Journal of Zoonotic Diseases
https://jzd.tabrizu.ac.ir/
Published by the University of Tabriz
Online ISSN: 2717-2910

Genotyping of *Echinococcus granulosus* specimens isolated from dogs in southeastern Iran

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Article type:
Original article

Keywords:
Dog
Echinococcus granulosus
Genotyping
Cystic echinococcosis
Southeastern Iran

Article history:
Received:
April 30, 2026
Revised:
July 5, 2026
Accepted:
June 22, 2026
Available online:
June 22, 2026

Abstract

Cystic echinococcosis is an important zoonotic disease caused by the larval stage of *Echinococcus granulosus* and remains endemic in Iran. Identification of circulating genotypes in definitive hosts is essential for understanding local transmission patterns and supporting molecular epidemiological surveillance (1). A total of 260 fecal samples were collected from owned, stray, and shelter dogs in Kerman, Zabol, and surrounding areas in southeastern Iran. Samples were examined microscopically for taeniid eggs, and DNA was extracted from microscopically positive samples. Polymerase chain reaction (2) assays targeted an approximately 450 bp fragment of the mitochondrial cox1 gene and an approximately 460 bp fragment of the nuclear ITS1 region. Amplicons were sequenced and analyzed using BLASTn and phylogenetic approaches to determine genetic identity and relationships (3). Among the 260 samples examined, 17 contained taeniid eggs and/or cestode proglottids, and 13 samples (5%) were confirmed by PCR as *E. granulosus*. Clear single amplicons were obtained for both markers. BLASTn and phylogenetic analyses demonstrated that all isolates belonged to *E. granulosus* sensu stricto (G1–G3 complex). The *cox1* sequences showed high similarity to reference strains (98.07%–99.76%), indicating marked mitochondrial homogeneity. In contrast, ITS1 sequences showed greater variability (97.71%–99.75%) and were distributed into shallow subgroups related to isolates from neighboring endemic regions (2).The coexistence of mitochondrial homogeneity and moderate nuclear variation reflects the established genetic structure of the *E. granulosus* sensu stricto(s.s.) population in southeastern Iran. It supports the predominance of the G1–G3 complex in this region.

Introduction

Cystic echinococcosis (CE) (4) is a major zoonotic helminthic disease caused by the larval stage of *Echinococcus granulosus* and remains a significant public health and veterinary problem worldwide. The

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https://doi.org/10.22034/jzd.2026.21812
https://jzd.tabrizu.ac.ir/article_21812.html
Cite this article: Khoshsima Shahraki M., Nourollahifard S., Golchin M., Foladi B., Dabirzadeh M. Genotyping of *Echinococcus granulosus* specimens isolated from dogs in southeastern Iran. Journal of Zoonotic Diseases, 2026, X

parasite has an indirect life cycle involving canids, especially dogs, as definitive hosts and a broad range of herbivorous mammals, as well as humans, as intermediate hosts. Human infection occurs through accidental ingestion of eggs excreted in dog feces, typically via contaminated food, water, soil, or direct contact with infected dogs (5). In humans, CE most commonly involves the liver and lungs and may remain asymptomatic for years; however, depending on cyst size and location, it can cause abdominal pain, hepatomegaly, cough, chest pain, dyspnea, and, in severe cases, cyst rupture, secondary infection, or anaphylactic reactions (6). CE is widely distributed in endemic regions, including the Middle East, North Africa, Central Asia, China, South America, and parts of Europe. Its persistence is influenced by environmental conditions, livestock management systems, home slaughtering practices, and uncontrolled dog populations. Because of its medical and economic impact, echinococcosis has been recognized as a neglected tropical disease requiring integrated surveillance and control (7). The species *E. granulosus* has been reclassified into four main groups: *E. granulosus* sensu stricto (comprising genotypes G1–G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (encompassing genotypes G6–G10). strategies (8). Iran is one of the endemic countries for CE, and infections have been reported in humans, dogs, sheep, goats, camels, and cattle across different provinces. Previous molecular studies in Iran have shown that *E. granulosus* s. s is the dominant genotype in most human and animal isolates, although other genotypes have occasionally been identified. Southeastern Iran is epidemiologically important because of extensive livestock breeding, the presence of free-roaming and shepherd dogs, and cross-border animal movement. However, molecular data from canine definitive hosts in this region remain limited (9, 10). Mitochondrial markers such as cox1 are commonly used for genotype identification because of their high copy number and discriminatory value. Nuclear markers such as ITS1 can provide complementary information and may reveal additional intraspecific variation. However, partial marker data should be interpreted cautiously, and phylogenetic patterns should not be overextended beyond the resolution of the analyzed fragments (8, 11). Therefore, the present study was conducted to characterize *Echinococcus granulosus* s.l.isolates obtained from dogs in southeastern Iran using mitochondrial (cox1) and nuclear (ITS1) markers. The aims were to identify the predominant genotype(s), compare local isolates with previously reported sequences from Iran and neighboring endemic countries, and assess marker-specific patterns of genetic variation (1), compare local isolates with previously reported regional sequences, and assess marker-specific patterns of genetic variation.Zoonotic diseases cause substantial public health concerns due to direct contact with animals and consuming contaminated food or water (1). Brucellosis and coxiellosis are among the most important zoonotic infections presenting with nonspecific clinical manifestations such as fever, chills, and malaise (2). Brucellosis, caused by the *Brucella* genus, infects a wide range of domestic and wild animals as well as humans. This disease has always been considered for both economic loss and public health aspects (3). The geographic distribution of brucellosis varies in different countries. However, many parts of the world are considered endemic areas, including Africa, Latin America, Central Asia, the Mediterranean, and the Middle East, such as Iran (4). According to the intensity of the infection, the provinces of Iran are categorized into four types: very high, high, moderate, and low, among which Fars province is denoted as moderate incidence (5). Due to the impairing complication of brucellosis infection, a preliminary diagnosis is essential to fulfil a precise and on-time treatment (4).

Coxiellosis, also known as Q fever and caused by *Coxiella burnetii*, is another globally distributed zoonotic infection except New Zealand (6). The disease may appear in acute and chronic forms in humans. The disease presents in acute and chronic forms, ranging from flu-like illness to severe complications such as endocarditis (7). Domestic ruminants, particularly sheep, goats, and cattle, serve as primary reservoirs, and transmission mainly occurs via inhalation of contaminated aerosols. Thus, it mainly affects rural people having close contact with infected animals (8). There are few studies on coinfection of brucellosis and Q fever in the world (9, 10).

Despite the recognized endemicity of both brucellosis and Q fever in Iran, there is a striking lack of molecular-based studies investigating their concurrent occurrence in human populations, particularly in southern regions such as Fars province (11, 12). Moreover, the substantial overlap in clinical manifestations between these two infections poses a serious diagnostic challenge, frequently leading to underdiagnosis or misclassification of Q fever in patients primarily suspected of brucellosis (13). Given their shared transmission routes, common animal reservoirs, and similar clinical presentations, elucidating the prevalence and determinants of coinfection is essential for improving diagnostic accuracy and guiding appropriate therapeutic strategies in endemic areas.
To the best of our knowledge, this study represents one of the first molecular investigations assessing the prevalence, associated risk factors, and phylogenetic characteristics of *Coxiella burnetii* among PCR-confirmed brucellosis patients in Southwest Iran. By integrating molecular detection with epidemiological analysis, this study aims to provide novel insights into the hidden burden of Q fever within a well-defined brucellosis cohort.

Materials and Methods

Study area

This study was conducted in Kerman and Zabol in southeastern Iran. Zabol is located in the Sistan region near the Afghanistan border and is characterized by an arid climate, low rainfall, and seasonal dust storms. Kerman has a semi-arid climate with hot summers and cold winters. These areas were selected because of their relevance to livestock production, presence of stray and shepherd dogs, and potential for sustained transmission of CE (12, 13).

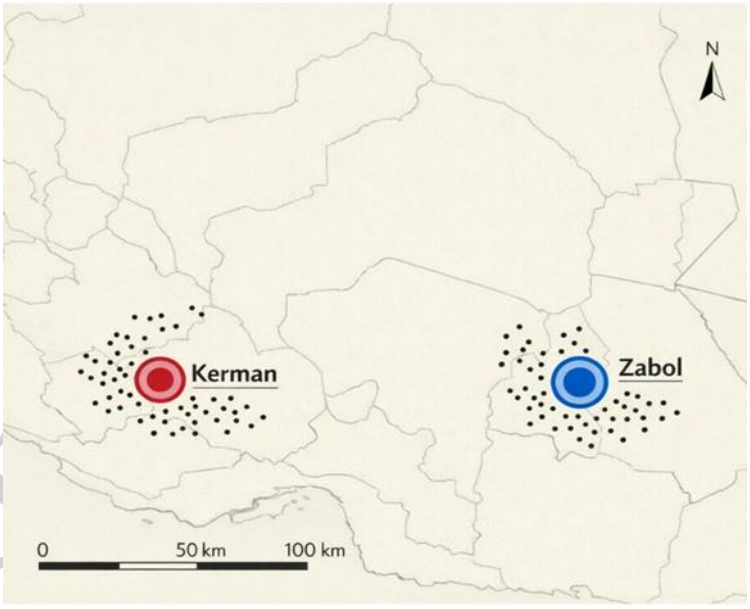

Fig. 1. Map of the study area in southeastern Iran showing Kerman, Zabol, and surrounding sampling locations.

Sample size and sampling

The sample size was estimated using Cochran's formula based on the prevalence reported in previous studies: $n=Z^2p(1-p)/d^2$ where Z=1.96, p=0.1787, and d=0.05 The minimum required sample size was calculated to be 227; therefore, 260 fecal samples were collected to compensate for possible sample loss (14). Sampling was conducted in during spring and summer 2024 in Kerman, Zabol, and the surrounding areas. A total of 260 dog fecal samples were randomly collected from stray dogs, herd dogs, and shelter dogs. Each sample was divided into two parts: one part was preserved in 10% formalin for parasitological examination, and the other in 70% ethanol for molecular analysis. The formalin–ether sedimentation method was used to concentrate taeniid eggs and examine fecal sediments microscopically (15).

DNA extraction

DNA was extracted from microscopically suspected samples using the MBST DNA extraction kit (MBST, Tehran, Iran) according to the manufacturer's instructions. Because taeniid eggs possess a thick and resistant wall, the samples were pretreated by repeated freeze–thaw cycles, enzymatic digestion with Proteinase K, and mechanical agitation to facilitate egg wall disruption and increase DNA recovery. The purified DNA was eluted based on the kit protocol and stored at −20°C until PCR amplification.

PCR amplification of cox1

A fragment of approximately 450 bp from the mitochondrial cox1 gene was amplified using primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′). PCR was performed in a total volume of 20 µL containing 2× master mix, template DNA, and primers. Cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 51°C for 45 s, extension at 72°C for 45 s, and a final extension at 72°C for 10 min. (16, 17)

PCR amplification of ITS1

The nuclear ITS1 region was amplified using primers EgF (5′-ATGGTTGTTATCGCTGCGA-3′) and EgR (5′-CAGAGCACTTTTGTATGCA-3′), yielding a fragment of approximately 460 bp. Cycling conditions included an initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s, with a final extension at 72 °C for 10 min. (17). Positive and negative controls were included in each PCR run.

Sequencing and phylogenetic analysis

The PCR products were sequenced bidirectionally using the Sanger method. The resulting chromatograms were manually inspected for quality, and low-quality terminal regions were trimmed. The edited sequences were compared with reference sequences available in GenBank using the BLASTn algorithm (18). Multiple sequence alignments were performed using MUSCLE (19). Phylogenetic trees were constructed in MEGA 11 using the Maximum Likelihood (ML) method (20). The best-fit nucleotide substitution model was determined within the software. To ensure accurate genotype assignment, representative GenBank reference sequences for the major *Echinococcus granulosus* s. l. genotypes (G1–G10) were included in the analysis. The reliability of the phylogenetic branches was assessed using 1,000 bootstrap replicates. The ML method was selected due to its robustness in estimating phylogenetic relationships and its suitability for analyzing genetically diverse genotypes included in this study.

Statistical analysis

Descriptive statistics were used to summarize microscopy and PCR findings. Percentages were calculated to estimate prevalence. Sequence comparisons and bootstrap analyses were performed using MEGA 11 (20), and additional statistical analyses were conducted using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA).

Results

A total of 260 dog fecal samples were examined. Seventeen samples contained taeniid eggs and/or cestode proglottids by parasitological examination. Of these, 13 samples were confirmed by PCR as *E. granulosus*, corresponding to an overall molecular positivity rate of 5%. All 13 PCR-positive samples generated amplicons of the expected sizes for both cox1 and ITS1. However, three samples yielded low-quality sequencing chromatograms and were excluded from downstream sequence analysis. Therefore, ten high-quality sequences for each marker were included in the final molecular and phylogenetic analyses.

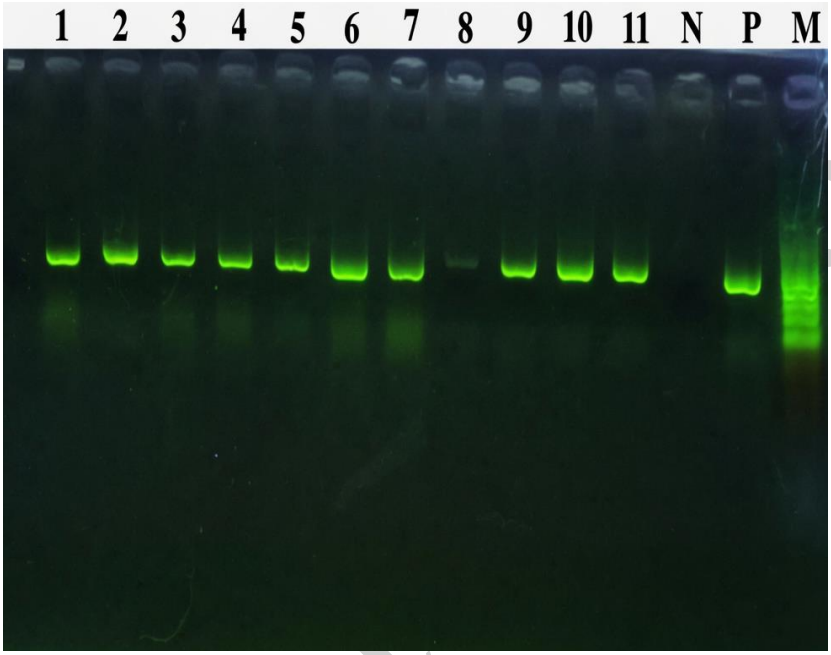

Fig. 2. Agarose gel electrophoresis of PCR products for the mitochondrial cox1 gene (~450 bp). M: 100 bp ladder; P: positive control; N: negative control.

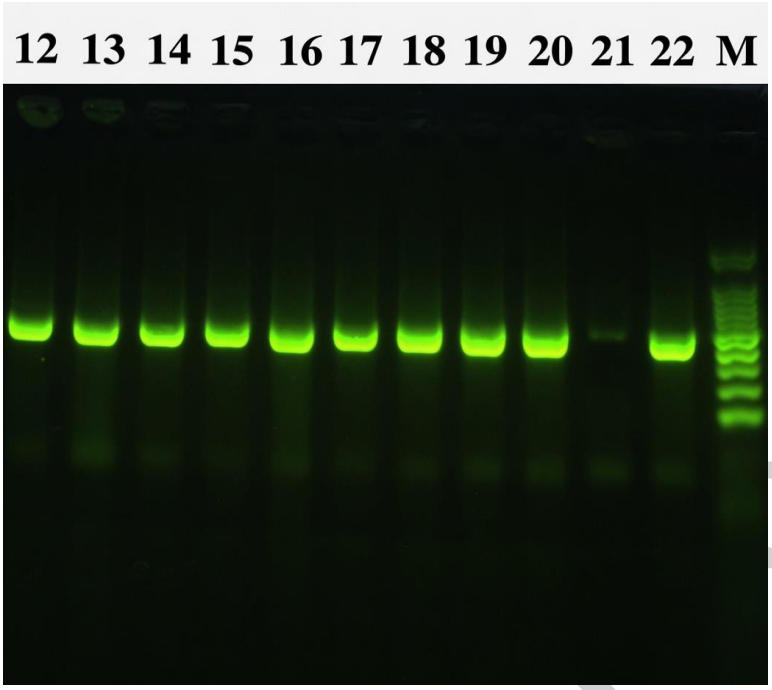

Fig. 3. Agarose gel electrophoresis of PCR products for the ITS1 region (~460 bp). M: 100 bp ladder; P: positive control; N: negative control.

Phylogenetic Analysis

The analyzed cox1 fragments exhibited limited nucleotide variation, consistent with a high degree of mitochondrial sequence conservation among the regional isolates. No non-synonymous substitutions were identified within the translated amino acid sequences of the analyzed fragments.The cox1 phylogenetic analysis revealed that all isolates from the present study clustered within the *Echinococcus granulosus s. s.* clade, specifically corresponding to the G1–G3 genotype complex. Similarly, in the ITS1 tree, the isolates were positioned within the *E. granulosus* s. l. complex and showed a close genetic relationship with the *E. granulosus s.s.* (G1–G3) reference sequences. However, due to the lower discriminatory power of the ITS1 marker and the relatively low bootstrap support observed for certain internal branches, genotype assignment was primarily determined based on the cox1 phylogeny, while ITS1 served as a complementary marker (Figs. 3 and 4).

Overall molecular findings

Both mitochondrial and nuclear markers consistently identified all analyzed isolates as belonging to *E. granulosus* s. s (G1–G3 complex). The mitochondrial cox1 locus displayed high conservation, whereas the nuclear ITS1 region showed broader variation. This mito-nuclear pattern is most appropriately interpreted as reflecting an established genetic structure and marker-specific diversity rather than direct evidence of recombination.

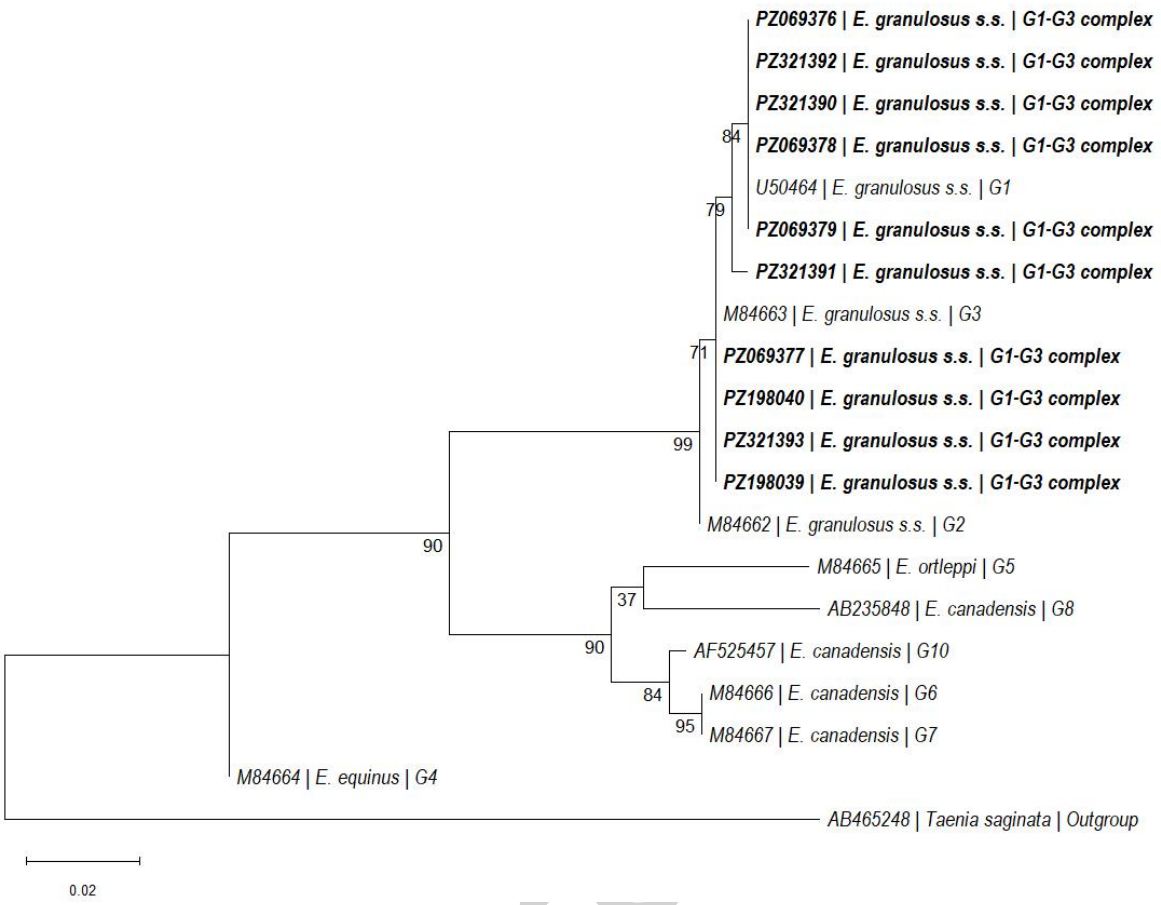

Fig. 4. Maximum Likelihood phylogenetic tree based on partial cox1 sequences of *Echinococcus granulosus* isolates. Representative GenBank reference sequences for the major *E. granulosus* s.l.genotypes, including G1–G8 and G10, were included for genotype assignment. *Taenia saginata* (AB465248) was used as the outgroup. Bootstrap values based on 1,000 replicates are shown next to the branches. Sequences generated in the present study are shown in bold. Tip labels include only the accession number, species name, and genotype/genotype complex. The isolates from the present study clustered within the *E. granulosus* sensu stricto G1–G3 complex.

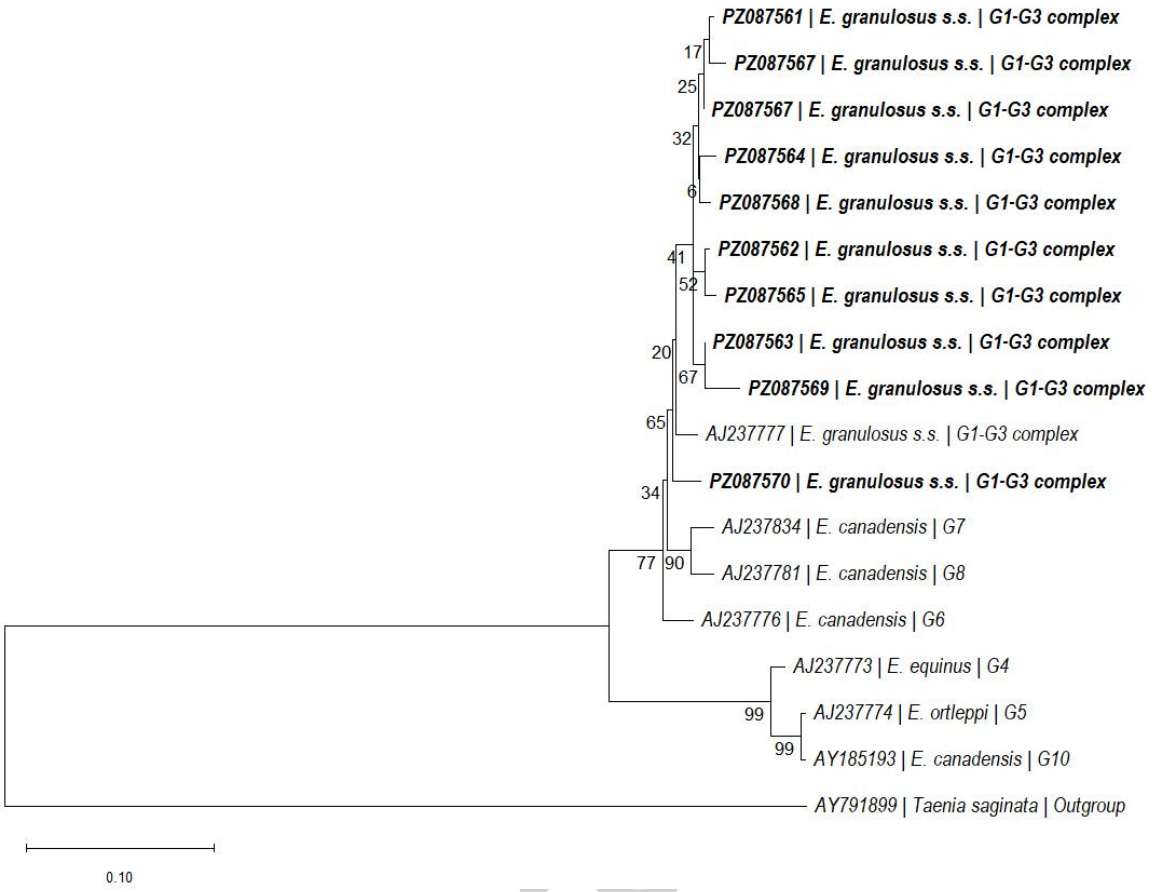

Fig. 5. Maximum Likelihood phylogenetic tree based on ITS sequences of *Echinococcus granulosus* isolates. Representative GenBank reference sequences of *E. granulosus* s.l.genotypes were included. Due to the limited discriminatory power of ITS for separating closely related genotypes within *E. granulosus* sensu stricto, AJ237777 was used as a representative of the G1–G3 genotype complex. *Taenia saginata* (AY791899) was used as the outgroup. Bootstrap values based on 1,000 replicates are shown next to the branches. Sequences generated in the present study are shown in bold. Tip labels include only the accession number, species name, and genotype/genotype complex.

Discussion

The present study demonstrated that *Echinococcus granulosus s.s.* (G1–G3 complex) is the predominant genotype circulating in dogs in southeastern Iran. This finding aligns with previous molecular studies from Iran and neighboring endemic areas, which have consistently shown the dominance of *E. granulosus s.s.* in both definitive and intermediate hosts (21, 22). The predominance of the G1–G3 complex reaffirms the continued importance of the synanthropic dog–sheep transmission cycle in this region. The cox1 sequences obtained in this study showed high similarity to previously published reference strains, with identities ranging from 98.07% to 99.76%. Phylogenetic clustering placed all isolates within a single well-supported mitochondrial clade. This result is consistent with earlier reports demonstrating limited mitochondrial variation among the G1 genotype across endemic regions (20). Importantly, while such clustering indicates strong genetic affinity at the analyzed locus, it should not be interpreted as direct proof of recent livestock exchange or specific transmission pathways

In contrast, ITS1 exhibited measurable nuclear variation (nucleotide diversity, $\pi = 0.017$), which may reflect a combination of factors, including mixed infections, multiple infection sources, or the introduction of regional variants. The apparent mito-nuclear divergence can be interpreted in light of the distinct biological properties of mitochondrial versus nuclear loci (e.g., uniparental inheritance pattern and smaller effective population size), rather than implying definitive recombination based on partial sequences. In addition, relatively short mitochondrial DNA sequences, such as the partial cox1 gene, may not always provide sufficient resolution to distinguish among genotypes within the G1–G3 complex clearly. Consequently, as previously suggested, substantially longer mtDNA sequences or mitogenomic approaches are needed to distinguish between these closely related genotypes reliably (4). Accordingly, the discordance between mitochondrial conservation and broader ITS1 variability should not be over-interpreted as evidence of recombination, especially when only partial fragments are analyzed. Rather, this pattern may reflect marker-specific evolutionary rates, standing intraspecific diversity, local demographic history, and the inherent population structure of the parasite (23, 24). In this context, the results are more appropriately understood as indicating moderate nuclear variation within an established regional population. Comparison with previously reported isolates from Iran and neighboring countries supports this interpretation. The cox1 identities observed here fall within the range commonly reported for G1-dominant populations in the Middle East and Western Asia. Likewise, ITS1 variation remained limited and compatible with the intraspecific diversity described in similar endemic settings. The presence of only a limited number of polymorphic nucleotide sites in the ITS1 alignment further supports the view that the detected variation reflects natural population-level diversity rather than deep taxonomic divergence (25).

From a public health perspective, the predominance of *E. granulosus s.s.* in dogs is of critical importance, as this genotype complex is the primary agent associated with human cystic echinococcosis (CE). In endemic rural settings, infected dogs contribute to significant environmental contamination with parasite eggs, thereby perpetuating transmission to livestock and humans. Therefore, control strategies in southeastern Iran should continue to emphasize routine deworming of dogs, strict prevention of dog access to infected offal, improvements in slaughter hygiene, and community education. These measures are particularly vital given that multiple studies from various regions of Iran have documented a range of *E. granulosus* genotypes in both intermediate and definitive hosts, including sheep, goats, cattle, and camels (25). The predominance of *Echinococcus granulosus s.s.* in the present study aligns with earlier molecular investigations conducted in dogs and other canids from different regions of Iran. A survey of free-roaming dogs in southeastern Iran (Kerman) identified genotype G1 in all cox1-sequenced isolates, supporting the dominant circulation of the sheep strain in this area (26) . Similarly, reports from northeastern and western Iran have described G1 as the predominant genotype in dogs, although G3 and G6 were also detected in some settings, suggesting a degree of regional genetic heterogeneity. Comparable patterns have been documented in wild canids from northern Iran, where *E. granulosus s.s.* (G1) was confirmed through multilocus sequencing (ITS1, cox1, and nad1), highlighting the potential role of wildlife reservoirs in maintaining zoonotic transmission cycles (10).

Differences among studies should be interpreted cautiously, considering geographic variation, host composition, sample size, and the molecular markers applied. For instance, the detection of G3 and G6 in northeastern and western Iranian dog's contrasts with the more uniform pattern observed in our isolates and in the Kerman study, where only G1 was reported (17, 26). This discrepancy may reflect local differences in livestock species composition and parasite transmission cycles, as well as methodological variation (e.g., PCR-RFLP versus direct sequencing, and mitochondrial versus nuclear loci) that may influence the resolution of genotype detection (10, 14, 27, 28). Moreover, prevalence estimates in Iranian canids vary widely across studies—from relatively low levels in wild canids to higher rates in stray dogs—which may be influenced by factors such as dog age structure, scavenging behavior, access to infected offal, and local management practices (26, 27, 29).Overall, while our findings support the predominance of *E. granulosus s.s.* in Iranian definitive hosts, the observed regional variation underscores the importance of continued molecular surveillance using standardized genetic markers and broader geographic sampling (28, 29).

Conclusion

This study confirms the predominance of *Echinococcus granulosus* s. s. (G1–G3 complex) in dogs from southeastern Iran. The observed mitochondrial homogeneity together with moderate nuclear variation suggests

the presence of an established genetic structure within the regional parasite population. These findings emphasize the epidemiological importance of the dog–sheep transmission cycle and highlight the value of combining mitochondrial and nuclear markers in molecular surveillance of cystic echinococcosis. This study has several limitations, including the relatively small number of successfully sequenced isolates and the analysis of only two partial genetic loci. In addition, the phylogenetic resolution of short marker fragments is limited for fine-scale population inference. Future studies should incorporate larger sample sizes, broader geographic coverage, and additional mitochondrial and nuclear markers or genomic approaches to better clarify transmission dynamics and parasite population structure in southeastern Iran.

Acknowledgements
The authors would like to thank Mahdi Rezaei Kahkha-zhaleh for assistance with the statistical analyses. The support of the parasitology laboratories in Kerman, Mashhad, and Zabol, as well as the College of Veterinary Medicine, University of Kerman, is gratefully acknowledged.

Ethical approval
This study was approved by the Ethics Committee of Zabol University of Medical Sciences (approval code: IR.ZBMU.REC.1402.169).

Conflict of Interest
The authors declare no conflict of interest.

Artificial Intelligence Statement
The authors declare that no artificial intelligence tools were used in the design of the study, data collection, data analysis, or interpretation of the findings. Artificial intelligence–assisted tools were used only for limited language editing. The authors take full responsibility for the final content of the manuscript.

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