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Original Article

Genetic Diversity and Phylogenetic Study of *Leishmania* Species in Iran by Multilocus Sequence Typing

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Abstract

Background: Leishmaniasis is an important public health parasitic infection, which is endemic in many parts of the world, including Iran. We aimed to investigate genetic diversity and phylogenetic relationship among different *Leishmania* isolates using multilocus sequence typing (MLST).

Methods: Totally, 41 isolates collected either from patients referred to Leishmaniasis Diagnostics and Treatment Center at Tehran University of Medical Sciences, Tehran, Iran or from animals during 2019-2021, were subjected to the study. They included *L. major* and *L. tropica* from human, *L. infantum* from canine, and *L. turanica* from rodents from different endemic foci of Iran analyzed using MLST including *gp63, g6pdh, lack, nagt,* and *hsp70* genes.

Results: A total of 5010 bps was analyzed from each isolate. The three targets, *nagt*, *lack*, and *g6pdh*, generated better topology comparing to the other genes. In the 44 isolates, 22 haplotypes (STs) were identified. *Leishmania tropica* contained the highest number of haplotypes (n=12) comparing to *L. major* (n=8), *L. infantum* (n=1) and *L. turanica* (n=1). All five genomic loci caused separation of Iranian *Leishmania* species at the species level, indicating conservation of these genes in the *Leishmania* parasite.

Conclusion: The highest number of haplotypes belonged to *L. tropica*, indicating that the genetic diversity of this species is higher than that of *L. major*. It was further confirmed that the MLST is a suitable method to examine genetic variation of *Leishmania* parasites with respect to evolutionary and epidemiological studies.

Introduction

Various species of *Leishmania* cause a spectrum of clinical features in human, i.e. cutaneous leishmaniasis (CL), muco-cutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL). According to the WHO estimation, 600,000 to 1 million new cases of CL and 50,000 to 90,000 new cases of VL with tens of thousands of deaths occur worldwide annually. (https://www.who.int/news-room/fact-sheets/detail/leishmaniasis).

CL is endemic in more than 90 countries and is the most common clinical form of leishmaniasis (1,2). The old-world CL is either anthroponotic caused by *L. tropica*, or zoonotic caused by *L. major* distributed in the Middle East and central Asia (3). CL accounts for almost 20,000 new cases per year in Iran (4). VL is reported sporadically in most parts of Iran and is endemic in northwestern and southern areas (5, 6) with about 100–300 new cases annually (7). The causative agents of human leishmaniasis in Iran include *L. major* and *L. tropica* for CL and *L. infantum* for VL; they are responsible for a wide spectrum of clinical manifestations in human and are of epidemiological importance (7, 8).

Several molecular assays have been described for *Leishmania* typing and/or phylogenetic characterization, based on the specific PCR, DNA probes, and sequences of various genetic markers including RNA polymerase II (9), 7SL RNA, ribosomal internal transcribed spacer (10-12), the N-acetylglucosamine-1-phosphate transferase gene (13), mitochondrial cytochrome b (14) and heat shock protein 70 genes (15). Multilocus enzyme electrophoresis (MLEE) has been the gold standard for species identification of *Leishmania* for many years, but mass cultures of parasites, time-consuming, and poor discriminatory power are drawbacks of this approach (16, 17). Multilocus sequence typing (MLST) has recently been considered as an alternative to the MLEE technique. This provides data on the genetic variations of housekeeping genes and applying the MLST strategy led to analysis of genetic diversity of the whole genus on a global scale (18-20).

The target genes selected in the current study, consisted of heat shock protein 70 (*hsp70*), N-acetyl glucosamine phosphotransferase (*nagt*), glycoprotein 63 (*gp63*), glucose-6-phosphate dehydrogenase (*g6pdh*), and *Leishmania* homolog of receptors for activated protein kinase C (*lack*). They are housekeeping genes and are involved in metabolic pathways in *Leishmania* parasite. A reason, we selected these five genes, was that they encode enzymes considered in MLEE analysis for identifying polymorphisms that are capable for characterizing *Leishmania* spp. zymodemes most prevalent in Iran. Developing new techniques that would provide useful data in accordance with existing MLEE data, and decreasing the drawbacks of the MLEE laborious technique, could be of great values.

The present study was designed to genetically analyze by MLST a variety of leishmanial isolates from different spices (*L. major, L. tropica, L. infantum, L. turanica*) in order to improve the knowledge of evolutionary history and the taxonomic status of *Leishmania* spices from different endemic region of Iran. We compared species typing results from all polymorphic sites of sequences found in these five markers that are applied directly on the samples.

Materials & Methods

Ethics Approval

All procedures performed in this study were in accordance with the ethical standards with reference number ZUMS.REC.1394.176, released by Ethical Review Committee of Zanjan University of Medical Sciences, Zanjan. Iran.

Sample collection and DNA isolation

Samples were collected from patients referred to Leishmaniasis Diagnostics and Treatment Center at Tehran University of Medical Sciences, Tehran, Iran during 2019-2021, and from other animal hosts (canine and

rodents). From 44 samples, 33 isolates belonged to CL and 7 isolates were from VL cases (2 human and 5 canine samples); 4 CL specimens were collected from rodents (*Rubombis opimus*) as reservoirs (Table 1). The samples were microscopically confirmed and cultured in RPMI 1640, supplemented with 5% fetal bovine serum (FBS) and 200 IU/ml penicillin-streptomycin, as well as NNN (Novy-MacNeal Nicolle) media. Genomic DNA was isolated from parasite cultures using the QIAamp DNA mini kit (QIAGEN) following the manufacturer's instructions.

Table 1: The original *Leishmania* isolates from different districts of Iran used in the multilocus sequence typing

No.	*Strain ID*	*Species*	*Origin*	*Host*	*Disease*
1	C1	*L. tropica*	Bam	Human	CL
2	C2	*L. tropica*	Bam	Human	CL
3	C3	*L. major*	Esfahana	Human	CL
4	C4	*L. major*	Ardestan	Human	CL
5	C5	*L. major*	Mashhad	Human	CL
6	C6	*L. major*	Sistan va Baluchestan	Human	CL
7	C7	*L. major*	Shiraz	Human	CL
8	C8	*L. tropica*	Afghanistan	Human	CL
9	C9	*L. tropica*	Mashhad	Human	CL
10	C10	*L. major*	Esfahana	Human	CL
11	C11	*L. major*	Semnan	Human	CL
12	C12	*L. major*	Shiraz	Human	CL
13	C13	*L. major*	Dehloran	Human	CL
14	C14	*L. major*	Ilam	Human	CL
15	C15	*L. major*	Ilam	Human	CL
16	C16	*L. major*	Shiraz	Human	CL
17	C17	*L. tropica*	Shiraz	Human	CL
18	C18	*L. major*	Bandar_Abbas	Human	CL
19	C19	*L. major*	Golestan	Rodent	CL
20	C20	*L. turanica*	Golestan	Rodent	CL
21	C21	*L. major*	Agha Aliabas	Human	CL
22	C22	*L. major*	Kermanshah	Human	CL
23	C23	*L. tropica*	Esfahan	Human	CL
24	C24	*L. tropica*	Shiraz	Human	CL
25	C25	*L. tropica*	Afghanistan	Human	CL
26	C26	*L. tropica*	Zahedan	Human	CL
27	C27	*L. tropica*	Afghanistan	Human	CL
28	C28	*L. tropica*	Mashhad	Human	CL
29	C29	*L. tropica*	Mashhad	Human	CL
30	C30	*L. tropica*	Shiraz	Human	CL
31	C31	*L. infantum*	Meshkin Shahr	Canine	VL
32	C32	*L. infantum*	Meshkin Shahr	Canine	VL
33	C33	*L. infantum*	Meshkin Shahr	Canine	VL
34	C34	*L. tropica*	Tehran	Human	VL, CL
35	C35	*L. tropica*	Ardebil	Human	CL, CL
36	C36	*L. infantum*	Meshkin shahr	Canine	VL
37	C37	*L. infantum*	Meshkin shahr	Canine	VL
38	C38	*L. major*	Bandar jask	Human	CL
39	C39	*L. major*	Golestan	Rodent	CL
40	C40	*L. major*	Golestan	Rodent	CL
41	C41	*L. tropica*	Mashhad	Human	CL
42	C42	*L. tropica*	Mashhad	Human	CL
43	C43	*L. tropica*	Bam	Human	CL
44	C44	*L. tropica*	Bam	Human	CL

CL: cutaneous leishmaniasis; VL: visceral leishmaniasis

MLST

The MLST approach was based on the analysis of five loci coding for single-copy housekeeping genes, which are shown in Table 2. These genes exhibited the discriminatory power among *Leishmania* isolates according to previous studies in other areas (21-26).

Table 2: Oligonucleotide primers used for multilocus sequence typing of the *Leishmania* isolates

Target genes	Primer name	Primer Sequence (5' to 3')	Tm	Product size (bp)	Reference	Location of genome
G6PDH	G6PDH G6PDH-R	ATGTCGGAAGAGCAGTCT TCACAGCTTATTCGAGGGAA	55	1540	Zhang et al 2013 (27)	Chromosome 34
HSP 70	HSP70-F HSP70- R	GATTGTTCTCGTTGGTGGTTCG GCATTTGCTTGATATGGTGCTG	61	1420	Fraga et al 2010 (45)	Chromosome 28
Nagt	Nagt- F Nagt- R	TCATGACTC TTG GCC TGG TAG CTC TAG CGCACTTCATCG TAG	61	1400	Hajjaran et al 2014 (24)	Chromosome 36
Lack	Lack- F Lack- R	CTAGAGATCTATGAACTACGAGGGTCACCT CTAGGAATTCGCTCGGCGTCGGA-GATGGACC	63	939	Hajjaran et al 2016 (23)	Chromosome 28
GP63	GP63- F GP63 -R	GTCTCCACCGAGGACCTCACCGA TGATGTAGCCGCCCTCCTCGAAG	70	1330	Guerbouj et al 2001 (22)	Chromosome 10

Tm: annealing temprature

PCR amplification and nucleotide sequencing

For PCR amplifications, specific primers (Table 2) were synthesized by the Bioneer Corporation (Daejeon, South Korea). PCR products were examined by electrophoresis on 1.5% agarose gels stained with ethidium bromide. The five loci amplicons obtained from 44 *Leishmania* isolates were purified and were sequenced using an ABI PrismTM 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) by Macrogen Company (Seoul, South Korea). Ambiguous (heterozygous) sites were coded using the standard IUPAC codes for combinations of two or more bases. All samples were compared to GenBank sequences. Consensus sequences from forward and reverse reads were edited using the BioEdit 5.0.9 (27) and aligned using ClustalW program in the MEGA 6.0 package (28), available from: http://www.ddbj.nig.ac.jp/search/clustalwe.html

Phylogenetic and sequence analysis

Phylogenetic trees and relationships between sequence types (STs) were constructed. The concatenated nucleotide sequences of all five genes, generated from 44 *Leishmania* isolates, were aligned and analyzed. Different statistical methods (Maximum likelihood tree, UPGMA tree and neighbor-joining tree) in MEGA 6.0 (28) were used. The sequences are available in GenBank with accession numbers: MW549068-MW549196, MN523458-MN523476, MF326223-MF326266). The Kimura 2-parameter model of nucleotide substitution and bootstrap values with 1000 replicates were applied and a UPGMA tree was built by Splits Tree 4 (29). A network of haplotypes was drawn by PopARTS software using statistical parsimony (30). The diversity indices, haplotype diversity (Hd), nucleotide diversity (π), and neutrality indices (Tajimas′D statistic) (31)) were estimated by DnaSP v5 software version 5.10 (32). The discriminatory power of the MLST scheme was measured with Simpson's index of diversity (33), using

an online resource (http://darwin.phylovis.net/Comparing Partitions).

Results

PCR and sequencing results

PCR amplification of 5 loci resulted in amplicons with the expected sizes (Table 2). The number of edited nucleotide sequences is mentioned in Table 3. These sequences obtained from 44 isolates and were concatenated in a global alignment consisted of total 5,010 nucleotides for each isolate (Table 4). A number of 26 STs was identified based on the assignment of unique allelic profiles, numbered ST1-ST26 (Table 5).

Table 3: Diversity and neutrality indices of a 44-isolate population from 4 species, *L. major, L. tropica, L. infantum,* and *L. turanica* based on 5 housekeeping genes

Genes	*No. of nucleotide*	*S*	π	*H*	*Hd*	*Tajima's D*
Nagt	985	59	0.0190	8	0.828	1.19
Hsp70	1181	23	0.0065	5	0.721	1.5
Lack	647	17	0.0096	6	0.761	1.62
G6pdh	1403	91	0.01905	10	0.859	1.0
GP63	794	80	0.0382	5	0.710	2.1

S: Number of Segregation Sites; π: nucleotide diversity; H: number of haplotypes; Hd: haplotype diversity

Table 4: Diversity and neutrality indices of 44 *Leishmania* isolates based on the concatenation of 5 MLST targets

Species	*No. of Nucleotide*	*S*	π	*H*	*Hd*	*Tajima's D*
L. tropica	5010	13	0/0009	14	0/96	0/97
L.major	5010	10	0/0008	10	0/89	1/86
L. infantum	5010	0	0/000	1	0/000	---
L. turanica	5010	0	0/000	1	0/000	---

S: Number of Segregation Sites; π: nucleotide diversity; H: number of haplotypes; Hd: haplotype diversity

Table 5: Distribution of *Leishmania* isolates among sequence types

ST (Sequence type)	*Isolates*	*ST (Sequence type)*	*Isolates*
ST1	C1,C30	ST14	C23
ST2	C2,C8	ST15	C25
ST3	C3,C5,C10,C18,C19	ST16	C26
ST4	C4	ST17	C28
ST5	C6	ST18	C29
ST6	C7,C11,C13,C22	ST19	C31,C33,C36,C37
ST7	C9	ST20	C34
ST8	C12	ST21	C35
ST9	C14,C15	ST22	C38
ST10	C16	ST23	C39,C40
ST11	C17,C24,C27	ST24	C41
ST12	C20	ST25	C42
ST13	C21	ST26	C43,C44

ST: Sequences types; C: Strain ID; 26 ST obtained from concatenated sequences of 44 *Leishmania* isolates

Phylogenetic analysis of concatenated sequences

Four clades, including *L. major, L. tropica, L. infantum*, and *L. turanica* were recognized in the phylogenetic tree (Fig. 1). The presence of some STs, which belongs to the isolates responsible for different leishmaniasis phenotypes (VL and CL), in the same species complex (clade A), was confirmed in this study. ST20 and ST21 related to VL patients, together with the STs related to *L. tropica* (isolated from CL patients), were clustered in the same clade.

The clade representing *L. major*, contained the strains isolated from both humans and rodents (ST23). The phylogenetic tree showed that *L. turanica* and *L. infantum* formed separate groups. *L. turanica* (ST12), a rodent isolate (C20 sample), showed similarity with *L. major*. Five canine isolates, belonged to *L. infantum* species formed a separate clade with far distance from other species.

The unrooted Neighbor-Net (NN) network analysis of 44 concatenated sequences generated identical result with rooted tree. Four clusters were clearly identified. The validity of the four clusters was confirmed by Maximum-likelihood (ML) and showed very similar topological patterns (Fig. 1).

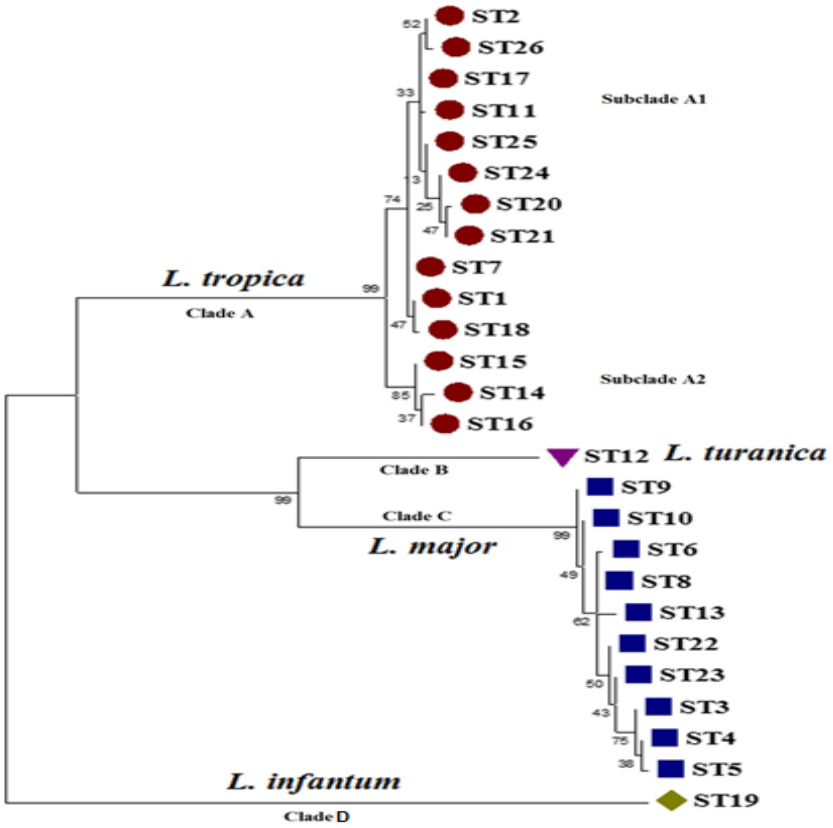

Fig. 1: Phylogenetic tree of Iranian *Leishmania* isolates based on concatenated nucleotide sequences of five genetic markers in MLST scheme by Maximum likelihood phylogenetic reconstruction. Branch values represent bootstrap values (1000 replications)

Haplotype network analysis

To investigate the relationship between selected genes, the TCS (Templeton, Crandall and Sing) networking was plotted. Haplotype network was built for each candidate locus separately (Fig. 2). Each of the clusters varied by several mutational steps, which were represented by variations between clusters. The TCS network of the entire set of the haplotypes from samples of different geographical locations from Iran, formed distinctive clusters. The number of haplotype clusters formed by individual loci included: 10, 8, 6, 5, and 5 for *g6pdh*, *nagt*, *lack*, *hsp70*, and *gp63* genes, repeatedly (Fig. 2) (Table 3).

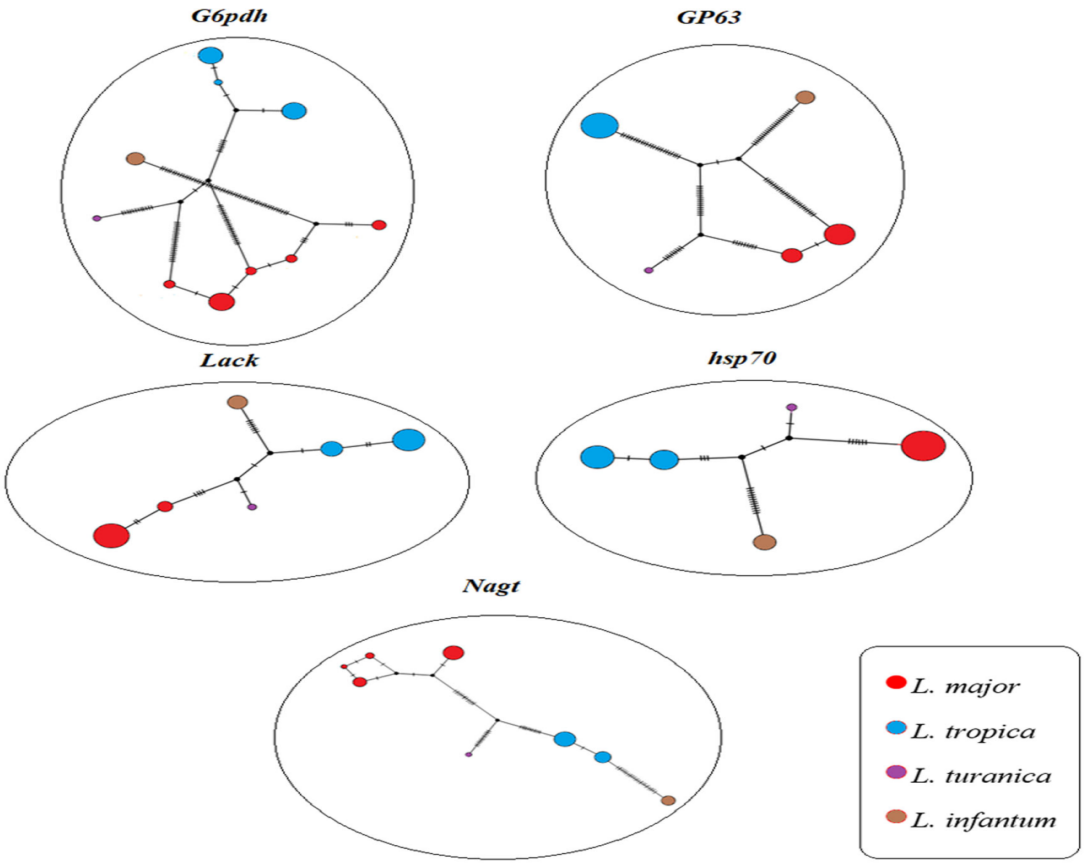

Fig. 2: The haplotype network in *Leishmania* isolates population implemented by PopART (Population Analysis with Reticulate Trees). *Leishmania* spices are shown by different colors

Diversity, neutrality and indices

Genetic diversity indices of *Leishmania* species based on single loci and concatenated sequences are shown in Tables 3 and 4. Haplotype diversity (Hd) indices for *g6pdh* and *nagt* targets were 0.859 and 0.828, respectively; it was lower in *lack, hsp70* and *gp63*, with 0.761, 0.721, and 0.710, respectively. Genetic diversity indices based on concatenated sequences from samples of *L. tropica* compared to those of *L. major*, showed greater Hd; while *L. infantum* and *L. turanica* showed lack of haplotype diversity (Hd = 0). Based on the nucleotide diversity indices (π), *g6pdh, nagt,* and *gp63* showed highest diversity (0.01905, 0.0190, and 0.0382), followed by the *hsp70,* and *lack* (0.0065 and 0.0096), respectively.

Discussion

Evaluation of the Simpson index showed the highest index (0.91) in MLST method comparing to those of single locus typing (Fig. 3). Both MLST and single locus analysis of the five loci showed similarly the existence of 5 clades in Iranian *Leishmania* isolates that were in accordance with the species level (34-36). For classification of strains within the major groups, discriminatory markers such as MLST, were shown to be more suitable (19). MLST provided a considerable contribution to understand the epidemiology, transmission, and phylogenetic of infectious diseases (18).

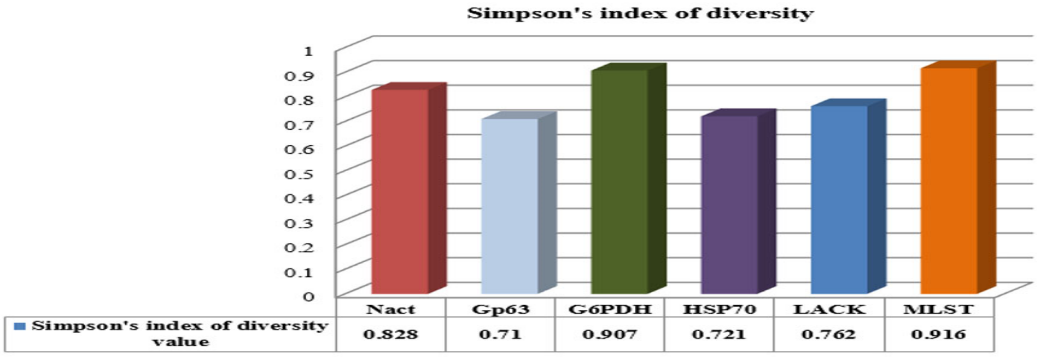

Fig. 3: Simpson index of diversity of five genetic markers used in MLST method for phylogenetic analysis of Iranian *Leishmania* species. Diversity indices and their 95% confidence intervals are presented in this figure

A Neighbour-Net in Splits Tree (Fig. 4) gives us the same information as that of NJ tree, but was more visual. The results of phylogenetic analysis showed that, all four *Leishmania* species were clearly separated in all analyses; *L. turanica* clustered between *L. tropica* and *L. major*, as it was mentioned before (37). All markers clearly distinguished *L. infantum* species complex, which included strains obtained from canines in Meshkin Shahr, Northwest Iran.

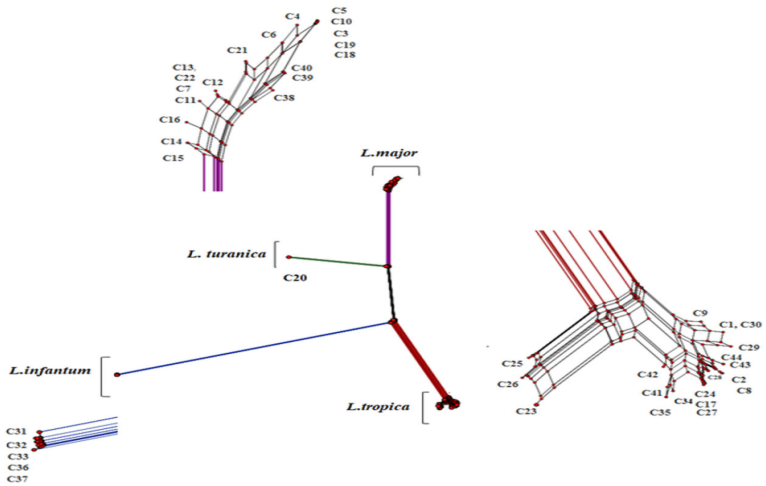

Fig. 4: Individual Neighbor net analysis of 44 *Leishmania* isolates based on concatenated nucleotide sequences of five housekeeping genes constructed by Splits Tree 4 software

Evaluation of these 5 loci (15, 21, 24-26) also showed that these genes contain sufficient information to distinguish among species/subspecies as well as human/non-human *Leishmania*.

Based on our sequences, *L. major* and *L. turanica* were found to be sister clades (Figs. 1-3). In another study, it was reported that *L. turanica* generally forms sister taxa with *L. major* (38), It is noteworthy that the animal reservoirs of these two species are rodents. Parasites in the body of different hosts present several mechanisms to escape from their immune system, which can cause different mutations (39).

From an evolutionary point of view, phylogenetic trees created four distinct classes (A, B, C, and D) with higher bootstraps, which are similar to the findings of Hajjaran et al. (37). The clades *L. major* and *L. tropica* located in relatively close situation (Fig. 1), with the common origin (40).

The *Leishmania* isolates have been collected from different endemic regions of Iran; however, they did not classify based on their geographical distributions. Two isolates, C35 and C34, were previously obtained from patients in Tehran and Ardabil with VL clinical manifestations, but they were unexpectedly misdiagnosed as *L. tropica* species. *L. infantum* is the major cause of VL. *L. tropica* is usually a causative agent of CL. however; it has been rarely representing the visceral symptoms in Iran and some other areas. In the phylogenetic tree, the C35 and C34 (Fig. 1), were placed in two separate STs (ST21 and ST20), but were placed in the same clade, indicating the close relationship with similar clinical manifestations. In addition, two isolates C14 and C15, obtained from the patients of Ilam province (west of Iran), and placed in ST9. No significant relationship between *Leishmania* isolates and geographical distribution was observed in the other STs. This can be due to the common origin of these populations or high gene flow between them. A study showed association between phylogenetic clusters of Chinese *Leishmania* isolates and their geographical locations rather than clustering based on clinical features (26).

The haplotype number and haplotype diversity showed the highest SNP variation in *g6pdh* and *nagt*. Recently, Hosseini et al (41) described SNP variations and the highest rate of heterozygous sites in *g6pd and 6pgd* genes (6 sites) for *L. tropica* and in aspartate aminotransferase (*asat)* and *6pgd* (7 sites) for *L. major* strains.

Conclusion

All five genomic loci caused separation of Iranian *Leishmania* species at the species level, which is an indication of conservation of these genes in the *Leishmania* parasite. The results provided more molecular information about the evolutionary history of Iranian *Leishmania* isolates. The main limitations of this study were the unavailability of the patients' medical history and precise geographical location of infection, and the loss of samples due to contamination of the parasite medium. This study confirmed further that MLST is a suitable method to examine genetic variation of *Leishmania* parasites with respect to evolutionary and epidemiological studies.

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Availability of data and materials

All sequences generated in this study were submitted in the GenBank database with accession numbers: MW549068 to MW549196, MN523458-MN523476, MF326223-MF326266.

Conflict of Interest

The authors have no conflicts of interest concerning the work reported in this paper

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