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Original Article

Species Identification and Genotyping of Cutaneous Leishmaniasis in Clinical Samples Based on ITS1-PCR- Sequencing in Southeast Iran

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Abstract

Background: Cutaneous leishmaniasis (CL) is one of the most common parasitic diseases in many regions of Iran. It has a major role in deprived societies. We aimed to identify *Leishmania* species based on molecular as ITS1-rDNA-PCR internal transcribed spacer 1 (ITS1) region, microscopy, and culture techniques in diagnosing cutaneous leishmaniasis.

Methods: From April 2018 to May 2020, we conducted a cross-sectional study involving 32 patients with suspected CL lesions in Sistan and Baluchistan Province, located in southeast Iran. Samples were subjected to microscopic examination, culture, and PCR amplification targeting the internal transcribed spacer 1 (*ITS1*) region. DNA sequencing was performed on PCR-positive samples for species identification and phylogenetic analysis.

Results: PCR demonstrated superior sensitivity (93.75%, 30/32) compared to culture (56.25%, 18/32) and microscopic examination (53.1%, 17/32). Molecular analysis revealed that *L. major* was the predominant causative agent of CL in the study area, with *L. tropica* occurring less frequently. Sequencing and phylogenetic analysis of the ITS1 region showed high intraspecies similarity among *L. tropica* isolates, while *L. major* isolates exhibited greater genetic diversity.

Conclusion: This study shows the co-existence of *L. major* and *L. tropica* in Mirjaveh, southeast Iran, with *L. major* as the primary cause. While *L. tropica* isolates displayed high genetic similarity, *L. major* samples were more diverse, indicating different epidemiological patterns. These findings highlight the importance of molecular methods for accurately identifying *Leishmania* species and understanding CL epidemiology in the region.

Keywords: Cutaneous leishmaniasis; Iran; Polymerase chain reaction; Sequence

Introduction

Leishmaniasis is an important parasitic disease caused by more than 21 species of the protozoan genus *L.* in 98 countries and threatens more than 350 million people (1). The WHO has listed leishmaniasis as one of the top 10 diseases common in tropical regions (2). Leishmaniasis can be transmitted by the bite of female dipterans of the family *Psychodidae*, subfamily *Phlebotominae*, gener-

ally known as sandflies (3). There are three clinical forms of leishmaniasis in humans namely cutaneous, mucocutaneous, and visceral involving the skin, mucous membranes, and visceral organs respectively (4). Cutaneous leishmaniasis (CL) is a dermal manifestation caused by various *Leishmania* species such as *L.* (*L.*) *tropica* and *L. (L.) major* in the Eastern Hemisphere and *L. (L.) mexicana, L. (Viannia) braziliensis, L. (V.) guyanensis,* and *L. (V.) panamensis* in the Western Hemisphere (5). CL has been witnessed in both anthroponotic (ACL) and zoonotic (ZCL) forms in Iran**.** The cause of the ZCL is *L.major*, with rodents serving as its main reservoir. On the other hand, in the ACL, the causative agent is *L. tropica*, and humans are the main reservoir (6). Recently, CL cases have been reported from some countries neighboring Iran, including Afghanistan, Iraq, Saudi Arabia, and Syria (7).

Species identification methods include clinical observation, microscopic examination, culture, and molecular techniques. PCR-based methods are both sensitive and specific for detecting *Leishmania* species, even at low parasite levels, and allow for accurate differentiation between species (8, 9). Among the molecular methods, multilocus microsatellite typing (MLMT) and PCR-restriction fragment length polymorphism (PCR-RFLP) of specific genetic markers, such as kDNA and rDNA, were highly effective in distinguishing between species and identifying intraspecies genetic diversity (10, 11). *ITS1* and *ITS2* sequencing is particularly suitable for species-level discrimination and has been widely used in phylogenetic studies of *Leishmania* (12-16).

Given the importance of molecular methods in epidemiological studies, this research focused on analysing *ITS1* sequences to understand the phylogenetic relationships of *L. major* and *L. tropica* in southeast Iran, where both zoonotic and anthroponotic forms of CL are endemic.

Materials and Methods

Study Area

This study was conducted in Sistan and Baluchistan Province, located in southeast Iran, between latitudes 25°3'N and 28°31'N and longitudes 58°48'E and 63°19'E. The province shares borders with Afghanistan and Pakistan to the north and east, the Sea of Oman to the south, and Kerman and Hormozgan provinces to the west (Fig. 1).

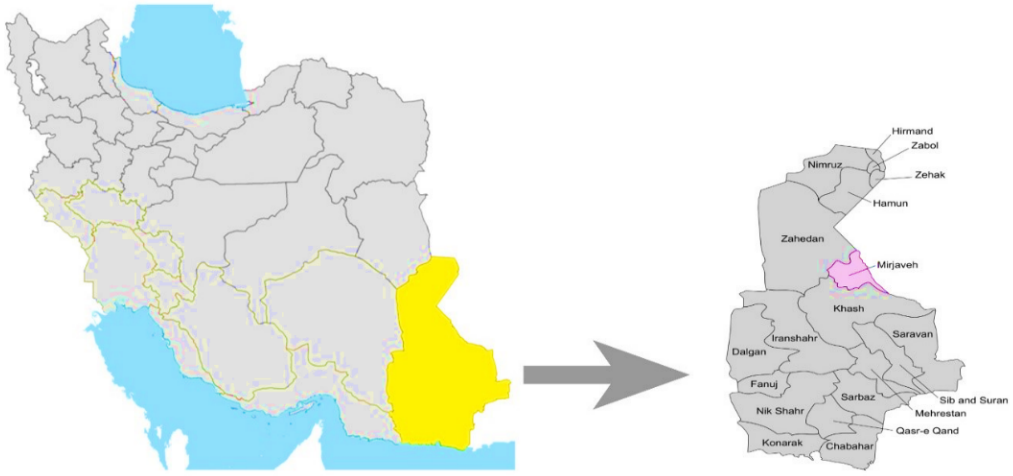

Fig. 1: Map of Sistan and Baluchistan province in Iran, Mirjaveh, and suburbs (Mellekan..), Southeast Iran(Investigation region) Mirjaveh City and suburbs on the map are seen in red), Aerial view of Mirjaveh City, Captured using Google Earth, (google Earth,2024). Retrieved from. https://www.google.com/about

Sistan and Baluchistan have a predominantly hot and dry climate, with moderate winter dryness, an average annual rainfall of 120 mm, and temperatures ranging from below 0° C in winter to a maximum of 40 °C in summer. Two areas in this province are considered endemic for cutaneous leishmaniasis (CL): Mirjaveh and its surrounding districts, such as Melekan, and Chabahar, the latter of which we have previously studied (17).

Mirjaveh, located 70 km southeast of Zahedan, the provincial capital, has a population of over 40,000 residents and comprises 313 villages near the borders of Iran, Pakistan, and Afghanistan. The area lies at an altitude of 1,373 meters above sea level, with a tropical to semi-tropical climate, an annual precipitation of 15.5 mm, relative humidity of 24%, and an average temperature of 21.7°C. The local population includes ethnic groups such as Baluch, Fars, Afghans, and Pakistanis (18).

Ethical Approval

This research complied with the ethical standards and national norms for medical research in Iran. The study was approved by the Institutional Ethics Committee of Zabol Medical University (Approval Statement no. IR-ZBMU.REC.1397.210).

Parasitological Procedures

Between April 2018 and May 2020, a total of 32 patients presenting with cutaneous lesions were examined for CL (Fig. 2). Eight patients were co-infections with bacterial and fungal pathogens, identified via additional diagnostic tests. All patients were recruited based on clinical suspicion of CL. The lesions were disinfected with ethanol before sample collection, and two impression smears were obtained from each patient. The smears were fixed with methanol, stained with Giemsa, and examined under a microscope for the presence of *Leishmania* amastigotes. Additionally, exudates from the lesions were cultured on Novy–MacNeal–Nicolle (NNN) medium for parasite isolation. Each patient also completed a questionnaire to record demographic and clinical information.

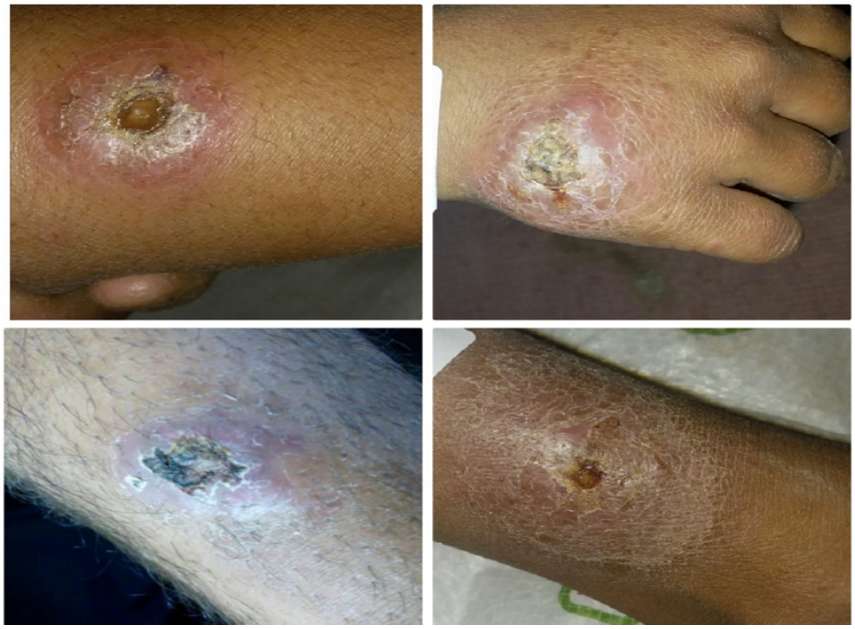

Fig. 2: The images depict ulcerative lesions with characteristic crusting and surrounding erythema, which are typical of infections caused by *L. tropica* and *L. major*, the predominant species identified in the region. The lesions show the progression of ulceration, a hallmark of the disease

DNA Extraction

After microscopic examination, genomic DNA was extracted from the stained slides and NNN medium. The slides were washed with phosphate-buffered saline (PBS) and gently scraped using sterile scalpel blades. The resulting solution was transferred to 1.5 ml Eppendorf tubes. DNA extraction was performed using the Genomic DNA Purification Kit (Azma Elixir Paiooh, Iran) following the manufacturer's protocol. The extracted DNA was stored at –20°C until further use in molecular analysis.

DNA Amplification

PCR amplification was performed using genus-specific primers targeting the internal transcribed spacer 1 (*ITS1*) region of ribosomal DNA (rDNA) to confirm species identification. Positive control strains of *L. major* (MHOM/IR/15/ER) and *L. tropica* (MHOM/SU/79/K27) were obtained from Isfahan and Tehran universities (16). Distilled water, in place of DNA, served as a negative control.

The PCR reaction was carried out in a total volume of 25 µl, which included 2.5 µl of 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2, 0.01% gelatin), 200 µM of each deoxynucleotide triphosphate (dNTP), 2 µM of each primer (Forward 5'-CTGGATCATTTTCCGATG-3' and Reverse 5'-TGATACCACTTATCGCACTT-3'), 0.3 U of Taq DNA polymerase, and 3 µl of template DNA, with sterilized water added to make up the final volume. The PCR protocol involved 30 cycles of denaturation at 92°C for 1 minute, annealing at 50 °C for 1 minute, and extension at 72 °C for 1 minute. A final extension was performed at 72 °C for 7 minutes, followed by a hold at 4 °C.

Amplicons were analyzed using 1.2% agarose gel electrophoresis in 0.5× TBE buffer. The gels were stained with ethidium bromide, and bands were visualized under ultraviolet light (UV) (Fig. 3).

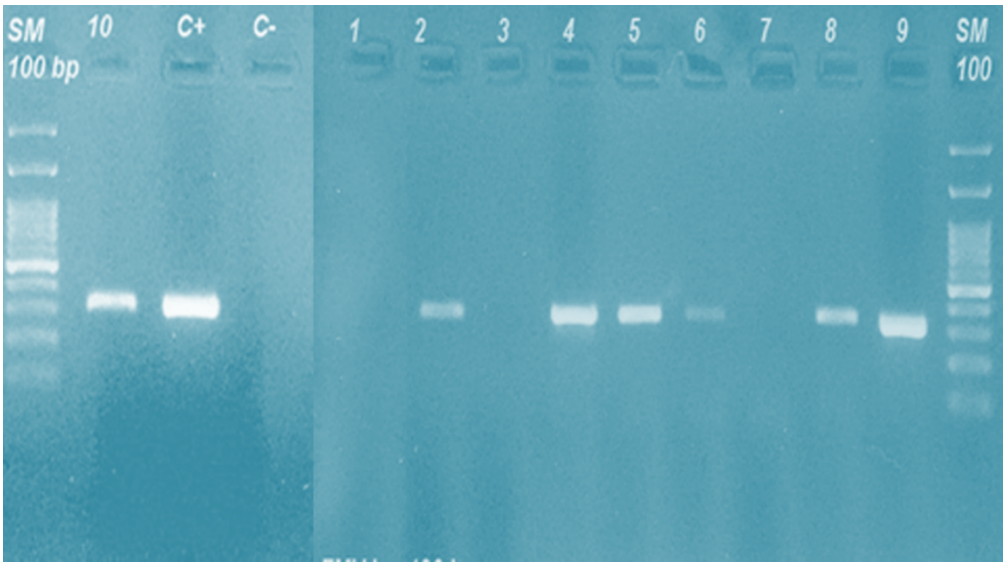

Fig. 3: Detection of cutaneous leishmaniasis in patient samples by PCR amplification. The image shows electrophoresis of the 303-bp PCR product on a 1.5% agarose gel. Lane SM contains the 100 bp molecular size marker (ladder). Lane C+ represents the positive control for cutaneous leishmaniasis, while Lane C- is the negative control. Lanes 2, 4, 5, 6, 8, 9, and 10 correspond to patient samples that tested positive for *Leishmania* DNA, indicating active infection. The clear amplification bands in these lanes confirm the presence of the target *Leishmania* sequence in the samples

Sequencing of *ITS1*

PCR products were commercially sequenced by Ref Gen (http://www.refgen.com) using Sanger sequencing technology. The obtained sequences were manually inspected and edited using Chromas software. Multi-sequence alignment was performed using ClustalW, and the results were compared with ITS1 sequences available in GenBank(http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide) to confirm species identification and assess genetic relationships.

Results

Diagnostic Performance of PCR, Culture, and Microscopy

A total of 32 clinical samples were collected and analyzed using PCR, culture, and microscopic examination to detect *Leishmania* species. PCR demonstrated the highest diagnostic sensitivity, with 30 of 32 samples testing positive (93.75%), compared to culture (18 of 32; 56.25%) and microscopy (17 of 32; 53.1%). Sensitivity was calculated based on the number of positive detections relative to PCR, considered the gold standard.

Patient Demographics

Among the 32 patients, the majority were male (n = 19, 59.4%), while females constituted 40.6% (n = 13) of the cohort. Patients' ages ranged from 5 to 65 years, with a median age of 32. October and November saw the highest number of patients, which coincides with the peak season for cutaneous leishmaniasis transmission in the region. Lesions were predominantly found on exposed areas of the body, such as the hands, arms, and face.

Sequencing and Phylogenetic Analysis

Of the 30 PCR-positive samples, six were selected for sequencing based on the quality of their PCR products. Isolates 2, 4, 5, 8, 9, and 10, which exhibited sharp and well-defined bands during gel electrophoresis, were sequenced and analyzed. Sequencing was performed using either the forward or reverse ITS1-specific primers. The amplified ITS1 regions, approximately 300 base pairs in length (Fig. 3), were sequenced and analyzed using *Geneious Prime software*.

The six sequenced isolates were identified as *L. tropica* and *L. major* based on comparison with reference sequences available in GenBank. The sequences were deposited in GenBank under accession numbers ON733094, ON733095, ON733096, ON733097, ON733098, and ON733099. Phylogenetic analysis was conducted using maximum likelihood methods, and relationships were visualized in phylogenetic trees.

Sequencing and Phylogenetic Analysis

L. tropica Sequencing and Phylogenetic Analysis

PCR products from *L. tropica* were sequenced using ITS1-specific primers, and the products were excised at approximately 300 base pairs (Fig. 3). Six samples were successfully sequenced using Geneious Prime software and submitted to NCBI via Azma Elixir Pajooh Company (Tehran, Iran). Among these, two isolates from Mirjaveh, ON733094 and ON733095, were identified as *L. tropica* based on BLAST analysis. These sequences were assigned the GenBank accession numbers ON733094 (BankIt2592060 41-34220-T_10-R_TOX-Reverse) and ON733095 (BankIt2592060 42-34219-T_9-R_TOX-Reverse).

BLAST comparison showed a high degree of similarity (≥99%) between ON733094 and ON733095 and other *L. tropica* isolates from Iran and neighboring countries. In particular, these isolates were closely related to those from Kerman City (MG694396.1, MG694415.1), Bam City (KX890196.1), Birjand (KC505434.1), as well as Pakistan (MN891726.1), and Afghanistan (KP335128.1) (Table 1).

Table 1: The most comparable *Leishmania tropica* ITS1 sequences in the World and Iranian species submitted in GenBank and Frequency of ITS1 sequence types

No	*Genbank*	*Isolation source*	*Total score*	*Species*	*Country*	*Per identity Percent*
1	MH347947.1	Human skin lesion-Amastigote	206	*L. Tropica*	Turkey	82.73
2	MH347942.1	Human skin lesion-Amastigote	178	*L. Tropica*	Turkey	78.23
3	KU680853.1	Human skin lesion-Amastigote	177	*L. Tropica*	Russia	78.07
4	MG694396.1	Human skin lesion-Amastigote	176	*L. Tropica*	Iran: Kerman	78.70
5	KT990131.1	Human skin lesion-Amastigote	173	*L. Tropica*	China	78.34
6	EU326226.1	Human skin lesion-Amastigote	173	*L. Tropica*	India	78.34
7	MG694415.1	Human skin lesion-Amastigote	171	*L. Tropica*	Iran: Kerman	78.24
8	KX890196.1	Human skin lesion-Amastigote	171	*L. Tropica*	Iran: Bam	78.24
9	KP335127.1	Human skin lesion-Amastigote	171	*L. Tropica*	Afghanistan: Herat	78.24
10	KM454145.1	Human skin lesion-Amastigote	171	*L. Tropica*	Morocco	78.24
11	KC505432.1	Human skin lesion-Amastigote	171	*L. Tropica*	Iran: Birjand	78.24
12	AB787190.1	Human skin lesion-Amastigote	168	*L. Tropica*	Ghana	77.88
13	MN891726.1	Human skin lesion-Amastigote	167	*L. Tropica*	Pakistan	77.78
14	KX890190.1	Human skin lesion-Amastigote	176	*L. Tropica*	Iran: Bam	78.70
15	KP335128.1	Human skin lesion-Amastigote	171	*L. Tropica*	Afghanistan: Herat	78.24
16	KC505434.1	Human skin lesion-Amastigote	171	*L. Tropica*	Iran: Birjand	78.24
17	MF926263.1	Human skin lesion-Amastigote	167	*L. Tropica*	Syria	77.78

Phylogenetic analysis (Fig. 4) demonstrated that the *L. tropica* isolates from Mirjaveh (ON733094 and ON733095) formed a monophyletic clade along with isolates from southeastern Iran (Kerman and Bam) and neighboring countries like Pakistan and Turkey. This clustering suggests that *L. tropica* from Mirjaveh shares genetic similarities with strains circulating in adjacent regions, indicating potential regional connectivity in transmission dynamics.

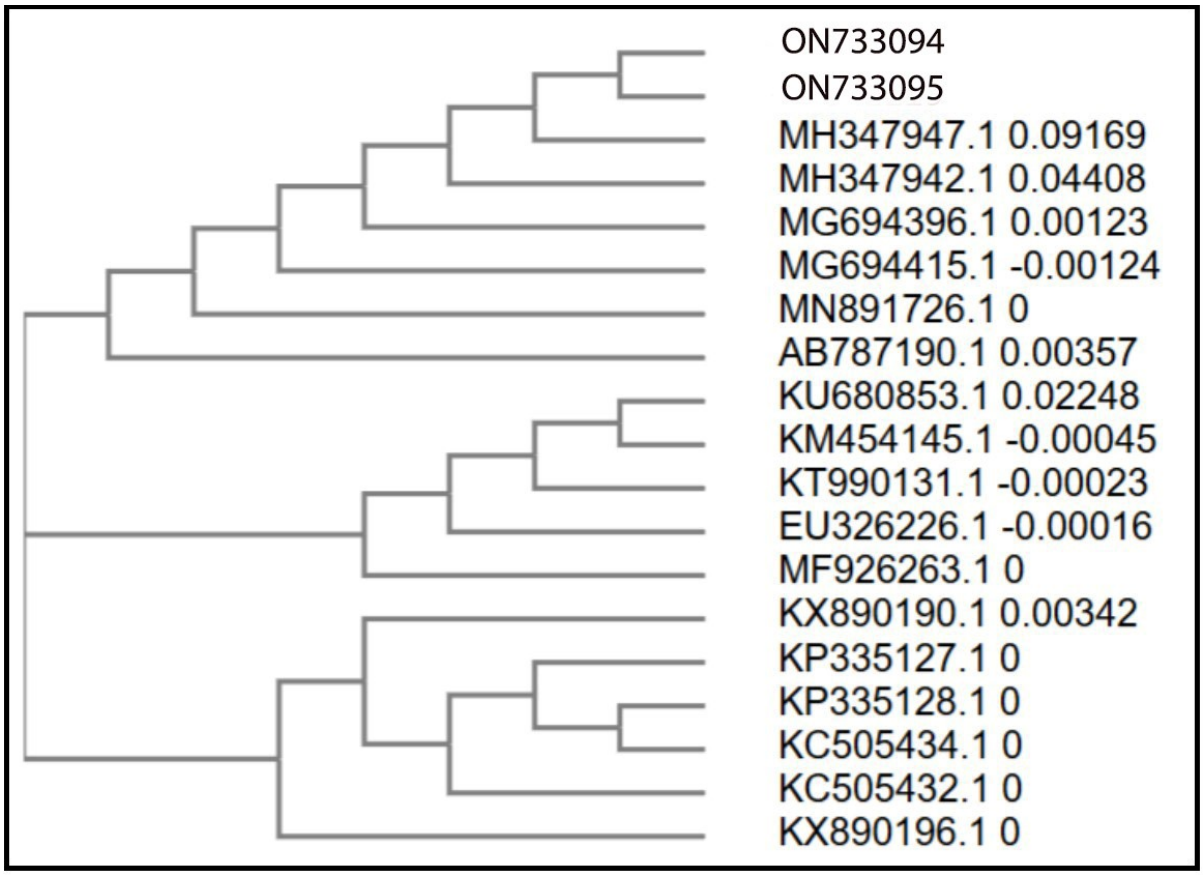

Fig. 4: Phylogenetic tree of various *L. tropica* isolates, illustrating the evolutionary relationships among different genetic sequences based on accession numbers. Sequences ON733094 and ON733095 are highlighted as they are directly related to this study

L. major Sequencing and Phylogenetic Analysis

Similarly, four other isolates from Mirjaveh were identified as *L. major* (ON733096, ON733097, ON733098, and ON733099) following BLAST and phylogenetic analysis. These sequences were deposited in GenBank with accession numbers ON733096 (BankIt2592060 43-34218-T_8-R_TOX-Reverse), ON733097 (BankIt2592060 44-34217-T_5-F_TOX-Forward), ON733098 (BankIt2592060 45-34216-T_4-F_TOX-Forward), and ON733099 (BankIt2592060 46-34215-T_2-F_TOX-Forward).

Compared to *L. tropica,* the *L. major* isolates from Mirjaveh exhibited greater genetic diversity, with sequence identities ranging from 90% to 98% when compared to reference strains from Iran and other countries. Phylogenetic analysis (Fig. 5) revealed that ON733096 and ON733098 clustered with *L. major* isolates from Mashhad (KP874100.1) and Aberkouh (KU680848.1) in Iran, and also with an isolate from Brazil (KU680857), forming a monophyletic group.

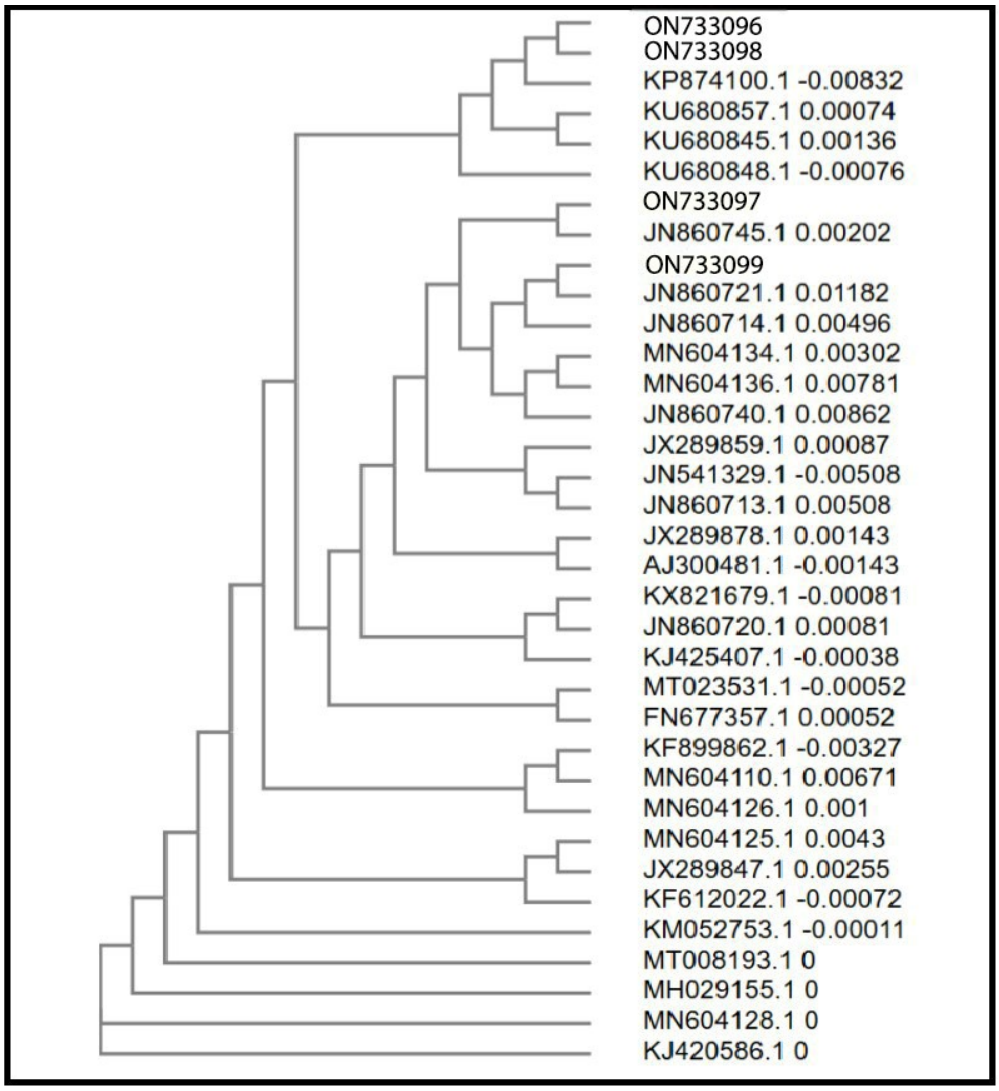

Fig. 5: The phylogenic tree of *L.major* is shown here and shows some species derived from polyclad clusters, illustrating the evolutionary relationships among different genetic sequences based on accession numbers. Sequences ON733096 ‘ ON733098 ‘ON733097 and ON733099 are highlighted as they are directly related to this study

ON733097 branched with *L. major* isolates from Kermanshah (JN860721.1), while ON733099 showed high similarity to an isolate from Isfahan (JN860721.1). The diversity observed among the *L. major* isolates may be attributed to the zoonotic nature of *L. major,* which has a wider host and vector range compared to *L. tropica*, contributing to more diverse transmission patterns in the region (Table 2).

Table 2: The most comparable *Leishmania* foremost ITS1 sequences in the World and Iranian species were submitted to GenBank

NO	*GenBank*	*Isolation source*	*Total score*	*Species*	*Country*	*Per Ident percent*
1	MN604136.1	Human skin lesion- amastigote	471	*L. major*	Morocco	97.85
2	MN604128.1	Human skin lesion- amastigote	442	*L. major*	Jordan	95.68
3	MH029155.1	Human skin lesion- amastigote	442	*L. major*	Iran: Bam	95.68
4	KU680857.1	Human skin lesion- amastigote	442	L. mexicana	Brazil	95.68
5	KF612022.1	Human skin lesion- amastigote	442	*L. major*	Thailand	95.68
6	KP874100.1	Human skin lesion- amastigote	442	*L. major*	Iran: Mashhad	95.68
7	KM052753.1	Human skin lesion- amastigote	442	*L. major*	Iran: Esfarayen	95.68
8	KJ420586.1	Human skin lesion- amastigote	442	*L. major*	Afghanistan	95.68
9	KF899862.1	Human skin lesion- amastigote	442	*L. major*	Iran: Ilam	95.68
10	KU680848.1	Human skin lesion- amastigote	513	*L. major*	Iran: Abrkouh	98.00
11	JX289878.1	Human skin lesion- amastigote	513	*L. major*	Kashan :Iran	98.00
12	MT023531.1	Human skin lesion- amastigote	512	*L. major*	Iran: Birjand	98.32
13	KU680845.1	Human skin lesion- amastigote	512	*L. major*	Russia	98.32
14	AJ300481.1	Human skin lesion- amastigote	512	*L. major*	Sudan	98.32
15	JX289859.1	Human skin lesion- amastigote	508	*L. major*	Esfahan :Iran	98.31
16	MN604110.1	Human skin lesion- amastigote	242	*L. major*	Morocco	76.87
17	JX289847.1	Human skin lesion- amastigote	218	*L. major*	Iran: Bam	76.41
18	KJ425407.1	Human skin lesion- amastigote	206	*L. major*	Iran: Esfarayen	76.47
19	JN860714.1	Human skin lesion- amastigote	387	*L. major*	Iran: Dehloran	96.73
20	JN860740.1	Human skin lesion- amastigote	383	*L. major*	Iran: Golestan	97.44
21	JN860721.1	Human skin lesion- amastigote	383	*L. major*	Iran: Kermanshah	97.44
22	JN860720.1	Human skin lesion- amastigote	383	*L. major*	Iran: Esfahan	97.44

Overall Phylogenetic and Geographic Insights

The phylogenetic trees (Figs. 4 and 5) demonstrate that *L. tropica* isolates from southeastern Iran, particularly Mirjaveh, are nearly identical genetically, suggesting a more conserved transmission pattern. In contrast, *L. major* isolates displayed more variation, possibly due to the broad-

er ecological and geographical range of this species.

In addition, alignments performed using Clustal Omega and Multalin software (Fig. 6) revealed high homology among *L. tropica* isolates, while *L. major* isolates showed more variability. These findings underline the distinct evolutionary dynamics of these two *Leishmania* species in the region.

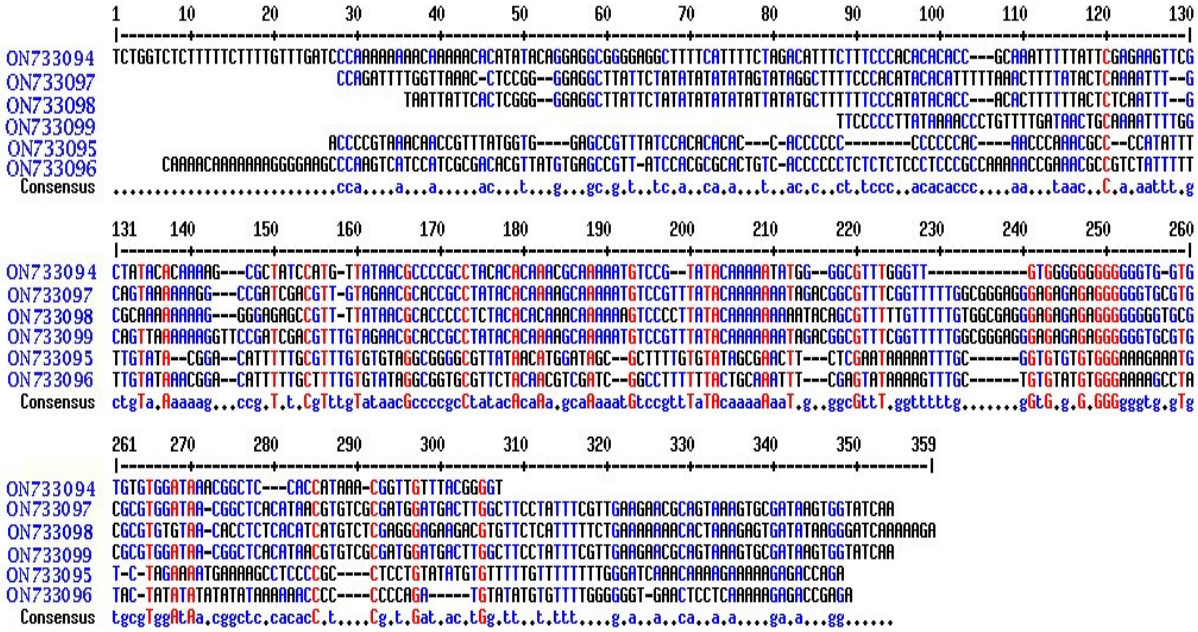

Fig.6: Sequence alignment of the ITS1- rDNA of *Leishmania* spp. isolated from patients, the region from Mirjaveh, Sistan, and Blugestan, Iran samples. Text size (for Image only): Medium bold, Text color (for Image only): black, Background color: white, High consensus color: red, Low consensus color: blue, Neutral color: black. This shows variable nucleotides and homology between samples isolated from Mirjaveh city and suburb, Southeast Iran

Geographic Distribution and Genetic Diversity

The geographic analysis of the sequenced isolates revealed that *L. major* exhibits greater genetic diversity than *L. tropica* in the study area. While *L. tropica* isolates from Mirjaveh formed a genetically homogeneous group with isolates from other parts of Iran and neighboring countries (Fig. 4), *L. major* showed more variation, with isolates clustering into distinct subgroups based on their geographic origin (Fig. 5).

The *L. major* isolate from Mirjaveh (ON733099) was closely related to an isolate from Isfahan (JN860721.1), while other isolates from the region shared closer genetic ties with isolates from Mashhad, Kermanshah, and even Brazil. This indicates that *L. major* may have multiple transmission foci in Iran, with possible introduction from distant regions or countries. The greater genetic diversity of *L. major* is consistent with its zoonotic transmission cycle, which involves multiple animal reservoirs and sandfly vectors, leading to more complex epidemiological patterns.

Comparison of Leishmania Species in the Region

The phylogenetic trees constructed for both *L. tropica* and *L. major* highlight important differences in the genetic diversity and transmission dynamics of these species. The *L. tropica* isolates from southeastern Iran showed a high degree of similarity, with almost 100% identity to each other and to other *L. tropica* strains from Iran and nearby regions, suggesting a more stable, human-to-human transmission cycle (Fig. 4).

In contrast, *L. major* isolates displayed greater genetic variation, with several distinct clades observed. This suggests a more complex zoonotic transmission cycle involving multiple reservoir hosts and vectors, which may explain the broader geographic distribution and genetic diversity observed among *L. major* strains in this region.

Alignment and Genetic Comparison

Sequence alignment using Clustal Omega and Multalin software revealed significant nucleotide variations between the *L. major* isolates compared to the *L. tropica* isolates (Fig. 6). The variable nucleotide sites identified in *L. major* may reflect the broader host range and ecological diversity of this species. In contrast, the *L. tropica* isolates displayed greater sequence conservation, reinforcing the hypothesis of a more restricted transmission cycle.

Discussion

The genetic diversity observed in *Leishmania* species is influenced by several factors, ranging from the parasite's distinct eco-epidemiological conditions to the molecular tools used for analysis (e.g., nuclear versus mitochondrial DNA markers) (19). These differences are essential for understanding variations in *Leishmania* populations and their transmission dynamics.

In this study, we found a higher prevalence of infection in males than females, likely because males engage more frequently in outdoor activities, thus exposing themselves to sandfly bites. This is consistent with the established understanding that outdoor workers are at higher risk for cutaneous leishmaniasis (CL) due to prolonged exposure to vectors. Additionally, most cases were observed in October and November, which aligns with the seasonal pattern of CL in the region. The disease likely peaks in the fall following a summer transmission period when sandfly activity is at its highest.

The current study confirms that both *L. tropica* and *L. major* are responsible for CL in Mirjaveh, a region that differs from Chabahar in southeastern Iran, where only *L. major* has been reported (19, 20). Parasitological methods, though considered the gold standard for diagnosing CL, depend heavily on the parasite load in clinical samples and require considerable technical skill, which can limit their sensitivity. A sensitivity range of 27% to 85% for parasitological methods in diagnosing leishmaniasis is reported (21).

The use of ITS1 gene sequencing provided valuable insights into the genetic diversity of *L. major* in this region. Phylogenetic analysis revealed that *L. tropica* isolates clustered with strains from Kerman (accession numbers MG694396 and MG694415), suggesting shared transmission routes or ecological conditions between these regions (Fig. 4). A broader phylogenetic analysis considering both host and vector evolutionary trends, in conjunction with geographic and climatic factors, would be essential for a more comprehensive understanding of *L.* transmission in this area (22). For instance, the main vector for *L. tropica*, *Phlebotomus sergenti*, has been shown to exhibit distinct genotypic variations across Iran, as reported using mitochondrial DNA markers (22).

Interestingly, the genetic similarity between *L. tropica* isolates from Mirjaveh and those from Bam in Kerman Province, both of which share desert-like climates, highlights the potential for geographic and climatic factors to influence the spread and evolution of the parasite. Bam has been recognized as a major focus of anthroponotic cutaneous leishmaniasis (ACL), further corroborating the close relationship between these regions (2, 23,24).

Our findings also revealed significant genetic diversity within *L. major* populations in Mirjaveh, which may be related to the diversity of zoonotic reservoir hosts. Considering that the reservoirs of important carriers of cutaneous leishmaniasis, namely *Ph. papatasi* and *Ph. salehi,* live in flat areas, and Mirjaveh and Rig Malek are in plain areas, perhaps that is why ZCL is seen more frequently as the predominant disease (25). The high genetic polymorphism observed in *L. major* isolates based on ITS1 and kDNA genes has also been reported in neighboring Afghanistan and other parts of

Iran (19). These polymorphisms suggest that *L. major* populations in this region are highly diverse, potentially due to the wide range of reservoir hosts and migration patterns.

The possibility of hybrid *Leishmania* strains emerging from cross-border transmission is also a concern, as hybrid strains have been reported in other regions such as Saudi Arabia and Ecuador (26). These hybrid parasites may combine the pathogenic traits of different species, potentially complicating treatment protocols and increasing disease severity.

Our findings further suggest that *L. major* strains from Mirjaveh may be spreading to adjacent areas, such as Kerman, and possibly extending into other parts of southeastern Iran and beyond. The close genetic relationship between *L. major* isolates from Kerman and Mirjaveh supports the hypothesis proposed that these strains may have originated in India, spread through Pakistan, and subsequently reached south-eastern Iran (27).

Conclusion

L. tropica and *L. major* are the primary causative agents of cutaneous leishmaniasis in the Mirjaveh region and surrounding villages near the Pakistan border. These species exhibit genetic similarities to isolates from neighboring countries like Afghanistan and Pakistan, as well as landlocked Iranian provinces such as Kerman and Birjand. This genetic connectivity highlights the importance of cross-border cooperation in controlling leishmaniasis and monitoring its spread.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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