylamide gel electrophoresis. Fragments of the same size, but differing in sequences, will co-migrate within one band in the gel and may be misinterpreted as the same sequence.

In similar study, the PPIP-PCR assay identified *L.major* as three distinct types, LmA and two subtypes (LmA1 and LmA2) in the Middle East.<sup>9</sup>

To our knowledge, this work is the first application of PPIP-PCR for genetic variation within *L.major* in Iran, and the results are summarized as follows:

i) all isolates have been identified as *L.major* by ITS-RFLP and PPIP-PCR with the exception of two mixed infections and ii) the best advantage of PPIP-PCR is the simple interpretation of results from PCR-products in gel agarose. These products enable us to discriminate among different intra-species variations and similarities. Profile bands of subtype A are similar to MHOM/IL/80/Friedlin, except that it has 220 and 180 bp bands instead of a 240 bp band. Profile band of subtype C is similar to MHOM/IL/86/Blum, but there is a 261 bp band instead of a 310 bp band (Table 4).

Different evolutionary mechanisms such as migration, selection, and genetic drift play a fundamental role in the genetic distribution in natural populations, but reproduction is the basic biological process that influences the population's genetic structure.<sup>10</sup>

Many authors have reported evidence of hybrid formation in *Leishmania*,<sup>11,12</sup> reinforcing the idea that sexual reproduction may occur in *Leishmania*, but at a level yet undefined. Obviously, occasional or rare bouts of sexual recombination in normally asexual organisms can have a profound effect on the extent of genetic diversity.

In conclusion, the results of this study show that *L.major* ZCL in Isfahan is genetically a highly polymorphic species. To consolidate these findings, additional *L.major* isolates from patients, reservoir hosts, and vectors from different locations need to be collected for further investigation.

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Jahan Nama Garden (Bagh-e Jahan Nama), Shiraz, Iran, built by Karim Khan Zand, the founder of the Zand Dynasty. He ordered construction of a monument (kooshk) in the central part of the garden in 1752 C.E. (Photo by M.H. Azizi MD, 2012)