Bionomics and Phylo-Molecular Analysis of *Leishmania* Species Isolated from Human Lesions Using ITS1 Genes in North-east of Iran

Running title: Bionomics and Phylo-Molecular Analysis of *Leishmania* Species

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Abstract

Backgrounds: Leishmaniasis is a zoonotic infectious disease caused by *Leishmania* species. The identification of parasite species and the type of disease is beneficial for treatment and preventive modalities. *Leishmania tropica* and *L. major* have been reported as the main etiological agents of cutaneous leishmaniasis (CL) in Iran.

Objectives: The incidence of zoonotic CL has increased and is different in distinct loci of Iran. Hence, we perused the *Leishmania* species and its genetic traits in the North East of Iran.

Materials and Methods: The investigation was conducted on 200 positive smears prepared from patients' lesions suffering from CL referred to the health care centers of northeastern provinces in Iran from 2013 to 2017. The obtained positive microscopy samples were divided to score the ranges from +1 to +6, of them 40 smears exhibited low-parasitemia. *Leishmania* species analyzed using PCR-RFLP, genetic diversity indices evaluation, phylogenetic analysis, and sequencing comparison with other species in the GeneBank based on ITS1 gene.

Results: The isolated *L. major* strains were similar to other Iranian isolates in this region. pairwise fixation index (F_{ST}) index was statistically significant in different *L. major* populations and showed the genetic differences in pairwise population of different geographical locations of Iran.

Conclusions: This is the first phylo-molecular study in this region which has used different methods to identify different parasite species. The current study confirmed an old pattern endemicity of zoonotic CL in North-east of Iran. Therefore, in order to assess the hybrid formation, more epidemiological, ecological, and gene polymorphism studies are needed to understand the pathogenic role of *Leishmania* species in Iran.

Keywords: *Leishmania*, Phylogenetic, genetic diversity, Sequencing, Iran

1. Background

Leishmaniasis refers to a group of vector-borne parasitic diseases caused by *Leishmania* species. These diseases are transmitted between mammalian reservoir hosts through the bites of female sand flies (1). In the world, about 350 million people are at risk of these diseases and more than 1.5 million new cases are reported annually causing 30,000 deaths (2). Leishmaniasis is associated with hygiene poverty and environmental conditions (urbanization, malnutrition, poor housing, etc.). It has been broadly reported in tropical and subtropical areas, including Iran (3).

Clinical forms of leishmaniasis are typically related to parasite characteristics, vector biology, and the hosts' immune responses. Consequently, different species of *Leishmania* cause various clinical manifestations ranging from self-curing cutaneous lesions to life-threatening visceral forms of the disease (4). Cutaneous Leishmaniasis (CL) is one of the 17 neglected tropical diseases as classified by the World Health Organization (WHO) (5). It is primarily a disease of low-income countries propagated and maintained by a complex lifecycle among *Leishmania* species, sandfly vectors, and mammalian hosts (5). CL is the most common clinical form of leishmaniasis and is endemic in different parts of Iran. *Leishmania major*, *L. tropica*, and *L. infantum* (rare cases) are the etiological agents of CL in Iran (6). In addition, the prevalence and incidence of the disease are considerable, and more than 30,000 new cases occur annually in different parts of the country (7). Zoonotic CL (ZCL) and Anthroponotic CL (ACL) are endemic in rural areas and some important cities of Iran, respectively. Mammals play an important role as the maintenance and transmission hosts of the parasite (8, 9).

Various specific and sensitive polymerase chain reaction (PCR) detections methods have been used to confirm the presence of *Leishmania* species in CL lesions. Internal Transcribed Spacer 1 (ITS1), notable individual ribosomal-DNA genes located between 5.8S and 18S coding regions, have been effectively used for characterization of *Leishmania* species. Despite the surrounding coding regions,

this gene does not undergo translation and is capable of identifying intra-species diversity (10, 11). kDNA consists of thousands of circular DNA transcripts (minicircle), each of which includes both conserved and variable regions. These regions are targeted in molecular and differential diagnosis (12).

2. Objectives:

Recently, the prevalence and incidence of ZCL have increased in different provinces of Iran, including North Khorasan. Hence, this investigation endeavored us to recognize *Leishmania* species by PCR assay based on ITS1, restriction fragment length polymorphism (RFLP), genetic diversity indices evaluation, phylogenetic analysis, and sequencing among the archived positive slides of CL patients referred to healthcare the centers of North Khorasan province, northeastern Iran from 2013 to 2017.

3. Materials and Methods:

3.1. Ethical statement

The experimental protocols were approved by the Ethics Committee of Maragheh University of Medical Sciences (No. IR.MARAGHEHPHC.REC.1397.006). The patients' names, personal information, illnesses, and medical information were kept confidential.

3.2. Study area

Bojnurd, Esfaraen, Garmeh, and Jajarm are the main cities in North Khorasan province located in the northeast of Iran with a variety of weather conditions from mostly moderate to warm with mountainous or plain conditions. This province is located among Golestan, Khorasan Razavi, and Semnan provinces, which are endemic provinces for CL in Iran (Figure 1).

3.3. Sample collection and Microscopic assays

In this study, 200 positive archived skin impression smears were studied. These smears were prepared from the lesions of suspected CL patients referred to healthcare centers of North Khorasan from 2014-2018. The slides were previously checked for leishman bodies and were reported as positive by microscopic examination. Afterward, they were again checked for *Leishmania* detections by microscopy. In all, 40 slides with low-parasitemia were selected for molecular assays. Smear samples prepared from the patients were previously fixed using methanol and stained with 5% Giemsa. The slides were then checked for detection of *Leishmania* via microscopy for at least 40 minutes.

3.4. PCR assay

3.4.1. DNA Extractions

The scraped smears of the glass slides were extracted by kDNA extraction kit (Takapouzist, Iran DynaBio®) procedure. Briefly, the samples were transferred to micro-tubes. Then, 20 µL proteinase K and 200 µL lysis buffer were added to the samples, mixed, and incubated at 60°C for 15 min to be

completely lysed. Afterwards 200 µL absolute ethanol was added to the samples and mixed by pluse-vortexing for 30 s. The mixtures containing some precipitates were carefully transferred to column micro-tubes, centrifuged at 8000 rpm for one min, and washed several times using buffers to remove impurities from the column micro-tubes. Finally, 100-200 µL of elution buffer or ddH2O was added to the membrane center of the column tubes and kept for three min. The tubes were centrifuged at 14000 rpm for two min to elute the DNA and were stored at -20°C for the PCR amplification (13).

3.4.2. PCR procedure & RFLP for ITS-rDNA gene detection

The extracted DNAs from the lesion smears were used for detection of different species of *Leishmania* differences in ITS1 gene in the samples. For each PCR reaction, the total volume of 25-µl reaction mixture contained five µl DNA sample, 12 µl master mix buffer [Cat No. A180301, Ampliqon taq DNA polymerase master mix red (containing 1.5 mM $MgCl_2$ and 2× concentration of taq DNA polymerase)], 1 µl of each primer of LITSV (5-ACACTCAGGTCTGTAAAC-3) and LITSR (5-CTGGATCATTTTCCGATG-3) (concentrations of 10 pico mol), and 6 µl of double distilled water. The samples were transferred to the thermocycler device (Eppendorf Master-cycler, Germany) for PCR amplification as follows: one cycle of initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 60 s, extension at 72°C for 90 s, and one cycle of final extension at 72°C for 7 min. Electrophoresis of samples were carried out using five µl of amplified PCR products (12). For the PCR-RFLP analysis, in order to detect the *Leishmania* species, the products of PCR (ITS-1 positive samples) were digested using restriction enzyme (RE) HaeIII (BUSRI) and the related buffer at 37ºC for two hours. Analyses of the RE digested products were performed on 1.5% agarose gel in TAE buffer. Subsequently, the agarose gel seprarated products were compared with the reference strains of *Leishmania*. For comparison purpose the 140 and 340 bp fragments were amplified from *L. major* strain, and 25, 38, 57, and 360 bp fragments from *L. tropica* detection (14).

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132 3.5. Sequencing assay

133 The random PCR products of *Leishmania* isolated from the CL patients were selected for sequencing
134 and the genomic homology studies. The relevant bands were extracted from the agarose gels using
135 QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany). In order to extract the bands, the gel
136 sections were transferred into 1.5 ml microfuge tubes. To each tube contaning gel slices, 750 µl of
137 sodium iodide was added and were placed in a 65°C incubator for 10 min for melting the gel slices
138 completely. The samples were loaded into the columns placed in 1.5 ml tubes and the tubes were
139 centrifuged at 14000 rpm for one min and the flow-throughs were discarded. To wash the DNAs on
140 the column, 750 µl washing buffer was added to the columns and placed in the new collection tubes
141 and centrifuged and the supernatants were discarded. The washing processes were repeated by using
142 350 µl washing buffer to the columns and transferring to the new collection tubes. To elute the DNAs,
143 50 µl distilled water was added to the columns that were placed in 1.5 ml sterile microtubes. The
144 tubes were centrifuged at 14000 rpm for one min to elute the DNA (13). The sequences of the samples
145 were obtained by sequencing the DNA products from both the directions through the sequencing
146 services of Macrogen Genomic Laboratories (Macrogen, Seoul, South Korea). The parasite species
147 were identified based on the homology with ITS1 gene sequences from *Leishmania* reference strains
148 deposited in the GenBank.

149 3.6. Genetic diversity indices, phylogenetic and sequences analyses

150 The chromatograms and raw nucleotide sequences of both reverse and forward directions were
151 analyzed using the Chromas 2.2 program. The nucleotides sequences were aligned and analyzed by
152 MUSCLE, and compared with the homologous sequences using the BLAST in the GenBank
153 database. The sequences were edited and assembled with the BioEdit 7.2.6 to identify Single
154 Nucleotide Polymorphisms (SNPs). Alignments were compared to the data related to *Leishmania*

155 species from Iran and other countries deposited in the GenBank database. Genetic distances were
156 calculated via the Maximum Composite Likelihood model using MEGA-7.
157 The number of segregating sites, diversity indices (Haplotype diversity: Hd and nucleotide diversity:
158 p) and neutrality values (Tajima's D and Fu's Fs tests) were calculated by DnaSP software version
159 5.10 (15). The degree of gene flow (gene migration) among the populations was evaluated using a
160 pairwise fixation index (F_{ST}). The haplotype network inferred by the common identified haplotypes
161 of ITS1 sequences from different location of Iran was constructed by PopART software and median-
162 joining algorithm (16).

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4. Results

4.1. Microscopic findings

In total, 200 slides of the patients from four different cities (Bojnurd, Esfaraen, Garmeh, and Jajarm) were examined for detection of *Leishmania*. All slides were reported as positive with different amounts of leishman bodies seen under light microscopes. As there were no reliable differential criteria between *Leishmania* species in the microscopic method, the positive slides of the 40 patients with low amounts of leishman bodies were randomly selected for the molecular method and were finally confirmed using PCR method.

4.2. Genetic diversity indices and sequencing findings

The resulting sequences of *Leishmania* species isolated from the patients from North Khorasan (Bojnurd) were aligned and compared with the *Leishmania* sequences existing in the GenBank. The nucleotide sequences were deposited in GenBank under the following accession number MT012484-MT012491. The presence of *L. major* detected by ITS1 gene analyses was confirmed by the achieved sequences with the GenBank database. Furthermore, the *L. major* strains isolated in the current study were compared with the similar Iranian isolates obtained from Esfahan, Birjand, Ilam & Kermanshah, Golestan and Khorasan provinces closer to the this study location or other geographical loci of Iran (Table 1). Haplotype diversity in North Khorasan (Bojnurd) samples is 0.875. In addition, by surveying of the samples in GeneBank, the most haplotype diversity (Hd: 0.866) with identified haplotypes (n: 9) belonged to Center of Iran (Esfahan and Kashan). Neutrality indices of the ITS1 gene in North Khorasan (Bojnurd) samples and other populations is not significant except Ilam, Kermanshah (Tajima's D: -2.33646, Fu's Fs: -3.27692) which indicated significant divergence from neutrality (Table 1).

4.3. Phylogeny

All the characters were equally weighted and alignment gaps were treated as missing data. The bootstrap analyses were conducted using 1000 replicates. F_{ST} values between various populations of *L. major* were calculated by Dnasp5 software package with the nucleotide data set of ITS1 gene. This index was statistically significant in different *L. major* populations and showed the genetic differences in pairwise population (Figure1). The nucleotide sequences for each haplotype were. The statistical parsimony network was drawn to differentiate a genealogical correlation among the common haplotypes that displayed a distinct geographical haplogroups from center north East of Iran (Figure 2). Phylogenetic analyses based on ITS1 sequence data were conducted by ML with *L. tropica* designated as outgroup is shown in Figure 3.

5. Discussion

Leishmaniasis is one of the major health problems in North Khorasan province in northeast of Iran. This was the first phylogenetic-based study in the field of leishmaniasis conducted in this region. Iran is one of the top ten countries with a high prevalence of CL (13, 17). *L. major* is the major etiological agent of ZCL and an extensive variety of creatures, especially rodents, have been distinguished as reservoirs (18). On the other hand, *L. tropica* is a well-known etiological agent of ACL in Asian countries (19). In a similar study from south of Iran, the sequences of *L. major* isolated from four patients showed 100% similarity to the Iranian isolate of AB678349. The *L. major* isolates showed 99% and 98% similarity to the published isolates from UK (AF308685) and Iran (KM555295), respectively.

This phylo-molecular study allows us to infer the genetic diversity and population structure of *L. major* in north east of Iran in comparison to population from neighboring provinces and areas farther than this area.

The fastidious information on the genetic structure of *L. major* metapopulations epitomizes the example of parasite sharing in consequence of outcome of natural modifications, vector and host mobility and imported cases from neighboring provinces.

In this study, the genetic migration differences (F_{ST}) in samples of North Khorasan province compared to Golestan province was zero (F_{ST} :0). This suggests that the origin of this species is the same in those regions and that there is a similar genetic pattern. In that case the population of Golestan and North Khorasan come together in a single set in the median-joining algorithm network haplotype (Figure 2). While the genetic differences between Birjand in South Khorasan province is 0.15455 (Figure 1). It is reported that by gene migration, genetic diversity of parasites can happen and expands the effective population size in a variety of geographical regions (20). These

differences between different loci are probably occurrence of bottleneck events, inter-trans regional of *L. major*, ecological alterations, vector and reservoir behaviors.

Notably, ITS-rDNA sequence analysis for species determination of *Leishmania* have been found to be highly conserved and stable. It should also be noted that the high evolutionary rate of ITS sequence (which even shows variability between the species of a single genus) leads to the high efficacy of PCR in detecting a variety of *leishmania* species. Despite the greater sensitivity of kDNA and ITS-rDNA in diagnosing infections, the high reliability of RFLP-PCR on the ITS-kDNA gene has been introduced as the most appropriate tool for highly effective determination of different *Leishmania* species (27-29). The existence of negative values of Tajima's D in Ilam, Kermanshah population implies evidence of some mechanisms including slippage and unequal crossing over/transposition, selective sweep hypothesis, the model of neutral mutation, population size equilibrium, genetic drift, purifying selection, and negative selection. But the neutrality tests in east population was not significant

Advanced molecular methods are needed due to the unreliability of the determination of *Leishmania* species based on microscopic assays in low-parasitemia smear samples (6). Considering the outcomes in microscopic and molecular assays, PCR has shown a remarkably high acceptance (21).

ITS-rDNA has been extensively used in molecular investigations. However, kDNA has been well-characterized as a useful tool for molecular diagnostics and species detections (22). Indeed, nested-PCR method has been previously used as a useful method for detection of positive samples (23). ITS-rDNA and kDNA detections were useful for identification of *L. major* in sandflies of Iran (11). Mirzaie et al. (2013) reported *Leishmania* species detection by RFLP-PCR targeting ITS with LITSR and SL58 primers. Moreover, three haplotypes of *L. major*, a similar haplotype of *L. tropica*, and two haplotypes of *L. major* were detected in the rodents of Esfahan and Fars provinces using

microsatellite genes, ITS-rDNA, nested-PCR, and sequencing methods (24, 25). Moreover, Parvizi and Ready (2008) designed ITS-rDNA and kDNA with the ability to identify the genetic strains of *L. major* in sandflies of Iran by amplification and sequencing (11).

In an attempt to determine CL species from the DNAs of the CL smears taken from patients by RFLP-PCR, *L. tropica* and *L. major* were detected in 20 and 27 samples, respectively (26). Based on the comparison with the sequences recorded in the global gene bank, the samples isolated from CL patients in Zahedan were shown to be *L. major* and *L. tropica*. Sharbatkhori et al. (2014) also reported consistent results in an attempt to identify *Leishmania* by using microscopic and molecular methods (ITS-rDNA sequencing, semi-nested PCR that amplified minicircle kDNAs) in the patients suspected for Leishmaniasis in northern cities of Iran (27).

North Khorasan province in northeast of Iran is one of the most important loci of zoonotic CL. Molecular epidemiologic evidence in the cities of this region has revealed the *L. major* as the most dominant parasite species. Identification of *Leishmania* species helps find appropriate prevention strategies. Besides molecular analysis in endemic areas of Iran, we also aimed at examination of genetic variations in the country.

6. Conclusion

This is the first phylo-molecular study in this region using the described methods. The current study revealed that *L. major* is the main causative agent of CL cases in many regions of North Khorasan, especially in rural areas. However, *L. tropica* is limited to the central areas of the province. The results of the study also indicated that the PCR method was beneficial especially in low-parasitemia cases, suspicious cases and species detection. Environmental factors, such as increased urbanization, population growth, and movements toward the vector reservoirs, pathogens of leishmaniasis, have changed the epidemiological pattern of CL during the last decades, which could complicate the strategy to control the aspects of CL.

Therefore, more epidemiological, ecological, and gene polymorphism (in order to assess hybrid formation) studies are needed to understand the pathogenic role of *Leishmania* species in Iran.

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Figure legends:

Fig. 1. Map of Iran showing the locations of study with F_{ST} values between various populations of *L. major* calculated by Dnasp5 based on ITS1 gene.

Fig. 2. The haplotype network of *L. major* constructed based on median-joining algorithm from various geographical foci of Iran.

Fig. 3. Phylogenic analyses based on ITS1 sequence conducted by maximum likelihood using MEGA7. Bootstrap analyses were conducted using 1000 replicates. *Leishmania tropica* was used as the outgroup.

Table 1

Diversity and neutrality indices of *L. major* from different geographical foci of Iran inferred ITS1 gene.

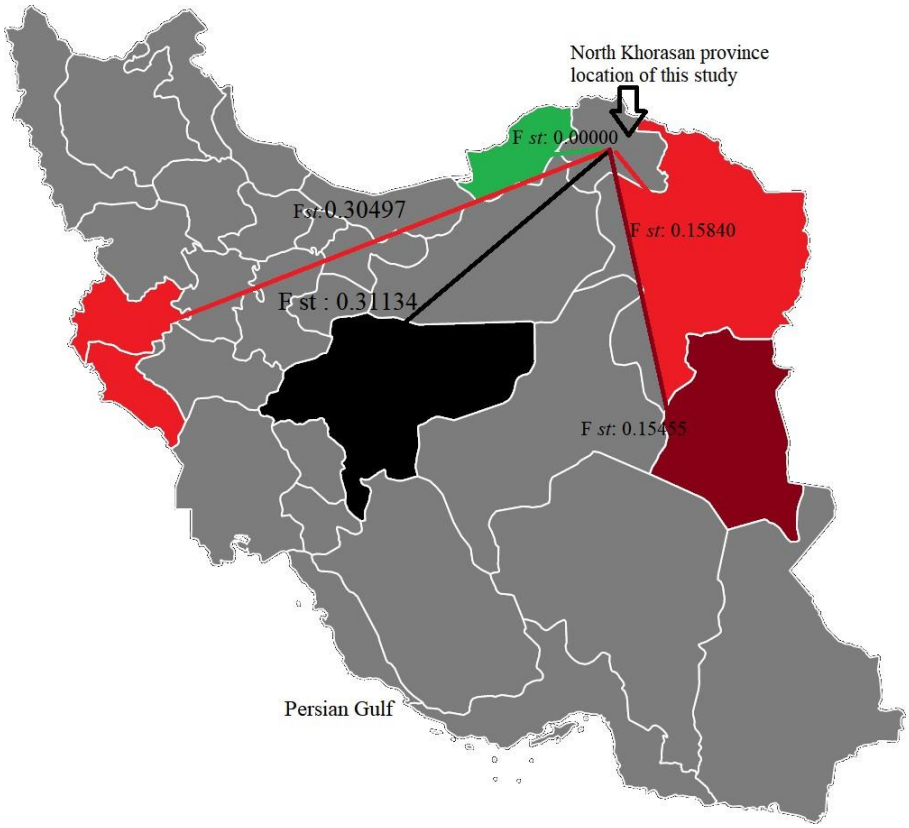
North Khorasan province
location of this study
F st: 0.00000
Fst:0.30497
F st: 0.15840
F st : 0.31134
F st: 0.15445
Persian Gulf

Figure 1.

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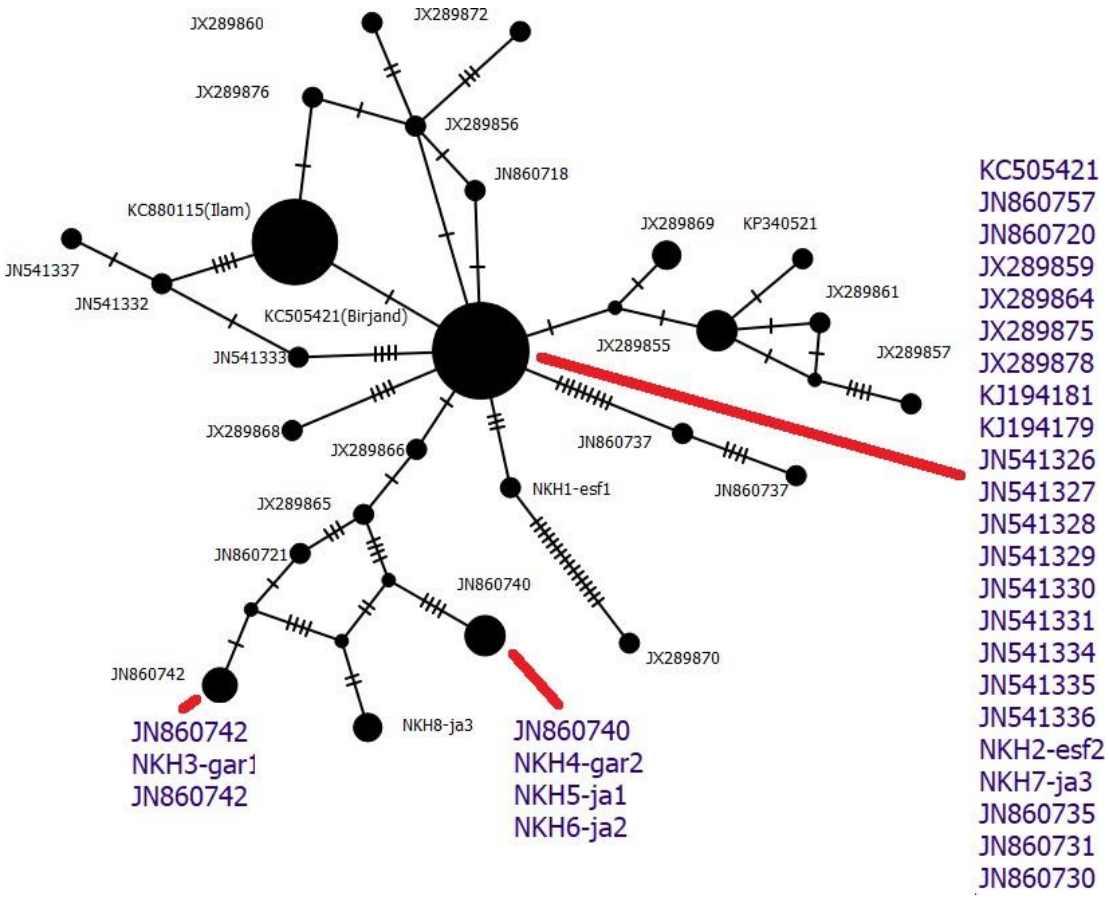

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428 **Figure 2**

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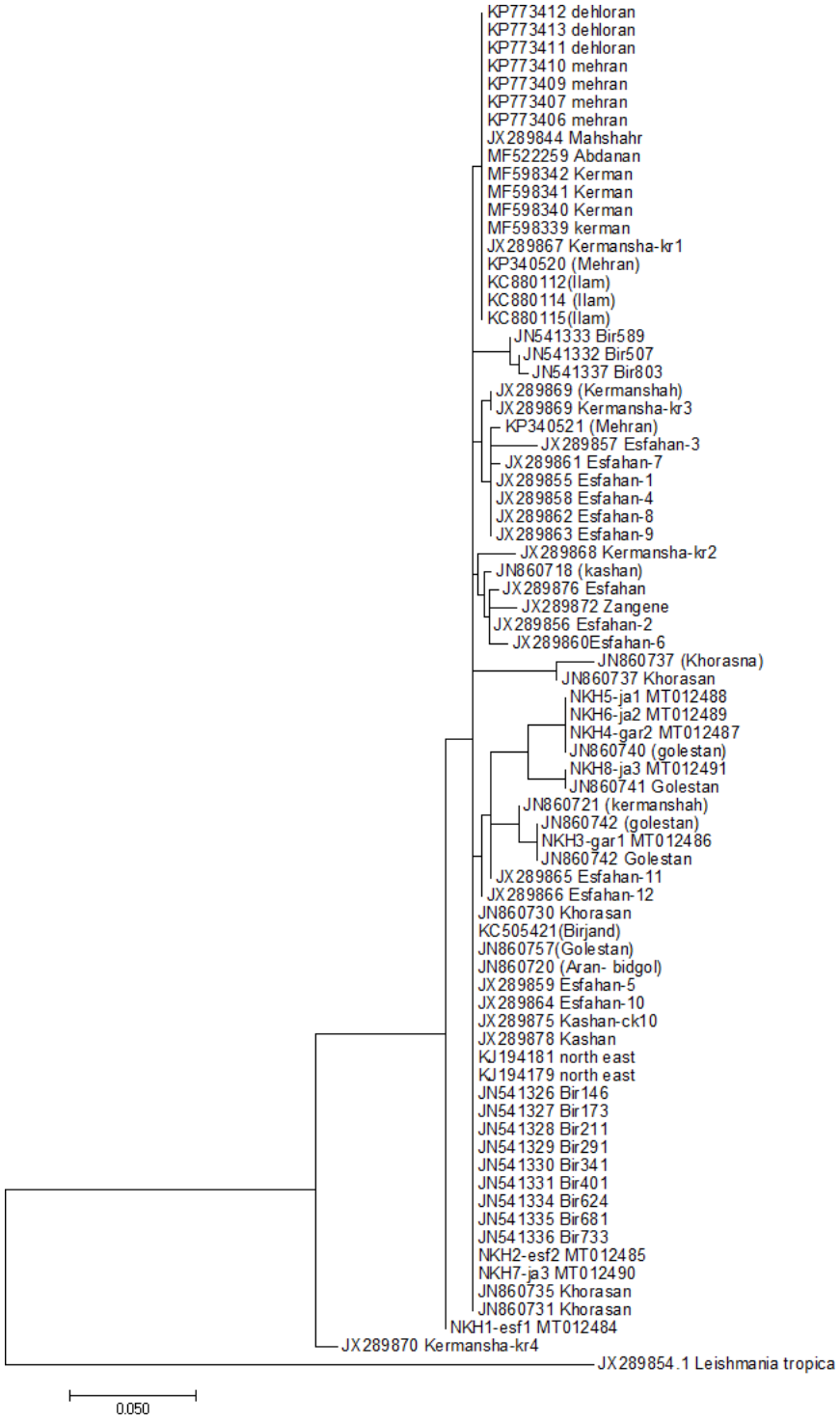

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Figure 3

Table 1

Population	Diversity indices					Neutrality Tests	
	Locations	**N**	**Nh**	**Hd ± SD**	**Nd (π)**	**Tajima's D**	**Fu and Li's D**
L. major	Esfahan	16	9	0.866± 0.066	0.00927	-1.10882	-1.31231
	Birjand	12	3	0.318 ± 0.031	0.00498	-0.61652	0.56268
	Ilam,Kermanshah	20	7	0.521± 0.141	0.001368	-2.33646*	-3.27692*
	Golestan	5	4	0.9± 0.164	0.02816	0.11455	0.11455
	Khorasan	7	3	0.523± 0.180	0.01960	0.12638	0.55991
	Bojnurd	8	5	0.857± 0.200	0.02888	0.47624	0.25974