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Tatera indica (Rodentia: Muridae) as the Prior Concern and the Main Reservoir Host of Zoonotic Cutaneous Leishmaniasis on the Border of Iran and Iraq

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Abstract

Background: Zoonotic cutaneous leishmaniasis (ZCL) is increasing in many parts of the world including Iran. Rodents are the most important reservoirs of *Leishmania* parasites in many remote areas of ZCL. Identification and molecular characterization of *Leishmania* parasites in reservoir hosts (rodents and dogs), potential vectors (sandflies), and suspected patients in leishmaniasis foci should be clarified for different controlling measurements and treatments.
Objectives: This study aimed to determine the main reservoir hosts of ZCL in Khuzestan province bordering Iran and Iraq.
Methods: Rodents were captured and identified using morphological and molecular techniques. *Leishmania* species were sampled from both ears of rodents and DNA was extracted. *Leishmania* detection was based on PCR and sequencing of ITS-rDNA of infected rodents. Phylogenetic analyses were conducted to understand the relationship, homology, and haplotype variations among *Leishmania major* parasites and identify the causative agents of leishmaniasis in the area. The maximum likelihood and neighbor-Joining with alternative Kimura 2-Parameter models were employed for phylogenetic analyses.
Results: *Leishmania major* was firmly recognized by conducting molecular analysis on 121 captured rodents, from which 45 samples unequivocally were identified as *Tatera indica*. *Leishmania* parasites obtained from *T. indica* were sequenced to analyze genetic polymorphism and/or similarity using ITS-rDNA genotype. Phylogenies revealed that one common haplotype of *L. major* (GenBank accession no. EF413075) was the most haplotype variant dominated among seven infected *T. indica*.
Conclusions: The widespread distribution of *L. major* parasites in human suggests not only *T. indica* was the main reservoir but also other rodents and mammalian hosts might be the reservoir hosts of ZCL in the region. Molecular and phylogenetic analyses confirmed the strength of haplotype variation maintaining the circulation of *Leishmania* species in their reservoir hosts.

Keywords: *Leishmania major*, *Tatera indica*, Zoonotic Cutaneous Leishmaniasis, Phylogenetic Analyses, ITS-rDNA, Iran

1. Background

Leishmaniasis as one of the emerging and neglected infectious diseases has been largely distributed in the world. Khuzestan province is situated on the border of Iran and Iraq, having a tropical climate with high prevalence rate of leishmaniasis in the five past years (1). Although, it is believed that three species of *Leishmania* parasites have been incriminated as the causative agents of human leishmaniasis in Iran, other mammals' *Leishmania* species have been isolated and identified from sandflies, rodents, and humans (2).

Zoonotic cutaneous leishmaniasis (ZCL) is an endemic disease in more than 80 countries in the world including Iran (3, 4). Zoonotic cutaneous leishmaniasis is distributed in more than half of Iranian provinces and Khuzestan province in the southwest has been affected by the disease with new and unknown foci. The life cycle of *Leishmania* parasites in ZCL depends on some criteria such as sandfly species as vectors, wild rodent species as reservoir hosts, and the geographical locations as natural important foci of the disease (5, 6). Among different species of wild rodents, a few are identified as the main species of ZCL reservoir hosts in Iran. A number of rodent species are widespread; some of them in Asia and some others in smaller areas (7).

There are many important species of rodents from different ZCL regions in Iran such as *Rhombomys opimus*, *Meriones libycus*, *M. persicus*, *Tatera indica*, *Nesokia indica*, *M. hurrianae*, and *Rattus norvegicus* which have been reported as the main hosts of ZCL parasites. But there is no sufficiently precise and consistent evidence to confirm that all of the

species are main reservoirs of ZCL in Iran and other regions (4, 8-20).

Currently, *T. indica* has drawn more attention not only as a principal ZCL reservoir but also due to our recent finding revealing that four subspecies of *T. indica* exist in Iran based on morphological and molecular characteristics. In spite of the expectation that two of which exist in Khuzestan province, only one subspecies was found (21).

2. Objectives

The objective of this investigation was to find the potential and/or prominent reservoir hosts of ZCL and define the role of *T. indica* in the transmission of *Leishmania major* in southwestern Iran. Another objective of this study was to explore (i) genetic variation, (ii) polymorphism, and (iii) genetic similarity as effective factors in maintaining equilibrium of mutation due to natural selection or genetic drift in the population of *L. major* isolated from sandfly species as vectors, wild rodent species as reservoirs and human at the border of Iran and Iraq (22).

3. Methods

3.1. Origin and Sampling of Leishmania From Reservoir Hosts

Rodents were collected from the active colonies of rodent burrows around the villages in the study area using 40 wooden and wire live traps in 2012 - 2014. Cucumbers and dates were used as baits for each location of ten villages in four districts of Khuzestan province. The areas' altitude was 18 meters above the sea level (a.s.l) with geographical coordinates as: 31.3273°N; 48.6940°E; (Figure 1). The rodent traps were set up in active colonies in 6 cities (12 villages) of Khuzestan Province early in the morning. The traps were left for several days in each location. However, *T. indica* was captured only from 6 villages. The samples was collected from the colonies of rodents' burrows located around the villages where ZCL was endemic using 50 wooden and wire live traps for each location.

The traps were checked and the baits were changed consecutively. Collected rodents were transferred to Pasteur Institute of Iran and maintained to be identified morphologically as well as molecularly.

3.2. DNA Extraction and Amplification of Leishmania Infection

The ears of each rodent were scratched and two impression smears were taken. Routine laboratory procedures and molecular methods of impression smears prepared from rodents' ears were followed according to the Parvizi et al. (2008) protocol to detect *Leishmania* infection. *Leishmania* sampling from rodents, isolation of parasites, impression smears from each ear of rodents, light microscope observation, culturing in Novy-MacNeal-Nicolle (NNN), and inoculation in Balb/C from the captured rodents were followed by the methods of Mirzaei et al. (2011) (17).

Whole genomic DNA of *T. indica* was extracted using Genet Bio kit (Takapoo Zist), Phenol-Chloroform (CinnaGen Co. Tehran. Iran) and ISH Horovize methods based on Parvizi et al. (13). The DNA of all *T. indica* samples was extracted and the ITS-rDNA gene fragment of *Leishmania* parasites was amplified using PCR to find the precise species of *Leishmania* parasite causing leishmaniasis. The forward primer IR1 and the reverse primer IR2 were exerted for the first-stage of PCR; while for the second-stage of the nested PCR, the forward primer ITS1F and the reverse primer ITS2R4 were used (23, 24).

3.3. Sequences and Phylogenetic Analyses

The positive PCR products obtained from *Leishmania* parasites were directly sequenced, aligned, and edited to determine *Leishmania* species and haplotype variations in individual rodents using Sequencher 4.1.4TM for PC. Phylogenetic analysis was performed in MEGA 6 software. Maximum likelihood (ML) and neighbor-joining (NJ) were the two statistical methods to draw trees and determine the genetic relationship of *Leishmania* species with different original hosts using alternative Kimura 2-Parameter (K2P) models (25). The DnaSP5 software was used to indicate polymorphisms and haplotype diversities.

4. Results

4.1. Morphological and Molecular Identification

Among 121 rodents sampled from six districts around 12 villages and sites in Khuzestan province, Iran, in 2012 - 2014, 45 were identified as *T. indica* (Table 1 and Figure 1). All *T. indica* samples (20 female, 25 male) were identified first morphologically and then molecularly using Cyt b gene in the method of Parvizi et al. 2008 (13). *Tatera indica* was captured more in Behbahan than other locations; but no significant difference was found in *Leishmania* infections based on different locations. 41 live-captured *T. indica* samples were examined for *Leishmania* infections using both conventional and molecular methods; but only 4 dead captured *T. indica* were used to isolate and detect *Leishmania* parasites in molecular methods.

Table 1. Distributions and *Leishmania* Infections in *T. indica*, Khozestan Province, Iran

Location	Habitat	*T. indica*		Age Groups			Season								Total (+ve)
Town		*Leishmania* (+ve)		0 - 2 (+ve)	2 - 4 (+ve)	> 4 (+ve)	Spring		Summer		Early Fall		Last Winter		
Ahvaz		F (+ve)	M (+ve)				F (+ve)	M (+ve)	F (+ve)	M (+ve)	F (+ve)	M (+ve)	F (+ve)	M (+ve)	
	Jadeh Hamideyeh[a]	2	5 (1) LR	1	2	4 (1)	1	2 (1)	1	3	0	0	0	0	10 (2)
	Jadeh Abadan[a]	1	2 (1) LR	0	1 (1)	2	1	0	0	1 (1)	0	0	0	1	
Shushtar	Ghalehnou[b]	0	1 (1) L	0	1 (1)	0	0	0	0	1 (1)	0	0	0	0	1 (10)
Ramhormoz	Darkhouyin[a]	0	1	0	1	0	0	0	0	1	0	0	0	0	1
Shush	Sorkheh[b]	1	0	0	1	0	1	0	0	0	0	0	0	0	1
Behbahan	Kharestan[a]	7 (1) LR	10 (1) LR	3 (2)	5	9	3	5	3	4 (1)	1 (1)	1	0	0	21 (3)
	Germez[b]	1	1	1	1	0	1	0	0	1	0	0	0	0	
	Ab Amir[b]	0	1	1	0	0	0	0	0	1	0	0	0	0	
	Heyat Abad[b]	1 (1) R	0	1 (1)	0	0	0	0	1 (1)	0	0	0	0	0	
Dezful	Gavmish Abad[b]	5	2	1	2	4	1	0	3	0	1	2	0	0	11 (1)
	Bonyeh Abad[b]	2	1 (1) R	1 (1)	2	0	2	0	0	1 (1)	0	0	0	0	
	Seyed Nur[b]	0	1	0	1	0	0	1	0	0	0	0	0	0	
Total No (+ve)		20 (2)	25 (5)	9 (4)	17 (2)	19 (1)	10 (0)	8 (1)	8 (1)	13 (4)	2 (1)	3 (0)	0	1 (0)	45 (7)
		45 (7)	45 (7)	45 (7)	45 (7)	45 (7)	18 (1)	18 (1)	21 (5)	21 (5)	5 (1)	5 (1)	1 (0)	1 (0)	

Abbreviations: F, female; M, male; L, left ear; R, right ear; LR, right and left ear.
[a] Rodent burrow, desert.
[b] Rodent burrow, around villages.

Figure 1. Location of Villages and Districts in Khuzestan Province, Iran, Where *T. indica* Was Sampled and Screened for *Leishmania* Infections

4.2. Detection of Leishmania Infection Using Molecular Analyses

Seven out of 45 *T. indica* were found with *Leishmania* infection (Table 1). Live-captured *T. indica* were more infected with *Leishmania* (7 out of 41) than those captured dead (zero out of 4). No significant difference was found in *Leishmania* infection between left and right ears of *T. indica*. But concurrent *Leishmania* infection in both ears was more prevalent.

This is the first time to detect *Leishmania* parasites focusing only on *T. indica* in large scales of Khuzestan province. *Leishmania major* firmly was identified first time in 7 *T. indica* by amplifying 460 bp fragment of ITS1-5.8S rRNA -ITS2 gene, RFLP, sequences, aligning, and comparing our sequences with homologous ones from the GenBank database. In addition, this finding was important because Khuzestan province has up to 1609 kilometres shared border with Iraq. Twenty one sequences of ITS-rDNA of *Leishmania* parasites were analyzed for genetic polymorphism and genetic similarity. *Leishmania* were isolated

from reservoir hosts of rodents, sandflies, and humans. 16 sequences of *L. major*, 4 sequences of *L. tropica*, and one sequence of *L. infantum* were employed for phylogenetic analysis trees reconstruction, and determination of the evolution of *Leishmania* parasites.

In terms of evolutionary relationships, molecular phylogenetic analysis by maximum likelihood and neighbor-joining methods revealed that taxa and two old world *Leishmania* species (*L. major* and *L. tropica*) had share common ancestors (Figures 2 and 3). In topology of trees, more variations were observed in *L. major* isolated from sandflies, followed by those from rodents and humans. *L. tropica* had more diversity than *L. major* and placed as out group (Table 2).

5. Discussion

Research on rodents as leishmaniasis reservoirs has revealed a diverse array of transmission cycles where epidemic cutaneous disease caused by *L. major* occurs near colonies of reservoir gerbil rodents in Asia including Khuzestan province on the border of Iran and Iraq (19). Rodents are not always well distinguished in literature; although distinguishing closely-related members of *T. indica* species is complex, it is important for transmission cycle and epidemiological aspects (13, 26). This is crucial to separate reservoirs that are biomedically important from the rodent species that are competent reservoirs but without reservoir capacity to cause much ZCL disease (7, 24). The ecological associations with infected reservoir hosts or humans, and the descriptive eco-epidemiology could suggest a potential reservoir role. Modeling of transmission cycling associations is required to identify the reservoirs that are a real public health priority (6, 27).

Most ZCL infections are diagnosed clinically and microscopically in patients; but in reservoir hosts, a combination of molecular, biochemical, and serological tests can demonstrate significant numbers of *Leishmania* infections in endemic areas of ZCL foci (28, 29). The incidence of ZCL associated with the transmission of *L. major* by rodents has declined in many foci where living standards have been improved (4). Only one report on *Leishmania* infection in one *T. indica* has been presented in a very small area of Khuzestan province named Roffaye, although it is not clear how authors confirmed *L. major* without sequencing and aliment and molecular analyses (19). *Rhombomys opimus* as the main reservoir host of ZCL has been trapped frequently in many areas of Iran while they were more with *Leishmania* infections; despite expecting the same situation in the case of *T. indica* as the second main reservoir host of ZCL in south of Iran, the minority of *T. indica* were found with *Leishmania* infection in the conducted investigations (17, 30, 31).

Despite low *Leishmania* infection in *T. indica*, the prevalence of human disease and infections is relatively high in some districts of Khuzestan province, so that these districts appear to have a transmission cycle typical of the ZCL, while *T. indica* were incriminated as the reservoir hosts of *L. major* (1, 10, 17, 20). Using cross tab, chi-square, and adjusted logistic regression statistical tests, some epidemiological factors such as age, gender, season, and habitat were analyzed to compare any epidemiological factor affecting *Leishmania* infection in *T. indica*. No significant factor was found to change the situation of disease; however, small changes in some factors were shown (Table 2). Statistical analyses showed the first age group of *T. indica* had significantly high *Leishmania* infection. This may be due to that younger *T. indica* search for food and show more activities around the rodent borrows and this can increase the chance for biting by sandflies.

Using ITS-rDNA gene and two statistical methods (NJ and ML), a few old world *Leishmania* species were identified in Iran and elsewhere under similar conditions (22, 32-34).

The number of pairwise differences among sequences was compared with the expected number of segregating sites in *Leishmania* species and using Tajima's D index analysis, a negative evolution process was found; also, the number of observed mutations was lower than the number of expecting mutations. Majority of mutations were not informative but unique. The gape in alignments of ITS-rDNA gene increased with the number of different haplotypes while by ignoring or removing the gap, the number of haplotypes decreased (Table 3).

Using the maximum likelihood method based on the Kimura 2-parameter model, a tree with the highest log likelihood (-838.0810) was constructed. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2030)). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. There were a total of 373 positions in the final dataset (Figure 2). Using the neighbor-joining method, the optimal tree with the sum of branch length of 0.13416000 was drawn. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were com-

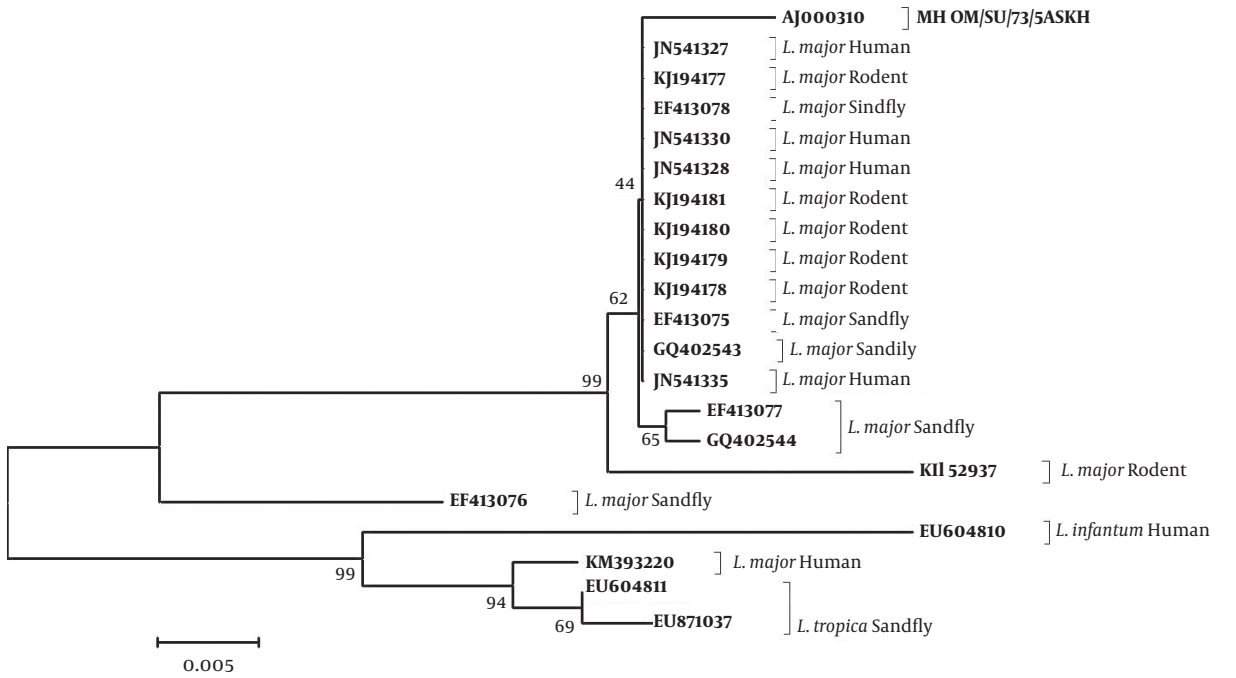

Figure 2. Maximum Likelihood Bootstrap Tree Showing the Relationships of the Haplotypes of the ITS1-rDNA Gene Fragment for the Isolates of Three *Leishmania* species

Table 2. Comparison of *Leishmania* Infection in *T. indica* Based on Epidemiological Factors Using Cross Tab, Chi-Square and Adjusted Logistic Regression Statistical Tests[a]

Variables	Uninfected, (n= 38)	Infected, (n = 7)	OR (CI 95 %)	P Value
Age groups				
0 - 2	5 (55.6)	4 (44.4)	1	0.105
2 - 4	15 (88.2)	2 (11.8)	0.27 (0.026 - 2.916)	0.284
> 4	18 (94.7)	1 (5.3)	0.052 (0.003 - 0.81)	0.035
Gender				
Female	17 (85.0)	3 (15.0)	1	1
Male	21 (84.0)	4 (16.0)	1.05 (0.090 - 12.228)	0.969
Season				
Spring	17 (94.4)	1 (5.6)	1	0.480
Summer	18 (78.3)	5 (21.7)	7.1 (0.43 - 111.15)	0.170
Late fall	2 (66.7)	1 (33.3)	20.35 (0.22 - 1861.4)	0.191
Early winter	1 (100)	0	1	1
Habitat				
RBAV[b]	15 (88.2)	2 (11.8)	1	1
RBD[c]	23 (82.1)	5 (17.9)	2.96 (0.33 - 26.1)	0.328

[a] Values are expressed as No. (%).
[b] Rodent burrow around village.
[c] Rodent burrow desert.

puted using the Kimura 2-parameter method in the units of the number of base substitutions per site. The rate vari-

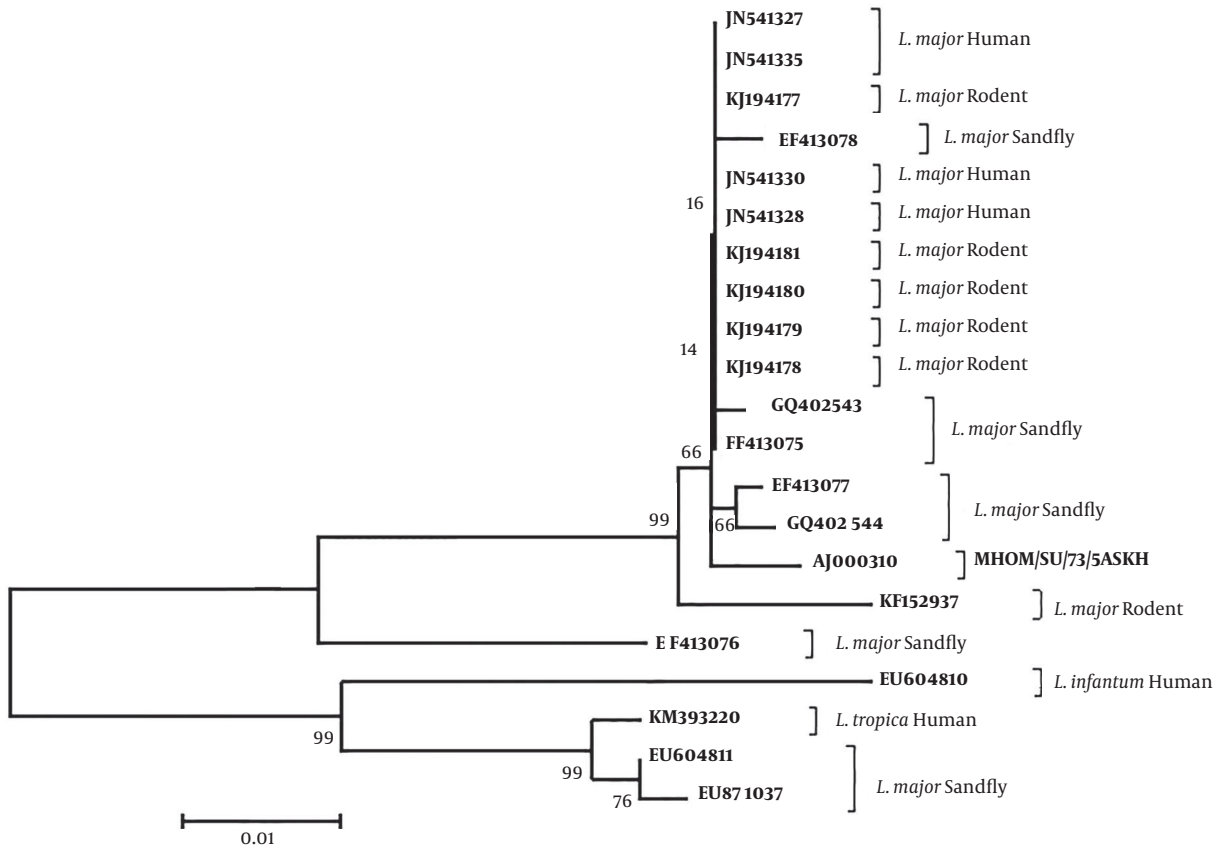

Figure 3. Neighbor-Joining Tree Showing the Relationships of the Haplotypes of the ITS1-rDNA Gene Fragment for the Isolates of *Leishmania* Species

Table 3. The Role of ITS-rDNA Gene Mutations in the Specified Host of *Leishmania* Species, and Comparison of Nucleotide Diversity and Genetic Variation[a]

Host	Parasite Sp.	No. Seq	No Nucleotide2	S (%)	K	Per Seq[b]	π[c]	Per Site[b]	Tajima's D	Singleton Variable	Parsimony Variable	H		Hd	
												With Gap	Without Gap	With Gap	Without Gap
Rodent	*L. major*	6	373 (336)	5 (1.488)	1.66	2.18	0.0049	0.006	-1.33	5	0	4	2	0.8	0.33
Sandfly	*L. major*	6	373 (333)	15 (4.504)	5.2	6.56	0.015	0.019	-1.22	15	0	6	5	1	0.93
	L. tropica	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Human	*L. major*	4	373 (337)	0	-	-	0	-	-	-	-	3	1	0.83	0
	L. tropica	3	373 (332)	17 (5.120)	11.33	11.33	0.034	0.034	1	17	0	3	3	1	1
	L.infantum	1	-	-	-	-	-	-	-	-	-	-	-	-	-
Total	All	21	373 (306)	33 (10.784)	7.6	9.17	0.025	0.029	-0.64	15	18	16	10	0.97	0.68

Abbreviations: S, segregation of variable nucleotide sites; K, average number of pairwise nucleotide difference between pairs of sequences; H, No. of haplotype; Hd, haplotype diversity.
[a] Number of sequence used in this study.
[b] The amount of genetic variation.
[c] Nucleotide diversity, Tajima's D: the D test statistic proposed by Tajima, (35).

ation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 306 positions in the final dataset (Table 3, Figures 2 and 3). Phylogenetic trees for *L. major* are supported on their specific clades and *L. tropica* on own clades.

The current report established that *T. indica* is not

the only reservoir host of ZCL circulating in Khuzestan province. Our investigation raises the possibility that the role of some rodents or other mammals in the incidence of ZCL might be due to some changes in the transmission rate of *L. major*. Phylogenetic analysis of ITS-rDNA gene is recommended for firm identification and separation of *Leishmania* species.

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Footnote

Authors' Contribution: Parviz Parvizi and Somayeh Mohammadi conceived and designed the Manuscript, Reza Fotouhi-Ardakani, Aref Amirkhani, Elnaz Alaee Novin and Somayeh Mohammadi analyzed the molecular and statistical data, Somayeh Mohammadi, Babak Vazirianzadeh and Javad Samii contributed to field works, Somayeh Mohammadi performed morphological and molecular laboratory experiments, Parviz Parvizi and Somayeh Mohammadi wrote the manuscript. All authors read and approved the final manuscript.

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