

## Original Article

# Rapid Identification of *Echinococcus granulosus* and *E. canadensis* Using High-Resolution Melting (HRM) Analysis by Focusing on a Single Nucleotide Polymorphism

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**SUMMARY:** High-resolution melting (HRM) is a reliable and sensitive scanning method to detect variation in DNA sequences. We used this method to better understand the epidemiology and transmission of *Echinococcus granulosus*. We tested the use of HRM to discriminate the genotypes of *E. granulosus* and *E. canadensis*. One hundred forty-one hydatid cysts were collected from slaughtered animals in different parts of Isfahan-Iran in 2013. After DNA extraction, the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene was amplified using PCR coupled with the HRM curve. The result of HRM analysis using partial the sequences of *cox1* gene revealed that 93, 35, and 2 isolates were identified as G1, G3, and G6 genotypes, respectively. A single nucleotide polymorphism (SNP) was found in locus 9867 of the *cox1* gene. This is a critical locus for the differentiation between the G6 and G7 genotypes. In the phylogenetic tree, the sample with a SNP was located between the G6 and G7 genotypes, which suggest that this isolate has a G6/G7 genotype. The HRM analysis developed in the present study provides a powerful technique for molecular and epidemiological studies on echinococcosis in humans and animals.

## INTRODUCTION

*Echinococcus granulosus*, which causes hydatid cysts in humans and animals, is one of the most important zoonotic parasites. This parasite has a global distribution in many countries in Europe, Africa, America, and Asia, including Iran (1–4). Echinococcosis causes high mortality in humans and economic losses in livestock (1). Recent studies on partial sequences of the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene, and other regions of the genomic DNA, such as internal transcribed spacer 1 (*ITS-1*) in the ribosomal DNA, suggested that *E. granulosus* has 10 different genotypes, G1–G10 (5–9). Recent taxonomic categorization has classified 5 species of *E. granulosus*, including *E. granulosus sensu stricto*, (G1–G3 genotypes), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6–G10), and *E. felidis* (10,11).

High-resolution melting (HRM) analysis monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time), as opposed to the endpoint detection (12). HRM provides a rapid, low-cost, and sensitive scanning

method to detect variation in DNA sequences using a single step (13). In particular, HRM based on EvaGreen (Biotium, Hayward, CA, USA) can be used to detect variations, such as, single nucleotide polymorphisms (SNPs) in DNA (14). The presence of SNPs is characterized by changes in protein expression and the phenotypes of organisms. When a SNP is located in a critical loci, for example, in the promoter or enhancer regions of the DNA, gene function may be altered, resulting in expected changes in the protein structure and/or biological effects (15–18). In different amplicons, melting temperature ( $T_m$ ) is very different, and the results are associated with length of the nucleotide and the percentage of guanine-cytosine (GC) content (16).

The aim of this study was the rapid identification of *E. granulosus* and *E. canadensis* genotypes using HRM analysis, by focusing on the SNP in locus 9867 of the *cox1* gene.

## MATERIALS AND METHODS

**Sample collection:** One hundred forty-one hydatid cysts were collected from slaughtered animals in different regions of Isfahan, including Fasaran, Khomeinishahr, and Najafabad-Iran, between January 2013 and March 2013. Of the 141 isolates, 11 cattle isolates were sterile and excluded from further investigation. Molecular survey and PCR amplification were successfully performed on the remaining 130 isolates. Of these 130 isolates, 116 were collected from sheep (72 from the livers and 44 from the lungs), 7 from goats (4

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from livers and 3 from the lungs), and 7 from cattle (all from the lungs). Separation of hydatid cysts was performed under sterile conditions, and hydatid cyst protoscoleces and/or germinal layers were collected from each hydatid cyst. Protoscoleces and germinal layers were preserved in 70% (v/v) ethanol and stored at  $-20^{\circ}\text{C}$  until DNA extraction.

**DNA extraction:** Before DNA extraction, the ethanol in the samples of protoscoleces and/or germinal layers was removed by washing 3 times with sterile distilled water. Genomic DNA was extracted using a genomic DNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's instruction with minor modification. The concentration of the extracted DNA was determined by Nano Drop (Thermo Scientific, Rockford, IL, USA), and the samples were stored at  $-20^{\circ}\text{C}$  for further analysis.

**PCR and DNA sequencing:** The *cox1* gene was amplified using specific primers (forward JB3 primer [5'-TTT TTT GGG CAT CCT GAG GTT TAT-3']) and reverse JB4.5 primer [5'-TAA AGA AAG AAC ATA ATG AAA ATG-3']) as previously described by Bowles et al. (5). PCR was performed in 25  $\mu\text{L}$  final reaction volume containing 10  $\mu\text{L}$  master mix (Type-it HRM PCR Kit; Qiagen, Hilden, Germany), 10  $\mu\text{L}$  distilled water, 1  $\mu\text{L}$  of each primer, and 4  $\mu\text{L}$  of template DNA. The enzymatic reaction was performed as follows: an initial denaturation step at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles at  $95^{\circ}\text{C}$  for 10 s (denaturation),  $55^{\circ}\text{C}$  for 30 s (annealing),  $72^{\circ}\text{C}$  for 27 s (extension), and a final extension step at  $72^{\circ}\text{C}$  for 5 min. The kit contained the novel double-stranded DNA-binding fluorescent dye, EvaGreen, and an optimized HRM PCR master mix buffer, consisting of HotStarTaq *plus* DNA polymerase, Q-Solution, and dNTPs. Real-time PCR was carried out in a Mini Opticon real-time PCR detection system (Rotor-Gene 6000; Qiagen) under the following conditions: the reaction mixture was heated at  $95^{\circ}\text{C}$  for 3 min for denaturation of DNA. Next, 40 cycles of amplification were performed, each cycle included  $95^{\circ}\text{C}$  for 10 s,  $55^{\circ}\text{C}$  for 30 s, and  $72^{\circ}\text{C}$  for 27 s, followed by a final extension at  $72^{\circ}\text{C}$  for 5 min. Fluorescence signals were measured after each amplification cycle. The melting temperature was raised from  $65^{\circ}\text{C}$  to  $85^{\circ}\text{C}$  at  $0.2^{\circ}\text{C}$  per 2 s. The quantitative detection and  $T_m$  were obtained using the Rotor-gene 6000 series software ver. 1.7 (Qiagen).

Two replications of each sample were analyzed.  $T_m$  analysis was repeated thrice in each run to confirm the repeatability of the  $T_m$  assay by estimating the  $T_m$  variation within a PCR amplification (intra-assay), and between PCR amplifications (inter-assay). The coefficient of variation (CV) was calculated by dividing the standard deviation (SD) by the arithmetic mean of the measured values of  $T_m$  ( $\text{CV} = [\text{SD}]/\text{mean value}$ ). Further, to check the uniformity of temperature in the cycler block, a number of genotype samples were re-amplified at different positions of the cycler block during the same amplification cycle. The intra-assay CVs represent the mean CVs of the results obtained from the replications of all the *E. granulosus* genotypes in the same run. The inter-assay CVs represent the mean CVs for the results obtained from 3 separate runs.

To confirm the genotype identification performed by

the EvaGreen real-time PCR assay, and due to the similarity of HRM PCR results for isolates in each genotype, 22 samples were randomly sequenced for *cox1* mitochondrial DNA. Three samples that were sequenced for *cox1*, exist in GenBank and were identified as G1, G3, and G6 (accession numbers: HM563013, HM563017, and HM563018, respectively). G1, G3, and G6 were included in each PCR set as positive controls.

**Sequence homology and phylogenetic analysis:** The obtained continuous sequences were compared with previously published sequences of the mitochondrial *cox1* gene for *E. granulosus* genotypes in NCBI using BLAST. Phylogenetic analyses of the sequence data were inferred with maximum likelihood and multiple alignments were done using MEGA 5 of the computer program CLC main workbench software (ver. 5.2.1, 2013; CLC-Bio, Waltham, MA, USA) (19–21). Therefore, the Kimura 2-parameter model was selected by best model search using Find Best DNA Model.

## RESULTS

According to the HRM PCR analysis, 93, 35, and 2 isolates were identified as G1, G3, and G6 genotypes, respectively. The percentages of each genotype from animals were as follows: sheep (G1, 71%; G3, 28%; and G6, 1%), goats (G1, 71%; G3, 14%; and G6, 15%), and cattle (G1, 86%; G3, 14%; and G6, 0%). The distribution of *E. granulosus* genotype in different parts of Isfahan province is shown in Fig. 1. As shown in Table 1, the real-time PCR melting curve results indicated that the mean  $T_m$  of the G1, G3, and G6 genotypes was  $79.50^{\circ}\text{C}$ ,  $79.24^{\circ}\text{C}$ , and  $77.70^{\circ}\text{C}$ , respectively. Assessment of intra- and inter-assay variability showed low and acceptable CVs. (Table 1). The melting curve and HRM curve analysis of the hydatid cysts identified by sequencing are shown in Fig. 2 and 3. The sequencing results confirmed that all isolates were correctly differentiated by HRM analysis. All of the isolates identified by post real-time PCR (HRM analyses) were clustered along with the corresponding reference genotypes and are shown in Fig. 4. The phylogenetic tree was divided into 2 main clades: the first clade contained 2 subclades corresponding to the G1 and G3 genotypes (*E. granulosus sensu stricto*), and the second clade corresponded to the isolates identified as G6/G7 (*E. canadensis*) along with the individual reference genotypes.

Phylogenetic analysis also revealed that 7 isolates of the G1 genotype had microvariants compared with the original sequences of the *cox1* gene (Accession No. M84661), and one of them (IREG.SH18) was entirely consistent with the original Iranian sequences of the

Table 1. Mean  $T_m$ , SD, and CV calculated based on intra- and inter-assay of each genotype of *E. granulosus*

genotype	Mean $T_m$ ( $^{\circ}\text{C}$ )	SD	Intra-assay CV (%)	Inter-assay CV (%)
G1	79.50	0.14	0.07	0.12
G3	79.24	0.14	0.09	0.15
G6	77.70	0.10	0.05	0.10

CV, coefficient of variation.

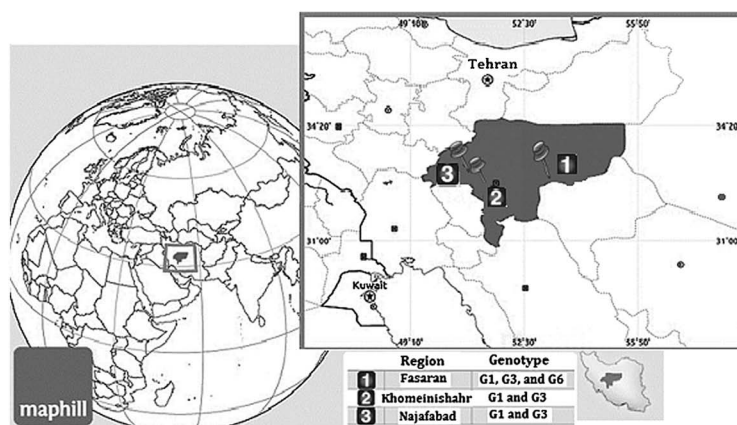


Fig. 1. Map of the study areas. Isfahan province is indicated by gray color and the study areas, Fasaran, Khomeinishahr, and Najafabad, are marked.

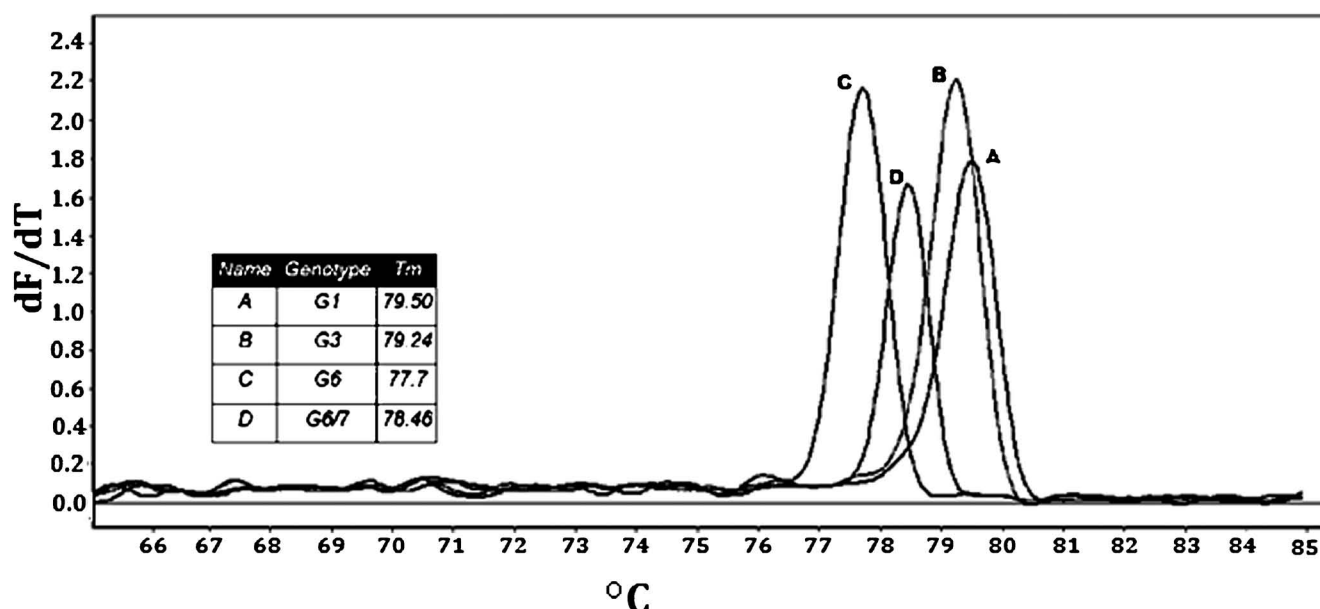


Fig. 2.  $T_m$  of the analyzed hydatid cysts identified by sequencing (A-D). (A) G1, (B) G3, (C) G6, and (D) G6/7 genotypes.

*cox1* gene (Accession No. AF297617). These variations were located in 3 subtypes and named G1 $\alpha$ , G1 $\beta$ , and G1 $\gamma$ . Four samples were identified as G3 and 1 sample as G6 and were completely identical to the reference sequences for the *cox1* gene ([Accession No. M84662] of the G3 and [Accession No. M84666] of the G6). In one isolate (IREG.GO3) of the 22 samples, sequencing confirmed the SNP in locus 9867.

## DISCUSSION

In the present study,  $T_m$  analysis was used to determine the *E. granulosus* genotypes (G1, G3, and G6) in Isfahan province, Iran. The mean  $T_m$  differences between the G1 and G3 genotypes were  $>0.2^\circ\text{C}$ . Our strategy to raise the  $T_m$  in intervals of  $0.2^\circ\text{C}$  allowed accurate separation of the 2 genotypes. The G1 and G3 genotypes can easily be distinguished from the G6 geno-

type, based on the differences in the  $T_m$  for this genotype (about  $1.5^\circ\text{C}$ ).

One sample sequence has a  $T_m$  different from G1, G3, and G6 and is located among them (Fig. 3). This isolate is similar to the G6 genotype; however, its  $T_m$  was different ( $78.46^\circ\text{C}$ ) diverging about  $0.8^\circ\text{C}$  from the  $T_m$  of the G6 genotype. This sample had a SNP in the critical locus 9867 in the complete mitochondrial genome. This position is a critical locus for differentiation between the G6 and G7 genotypes. In this locus, nucleotides thymine (T) and C have been located in the G6 and G7 genotypes, respectively. However, in this isolate, neither nucleotide T nor C was present, instead, the nucleotide G was found, and this was confirmed with 2 repetitions of the sequencing. In the phylogenetic tree, this isolate was located between the G6 and G7 genotypes, which suggest that it may be the G6/G7 genotype. As the G7 genotype has not been reported previously in Iran, this

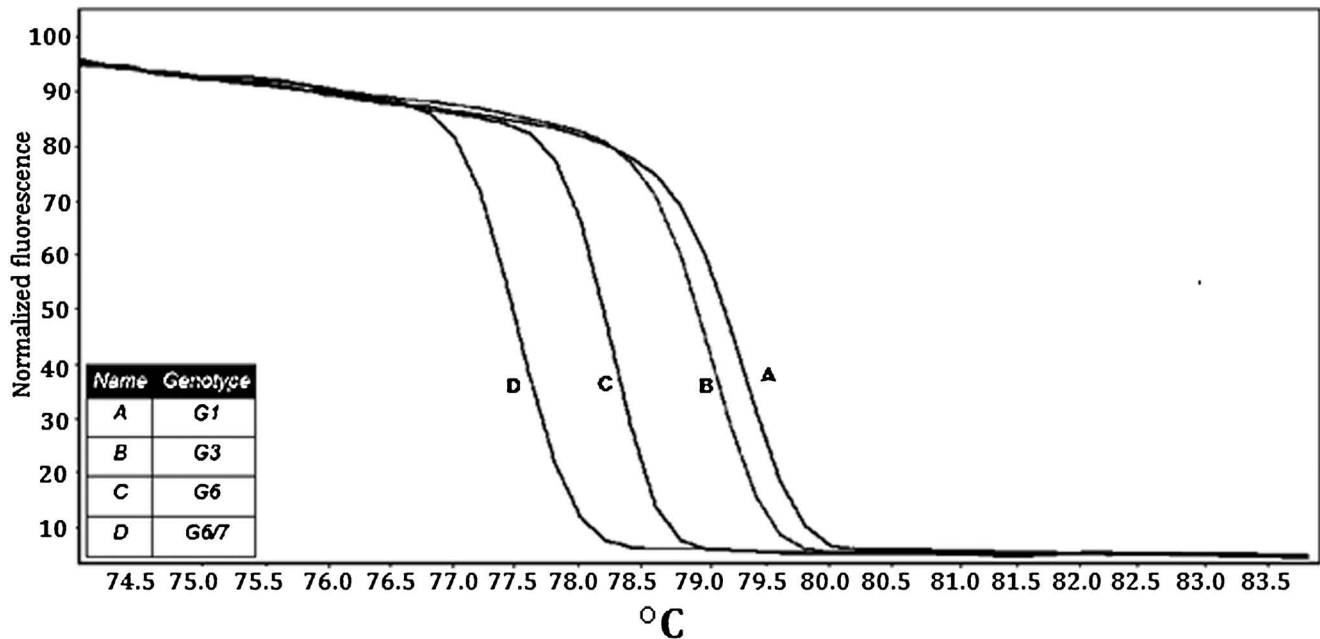


Fig. 3. HRM based on (EvaGreen) curve analyses of *E. granulosus* identified by sequencing (A-D). (A) G1, (B) G3, (C) G6, and (D) G6/7 genotypes.

intermediate genotype suggests the existence of the G7 genotype. However, in this study, the G7 genotype was not confirmed, but this variation could suggest a new phenotype in Iran. Hence, future studies are recommended to detect other latent genotypes, or other SNPs, in critical loci of the *cox1* gene in Iran.

HRM analysis has some advantages. For example, it is faster and less complicated than other methods (22). It can be used to identify any changes in the gene sequences and is more powerful than other methods that detect only a single allele (23). This close-tube method allows detection of any alteration in DNA with simple screening. However, there are some disadvantages, such as the requirement for a small amplicon (preferably <300 bp), which makes the selection of primers less flexible (24).

Recently, many epidemiological studies reported different genotypes of *E. granulosus* in diverse regions, including Asia, Europe, Africa, and the Americas (25–36). For instance, Boubaker et al. showed that *E. granulosus sensu stricto* (G1/G2/G3), *E. equinus* (G4), and *E. canadensis* (G7) were the predominant genotypes in Tunisia, Algeria, Argentina, Spain, and Bulgaria (25). Current studies in the Middle East showed that the G1–G3 genotypes in Palestine and Turkey and G1, G3, G5, and G6 genotypes in India were the most common genotypes in these regions (26–28).

Several studies regarding *E. granulosus* genotypes in the intermediate and definitive hosts, such as sheep, goats, cattle, camels, buffalo, humans, and dogs, have been conducted in various parts of Iran (4,29–31). The geographical distribution of *E. granulosus* genotypes in Iran is shown in Fig. 5. G1, G3, and G6 genotypes have been reported in the intermediate hosts, and the G1 genotype is the most predominant genotype in Iran (29–37). The result of the present study is similar to the previous studies conducted in Iran.

So far, only 2 studies have reported the *E. granulosus* genotypes using HRM analysis (13,32). In the first study, Santos et al. distinguished between *E. granulosus* and *E. ortleppi* as well as *E. vogeli* and *E. oligarthrus* using HRM melting curve. The authors recommended this method as a useful tool for routine detection and diagnosis of echinococcosis in endemic regions (13). Secondly, Rostami et al. used the HRM technique for molecular studies on cystic echinococcosis in the Kerman province in Iran: G1, G3, and G6 genotypes of *E. granulosus* have been differentiated in this region (32). The result of this survey is similar to our study, but there was no reported case of any SNPs in their findings.

In summary, our findings suggest that HRM analysis provides a powerful mean for molecular and epidemiological studies on echinococcosis in humans as well as domestic animals. Therefore, it is suggested that the extensive use of this technique with greater sample size can aid in identifying undetected genotypes using different molecular targets.

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**Conflict of interest** None to declare.

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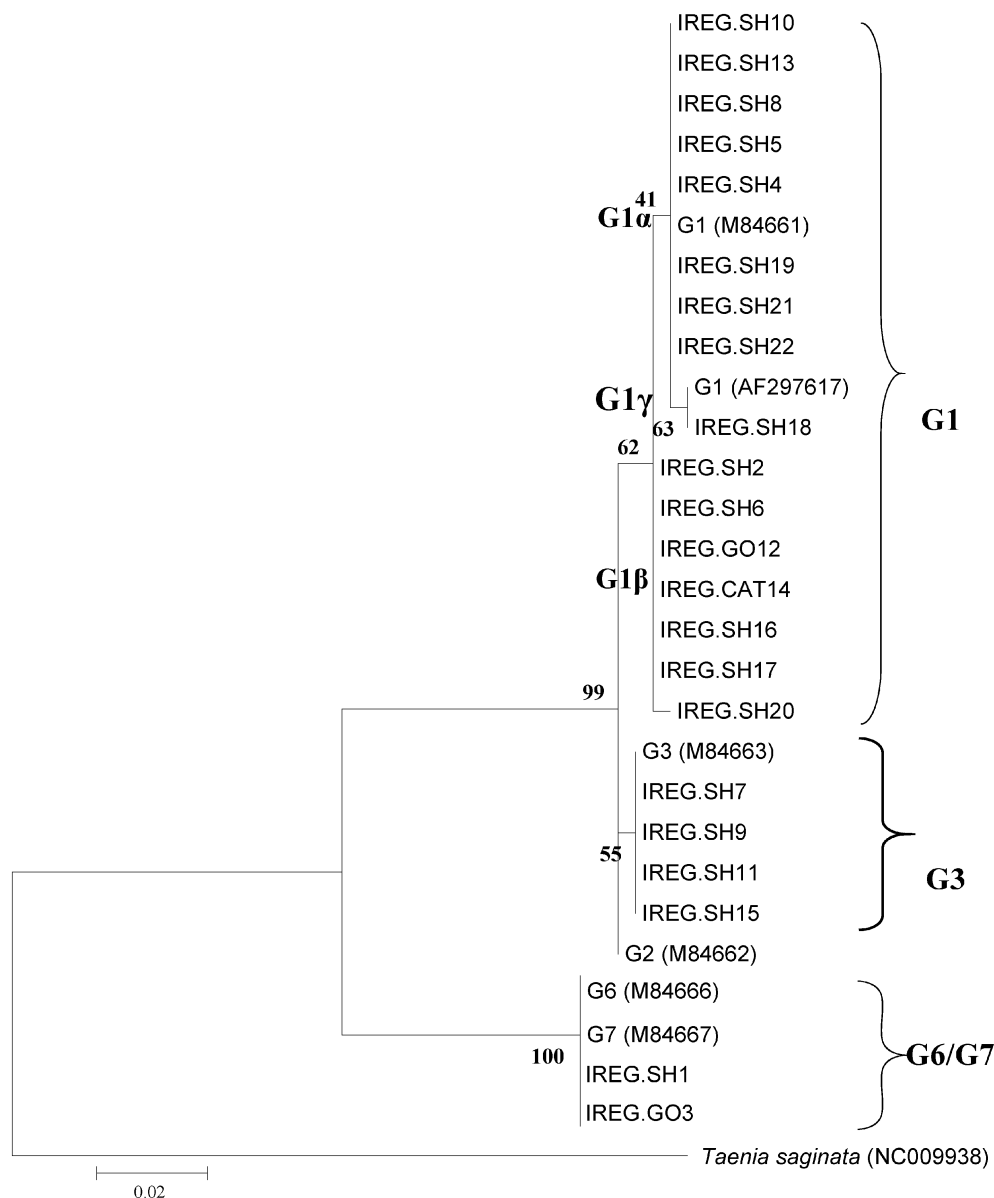


Fig. 4. Molecular phylogenetic tree of 22 *E. granulosus* isolates of sheep, goats, and cattle along with reference isolates based on *cox1* gene sequences. The Kimura 2-parameter model was used to best model search, and the evolutionary history was inferred by using the maximum likelihood method (18). The tree with the highest log likelihood (-814.2241) is shown. GenBank accession numbers: G1, M64661; G2, M64662; G3, M64663; G6, M84666; and G7, M64667.

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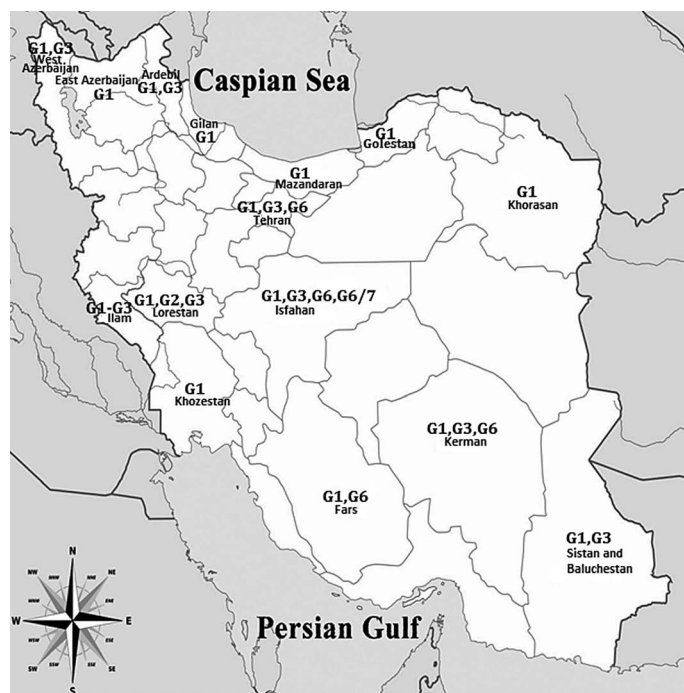


Fig. 5. Geographical illustrations of the distribution of each genotypes of *E. granulosus* in Iran based on the present and previous studies. G1, G2, G3, and G6 genotypes are distributed across the whole extent of Iran.

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