Molecular Phylogeny of *Theileria equi* in horses of Khuzestan province based on 18S ribosomal RNA

Alireza Rocky[1], Abbas Jolodar[2*]

1. PhD student in the Faculty of Veterinary Medicine at the Shahid Chamran University of Ahvaz, Ahvaz, Iran.
2. Department of Basic Sciences, Biochemistry and Molecular Biology Section, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran.

Running title: Phylogeny of *T. equi* in horses of Khuzestan using 18S rRNA

[*]**Corresponding author:** Dr. Abbas Jolodar, Associate Professor in Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, 61355-145, Ahvaz, Iran. Phone: +98-61-33330073; Fax: +98-61-33360807; jolodara@scu.ac.ir

ORCID number of Abbas Jolodar: 0000-0001-9994-2281

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Abstract

Equine piroplasmosis is a tick-borne disease caused by two protozoan parasites, *Babesia caballi* and *Theileria equi* (formerly *Babesia equi*), transmitted by *Ixodid* ticks. In this study, a 435 bp fragment of the 18S ribosomal RNA (rRNA) gene was amplified from horse blood samples collected in Khuzestan Province, Iran, to assess the parasite's molecular phylogeny and predicted RNA secondary structure. Taxonomic analysis of the amplified sequence designated *Te*Kh, yielded 108 matches within the phylum *Apicomplexa*. Of these, 104 hits belonged to the family *Theileriidae*, and 86 were affiliated with the genus *Theileria*. Comparative analysis revealed that TeKh is identical to the only reported T. equi sequence from Fars Province, Iran (MK615933). Further multiple sequence alignment showed high similarity: 98.71% to a *T. equi* isolate from Russia (OM475523) and 99.74% to one from Turkmenistan (OL638195). Structural predictions using the RNAfold algorithm indicated a

thermodynamic free energy of −121.98 kcal/mol, suggesting a stable RNA secondary structure. The phylogenetic tree revealed that sequences in Cluster A, which included isolates from diverse countries, showed minimal divergence, indicating high genetic conservation of *T. equi* across geographic regions. In contrast, Cluster B exhibited greater genetic variability, including *T. bicornis* (AF499604) from South Africa and *Theileria* sp. (KF597078) from Kenya, while *T. cervi* (KT959227) from China formed a separate branch with moderate bootstrap support (44%). In conclusion, the *T. equi* sequences isolated from Khuzestan and Fars provinces in Iran showed no detectable genetic differences, highlighting strong intraspecific similarity that may reflect animal movement between these regions. Although the sample size in this phylogenetic analysis was limited, the consistently high sequence identity among *T. equi* isolates from various geographic regions suggests that further investigation is warranted before the 18S rRNA gene can be confidently employed as a species-specific molecular marker for diagnosing *T. equi* in equine hosts.

Key words: 18S ribosomal RNA, *Babesia*, Horse, Phylogeny, *Theileria equi*,

1. Introduction

Equine piroplasmosis is a tick-borne disease of equids caused by two protozoan parasites, *Babesia caballi* and *Theileria equi* (formerly *Babesia equi*), which are transmitted primarily by *ixodid* ticks. Clinically, the disease manifests with fever, inappetence, anemia, icterus, hemoglobinuria, and elevated respiratory and heart rates; in severe cases, mortality may occur. Equine piroplasmosis is endemic to most tropical and subtropical regions, posing a significant threat to equine health and international trade. According to the World Organization for Animal Health (WOAH), formerly known as the Office International des Epizooties (OIE), endemic areas include Africa, Asia, Central and South America, Cuba, Southern Europe, and the Middle East (1).

The taxonomic classification of the protozoan parasites responsible for equine piroplasmosis has been debated since their initial discovery, with the status of *T. equi* remaining particularly controversial (2). Currently, both *B. caballi* and *T. equi* are placed within the phylum *Apicomplexa*, which also includes other hemoprotozoa such as *Plasmodium* and *Theileria* (3). While *B. caballi* is widely accepted as a typical member of the genus *Babesia*, *T. equi* exhibits morphological and developmental features that align more closely with members of the genus *Theileria* (1). Molecular phylogenetic analyses have

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revealed that *T. equi* shares characteristics with both *Babesia* and *Theileria*, suggesting that it does not fit neatly into either genus. Several studies have suggested making a new genus to fit its special place in family tree, which lies intermediate between the two lineages (4-6).

Nuclear rRNA genes are widely regarded as suitable molecular targets for species-level identification (7, 8). However, sequence heterogeneity within the 18S rRNA gene of *T. equi* has been documented in various countries (9-11), raising questions about its taxonomic stability. Although Iran is located within a known endemic region for equine piroplasmosis, and infections caused by *T. equi* and *B. caballi* have been reported in equids from multiple provinces (12-14), no molecular characterization or phylogenetic analysis of these parasites has yet been conducted on Iranian isolates.

The purpose of this study was to explore the molecular characteristics and phylogenetic placement of *T. equi* isolates collected from horses in Khuzestan Province, Iran

2. Materials and methods

2.1. Sample collection.

EDTA-treated blood specimens were collected from five pure-bred Iranian Arab horses located in Ahvaz, Khuzestan Province, Iran. Blood was drawn from the jugular vein of each animal using sterile syringes and immediately transferred into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. All specimens were handled under standardized conditions to preserve DNA integrity for downstream molecular analyses in the Molecular biology laboratory of the Veterinary Medicine at Shahid Chamran University of Ahvaz.

2.2. DNA extraction.

Genomic DNA was isolated from 50 ml of EDTA-treated whole blood samples using the Rapid Genomic DNA Isolation Kit (MBST, Tehran, Iran), following the manufacturer's instructions. The concentration and purity of the extracted DNA were evaluated by measuring the A260/A280 absorbance ratio using NanoDrop microvolume spectrophotometers. Purified DNA was aliquoted and stored at -20 °C until further molecular analyses.

2.3. PCR amplification and sequencing.

To detect *T. equi*, primers targeting the 18S rRNA gene, Bec-UF2 (5′-TCGAAGACGATCAGATACCGTCG) and Equi-R (5′-TGCCTTAAACTTCCTTGCGAT) were used

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as described (15). PCR amplification was carried out in 25 µL reaction volumes, including 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.6 µM of each primer, 0.4 mM of each dNTP, 2 mM $MgCl_2$, 100 ng of genomic DNA, and 2.5 U of *Taq* DNA polymerase.

The Thermal cycling process was set up as follows: initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 60 °C for 50 seconds, and extension at 72 °C for 60 seconds, with a final extension at 72 °C for 5 minutes. The DNA fragments run on a 1% agarose gel stained with Safe Stain (Sinaclon, Tehran, Iran) and checked under UV illumination. PCR products showing positive amplification for *T. equi* were cexcised from the gel and purified using the GF-1 Gel DNA Recovery Kit (Vivantis, Malaysia). The Purified DNA fragments were then sequenced using dideoxy chain termination method on an Applied Biosystems 373 automated DNA sequencer.

2.4. DNA sequence analysis.

The amplified DNA fragments were subjected to sequencing in both strands using a dideoxy termination method and run on an Applied Biosystems 373 DNA sequencer. DNA sequence comparisons were performed using the blastn algorithm (16) through the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov) to identify species based on nucleotide homology. Homologous sequences were retrieved from the database for comparative analysis. Multiple sequence alignment of all retrieved and experimental sequences was carried out using the CLUSTAL_W program (17) hosted by the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). Phylogenetic analysis and genetic distance estimation were conducted by the neighbor-joining method with 1,000 bootstrap replicates, performed by MEGA11 software (18). The fractional GC content of the nucleotide sequences was calculated using the Sequence Manipulation Suite (http://www.bioinformatics.org/SMS/). The secondary structures of rRNA were visualized via the RNAfold web server (rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) was described (19).

3. Results

3.1. PCR amplification and sequence analysis

The quantity of nucleic acid extracted from blood samples tested was between 8 and 20 ng/µl.

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PCR-amplification was carried out to isolate an 18S rRNA gene fragment (Figure 1). Specifically, TeKh1, *Te*Kh3 and *Te*Kh4 were successfully amplified and sequenced in two directions. Final sequences were then determined using overlapping regions prior run against database.

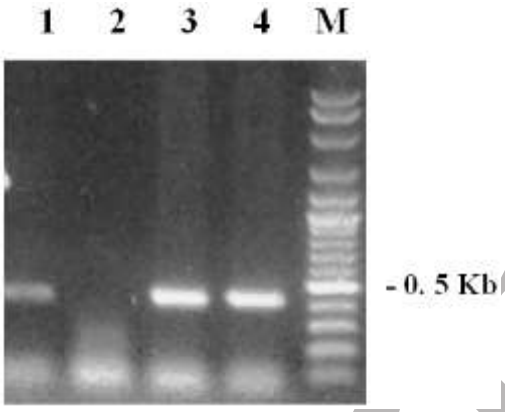

Figure 1. PCR amplification of partial 18S rRNA gene from *Theileria equi*, resolved on a 1% agarose gel**.** Lane M: GeneRuler DNA ladder size marker . Lane 1, 3, 4: PCR products corresponding to the *Te*Kh sequence. Lane 2: Negative control (no template)

Multiple alignment showed that these three sequences (*Te*Kh1, *Te*Kh3 and *Te*Kh4) were identical. This fragment was 435 bp and named *Te*Kh. The nucleotide composition for the amplified sequence was as follows: 111 A (25.52%), 84 C (19.31%), 125 G (28.74%) and 115 T (26.44%). Thus, the percentage of purine nucleotides (54.25%) in the fragment was slightly higher than the pyrimidine nucleotides (45.75%) and its GC count was 48%. In order to compare the amplified nucleotide sequence with the sequences in Genbank database, a comparison was made from the blastn program using the highly similar sequences (megablast) program.

Taxonomic analysis of *Te*KH found 108 matches in the order *Piroplasmida*, which is part of the phylum *Apicomplexa*. Of these, 104 hits were related to the family *Theileriidae*, and 86 were linked to the genus *Theileria*. Out of these 86, 74 were identified as *T. equi.* The results showed that the sequence of *Te*Kh is 100% identical to the only available sequence *Theileria equi* strain Fars 97 small subunit ribosomal RNA gene (MK615933) that was published in the Genbank database from Fars province of Iran in March 2019 as a direct submission.

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3.2. **Multiple alignment of *Te*Kh**

After removing the primers and trimming the sequence *Te*Kh, the 391 bp gene fragment was used for further analysis. In order to analyze the amplified sequence of *T. equi* from Khuzestan in the larger context, multiple alignments were conducted based on the *Te*Kh sequence with the retrieved sequences including the only *Theileria* sequence from Iran (strain Fars 97 small subunit ribosomal RNA; MK615933) using Clastal_W software. The amplified nucleotide sequence of *Te*KH in the aligned parts was 98.71% and 99.74% similar to sequence *T. equi* (OM475523l; Russia) and *T. equi* (OL638195; Turkmenistan), respectively. Among the all identical regions, only two differences were observed. A single G to A substitution found at position 234 of the *S. equi* from Turkmenistan (OL638195*)* which is the only non-conservative substitution within this stretch of nucleotide sequence. The Russian sequence (OM475523l) lacked a T at the position of nucleotide 361. *Te*Kh was identical to *T. equi* sequence isolated from the Fars province in Iran.

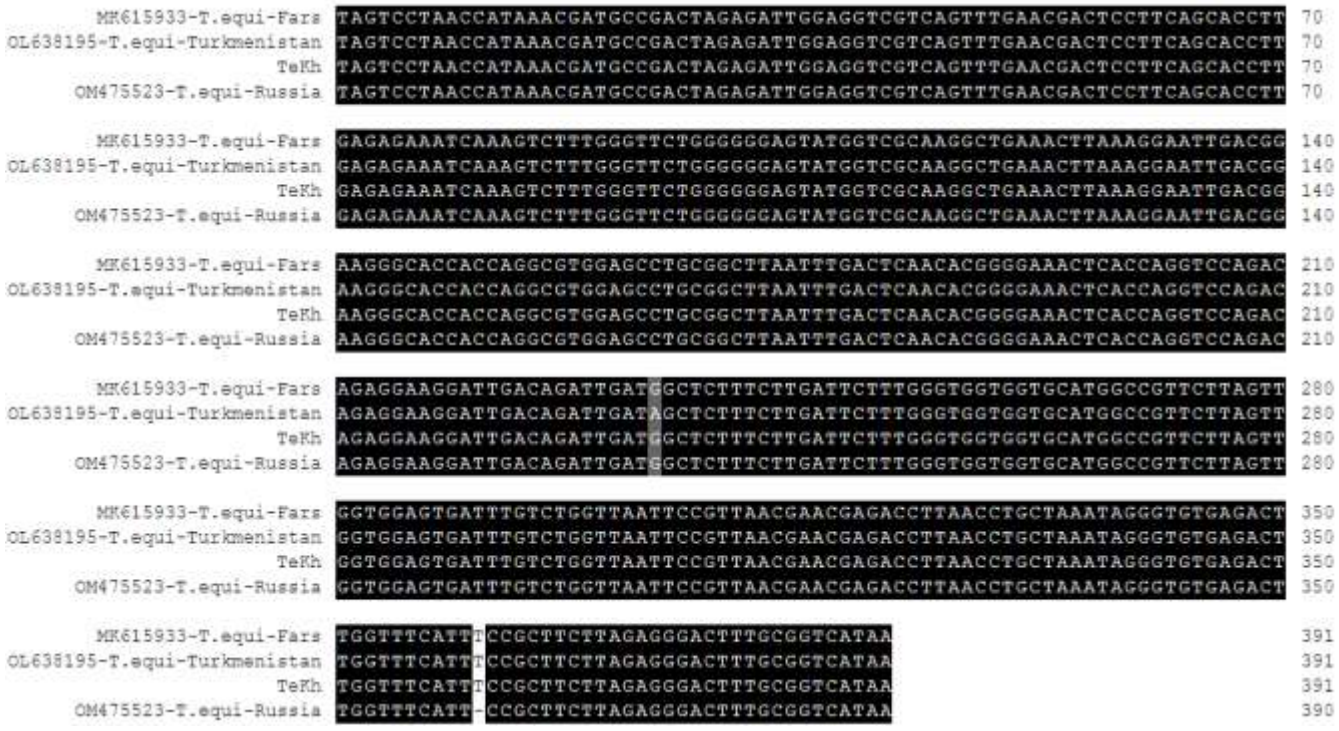

Figure 2: Alignments of the 18S rRNA nucleotide sequence *(Te*Kh) of Khuzestan with other *T. equi* sequences. Identical nucleotides are shown in dark color. Conserved nucleotides are shown in gray.

3.3. Secondary structure prediction of *Te*Kh

The secondary structure of the 18S rRNA sequence from *Te*Kh was predicted using the RNAfold algorithm (Figure 3). The analysis yielded a thermodynamic free energy of -121.98 kcal/mol, indicating that the RNA can adopt a highly stable folding conformation. However, the most stable configuration referred to as the minimum free energy (MFE) structure, was rarely populated, exhibiting a zero percent frequency in the predicted ensemble. This observation suggests that the RNA molecule possesses substantial structural flexibility, with multiple energetically favorable folding alternatives. This was further supported by a high ensemble diversity score of 156.57, reflecting a broad range of possible secondary structures within the thermodynamic ensemble.

A)

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AGUCCUAACC AUAAACGAUG CCGACUAGAG AUUGGAGGUC GUCAGUUUGA ACGACUCCUU      60
CAGCACCUUG AGAGAAAUCA AAGUCUUUGG GUUCUGGGGG GAGUAUGGUC GCAAGGCUGA     120
AACUUAAAGG AAUUGACGGA AGGGCACCAC CAGGCGUGGA GCCUGCGGCU UAAUUUGACU     180
CAACACGGGG AAACUCACCA GGUCCAGACA GAGGAAGGAU UGACAGAUUG AUGGCUCUUU     240
CUUGAUUCUU UGGGUGGUGG UGCAUGGCCG UUCUUAGUUG GUGGAGUGAU UUGUCUGGUU     300
AAUUCCGUUA ACGAACGAGA CCUUAACCUG CUAAAUAGGG UGUGAGACUU GGUUUCAUUU     360
CCGCUUCUUA GAGGGACUUU GCGGUCAUAA                                      390
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B)

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.......... .......... .......... .......... .......... ......((((      60
(((.((((.( (((....... ...)))).)) )).)))))) .......... ..........     120
.......... .......... ...((((((( .......... .......... ..........     180
.......... .......... .......... .......... .......... ..........     240
.......... ......)))) ))).....(( .......... .......... ..........     300
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)).))..))) )..))...)) ).))))....                                      390
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C)

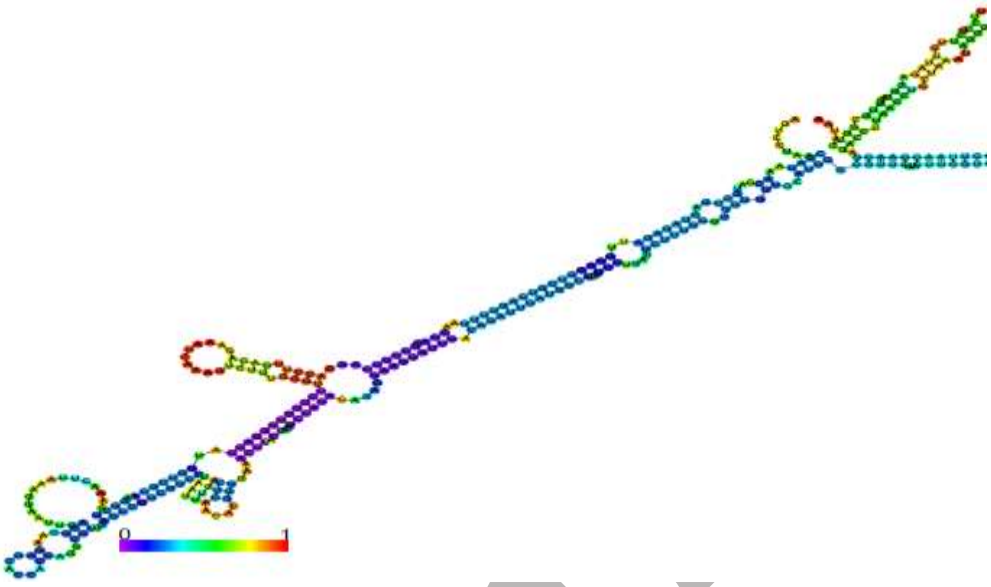

Figure 3. Predicted secondary structure of *TeKh*. (A) Primary RNA sequence of *TeKh*. (B) Secondary structure representation using dot-bracket notation, where dots indicate unpaired nucleotides, and paired parentheses denote base pairing. (C) Optimal secondary structure prediction based on minimum free energy (MFE), encoding base-pair probabilities.

3.4. **Phylogenetic analysis and genetic distance**

The phylogeny tree of *Te*Kh isolated from Khuzestan was created using the nucleotide sequence of the 18S rRNA gene. This was done using two methods: Neighbor-Joining method and maximum likelihood. Both methods gave very similar trees with strong support values. The phylogeny tree showed that all *T. equi* species in Cluster A were grouped together. However, *T.equi* from Turkmenistan (OL638195) and Russia (OM475523) were in separate group with a bootstrap value of 72% and 44%, respectively. The sequence from Khuzestan (*Te*Kh) and the only sequence from Iran which was isolated from Fars province (MK615933) were both in cluster A were identical. Cluster B was separated from cluster A with a moderate support (69%). More genetic diversity was observed in cluster B. Group B included *T. bicornis* (AF499604) from South Africa and *T. cervi* (KT959227) from China. Sequences from Switzerland which was *Babesia equi* (KM046920), and Kenya which was (*Theileria sp* (KF597078), were collected. The support for these sequences was 79% and 42%, respectively. The tree was built using the18S rRNA gene sequence of *Candida odintsovae* (AB054570) as the out group.

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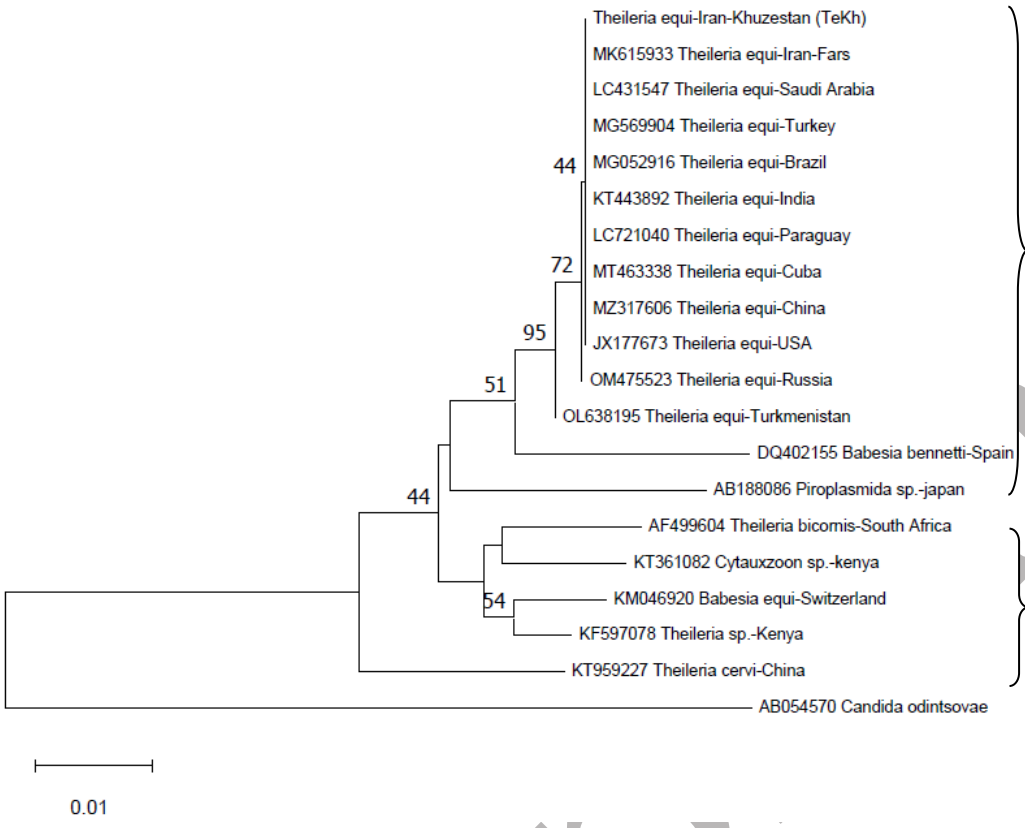

Figure 4: Phylogeny tree of *Te*KH isolated from Khuzestan and similar sequences of this species based on 18S rRNA gene using Neighbor -joining analysis. Bootstrap values are shown as percentages at each node based on 1000 replicates. The numbers before each species name are the accession numbers of the related genes in the GenBank database. The numbers above the lines indicate how the groups are connected. The length of each branch shows how many changes happened in the gene sequence, according to the scale provided.

The genetic distance between *T. equi* from Khuzestan (*Te*Kh) and other *T. equi* strains was calculated using MEGA11 (Table 1). The genetic diversity of *Te*Kh and other *T. equi* varied from 0% (*T. equi* strain Fars 97 small subunit ribosomal RNA gene; MK615933) to 0.03% (*T. equi* isolate UDSSR 91 small subunit ribosomal RNA gene; OL638195). The sequence of *Te*KH isolated from Khuzestan province was exactly the same sequence from Fars province in Iran. The genetic diversity of the *Te*KH with other sequences was between 2.4% and 4.2%. As expected, the genetic distances between *Te*Kh and the out group was 11.1%.

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Table 1: The genetic pairwise distances of *Te*Kh from Khuzestan using nucleotide sequence of 18S rRNA gene.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1- *T. equi*-Iran-Khuzestan (TeKh)										
2-MK615933 *T. equi*-Iran-Fars	0.000									
3-OL638195 *T. equi*-Turkmenistan	0.003	0.003								
4-AF499604 *T. bicornis*-South Africa	0.037	0.037	0.034							
5-KM046920 *B.equi*-Switzerland	0.026	0.026	0.024	0.037						
6-DQ402155 *B. bennetti*-Spain	0.024	0.024	0.021	0.053	0.045					
7-KF597078 *Theileria* Sp-Kenya	0.024	0.024	0.021	0.032	0.013	0.042				
8-KT959227 *T. cervi*-China	0.034	0.034	0.032	0.029	0.032	0.050	0.034			
9-KT361082 *Cytauxzoon* Sp-kenya	0.042	0.042	0.039	0.021	0.042	0.058	0.032	0.032		
10-AB188086 *Piroplasmida* Sp-japan	0.034	0.034	0.032	0.024	0.039	0.050	0.029	0.029	0.029	
11-AB054570 *Candida odintsovae*	0.113	0.113	0.111	0.126	0.121	0.113	0.118	0.118	0.129	0.124

4. Discussion

Phylogenetic analysis of Theileria based on the 18S rRNA gene showed a strong degree of connection in their evolution. relatedness among members of the *T. equi* species. In the resulting tree, the genera *Theileria* and *Babesia* are clearly delineated into two distinct clusters, reflecting their taxonomic separation. Cluster A comprises *T. equi* isolates recovered from ungulates, all exhibiting an exceptionally low genetic divergence of approximately 0.03%, underscoring their close evolutionary relationship. This cluster includes samples from Saudi Arabia, Turkey, Brazil, India, Paraguay, Russia, Cuba, China, and the USA, all showing minimal sequence variability. The high sequence conservation of the 18S rRNA gene in *T. equi* across diverse regions reflects its essential role in eukaryotic evolution. As part of a multi-copy gene family maintained through concerted evolution, its stability ensures consistent ribosome function. This conservation is thought to be evolutionarily advantageous, ensuring reliable ribosome function and cellular homeostasis (20). This evolutionary constraint is mirrored in the RNA's folding behavior, where thermodynamic analysis revealed a highly stable secondary structure (−121.98 kcal/mol) alongside an high ensemble diversity, suggesting both resilience and structural flexibility. Together, these features highlight the gene's functional importance and evolutionary persistence.

Phylogenetic analysis revealed that the *T. equi* sequence from Khuzestan clustered closely with the only available *T. equi* entry from Fars Province in the GenBank database, indicating high genetic

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similarity. The negligible divergence between these Iranian isolates suggests that animal movement between regions may contribute to the shared genetic profile, reflecting limited intraspecific variation within local equine populations. Cluster B was distinctly separated from Cluster A in the phylogenetic tree, supported by a 99% confidence level using the neighbor-joining method. While Cluster A comprised tightly grouped *T. equi* 18S rRNA sequences with minimal divergence, Cluster B encompassed a broader diversity of species, including *B. equi* and *B. bennetti*. Notably, a unique *Theileria* sp. (KF597078) isolated from a waterbuck in a region endemic for *T. parva* was also placed in Cluster B. Although no direct evidence implicates waterbuck in the transmission of *T. parva*, the detection of novel *Theileria* genotypes in this cattle-like species may have implications for wildlife-associated parasite reservoirs (21, 22).

The inclusion of *T. cervi* (KT959227), responsible for a piroplasmosis outbreak in Sika deer (*Cervus nippon*) in China, further highlights the complexity within Cluster B. That outbreak also marked the first detection of *T. annulata* and *B. motasi*, parasites typically affecting cattle and sheep, within Sika deer populations (23). Additionally, a lone sequence of *Cytauxzoon* sp. (KT361082), identified in a domestic cat from Kenya, was assigned to Cluster B. Although morphologically similar to *Theileria*, *Cytauxzoon* is distinguished by its ability to reproduce in macrophages, in contrast to *Theileria*, which replicates in lymphocytes (24).

In conclusion, the *T. equi* sequences isolated from Khuzestan and Fars provinces in Iran showed no detectable genetic differences, highlighting strong intraspecific similarity. Although the sample size in this phylogenetic analysis was limited, the consistently high sequence identity among *T. equi* isolates from various geographic regions suggests that further investigation is warranted before the 18S rRNA gene can be confidently employed as a species-specific molecular marker for diagnosing *T. equi* in equine hosts.

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Authors' Contribution

Study concept and design: A.J, A.R

Acquisition of data: A.J, A.R

Analysis and interpretation of data: A.J, A.R

Drafting of the manuscript: A.J, A.R

Critical revision of the manuscript: A.J

Statistical analysis: A.J

Ethics

The authors have observed all ethical points including non-plagiarism, double publication, data distortion and data manipulation in this article.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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