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Molecular and morphological characterization of the tapeworm *Taenia hydatigena* (Pallas, 1766) in sheep from Iran

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Abstract

Although *Taenia hydatigena* is one of the most prevalent taeniid species of livestock, very little molecular genetic information exists for this parasite. Up to 100 sheep isolates of *T. hydatigena* were collected from 19 abattoirs located in the provinces of Tehran, Alborz and Kerman. A calibrated microscope was used to measure the larval rostellar hook lengths. Following DNA extraction, fragments of cytochrome *c* oxidase 1 (CO1) and 12S rRNA genes were amplified by the polymerase chain reaction method and the amplicons were subjected to sequencing. The mean total length of large and small hooks was 203.4 μm and 135.9 μm, respectively. Forty *CO1* and 39 12S rRNA sequence haplotypes were obtained in the study. The levels of pairwise nucleotide variation between individual haplotypes of CO1 and 12S rRNA genes were determined to be between 0.3–3.4% and 0.2–2.1%, respectively. The overall nucleotide variation among all the *CO1* haplotypes was 9.7%, and for all the 12S rRNA haplotypes it was 10.1%. A significant difference was observed between rostellar hook morphometry and both *CO1* and 12S rRNA sequence variability. A significantly high level of genetic variation was observed in the present study. The results showed that the 12S rRNA gene is more variable than *CO1*.

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Introduction

Members of the genus *Taenia* are the most important cyclophyllidean tapeworms. Species of the genus *Taenia* are responsible for major medical and economic losses in humans and animals. *Taenia hydatigena* is a cosmopolitan and widespread parasite that can infect a wide range of herbivorous animals with its larval stage, *Cysticercus tenuicollis* (Murrell *et al.*, 2005). This tapeworm uses canids, mainly dog, fox, wolf and coyotes, as the definitive hosts. Canids shed proglottids in the faeces and eggs, which are dispersed in the environment are then accidentally ingested by ruminant intermediate hosts. The released oncospheres migrate to the liver via the bloodstream and cause haemorrhagic and fibrotic lesions known as hepatitis cysticercosa (Murat, 2005). The migration of the cysticerci to the liver may either result in condemnation of the liver at slaughter or the death of young animals resulting from severe infections and traumatic hepatitis (Jepson & Hinton, 1986).

Taenia hydatigena is an ubiquitous tapeworm found in domestic animals worldwide, having been reported with prevalences of 16.7% in sheep from Germany (Hasslinger & Weber-Werringhen, 1988), and 33.3% in sheep and 30.2% in goats from Nigeria (Fakae, 1990; Nwosu *et al.*, 1996). Taeniid tapeworms associated with the sheep–dog life cycle, are prevalent in many sheep-rearing countries, mainly because of a high number of farm/stray dogs, high rate of home slaughter and improper disposal of slaughter offal (Harandi *et al.*, 2010; Varcasia *et al.*, 2011). Previous studies have indicated that *T. hydatigena* is one of the most prevalent *Taenia* species in dogs and sheep in Iran, reported in 53.0% of dogs, 9.1% of foxes, 10% of jackals (Dalimi *et al.*, 2006), 12.8% of sheep, 18.2% of goats (Radfar *et al.*, 2005), and in 28.5 and 50.8% of dogs in different parts of the country (Hosseini & Habibi, 2000; Hejazi *et al.*, 2004).

An accurate characterization of the causative agents of different *Taenia* infections is essential for understanding the epidemiology and transmission dynamics, vaccine development, accurate diagnosis, treatment, and effective prevention and control of the taeniid species. Comparative genetic analyses provide reliable differentiation of genetic variants and improve our understanding of the nature and significance of intra-specific variation within the *Taenia* species of medical and veterinary importance. DNA sequence analysis of mitochondrial genes is a sensitive and reliable tool for estimating genetic relatedness within different helminth species. Mitochondrial cytochrome *c* oxidase 1 (CO1) is one of the universal markers for molecular identification, characterization and population studies of eukaryotic organisms. Incorporating morphological findings into DNA data provides a more comprehensive picture of the extent and significance of variation within individual species, although the scarcity of morphological characters is a problem for implementation of such an approach (McManus, 2002). Rostellar hook characters, particularly large and small hook lengths, have been used as a tool for morphological and taxonomic studies on different cyclophyllidean cestodes (Verster, 1969; Edwards & Herbert, 1981; Loos-Frank, 2000).

The genetic characterization of *T. hydatigena* isolates has been investigated in different parts of the world (Okamoto *et al.*, 1995; Kedra *et al.*, 2001; Zhang *et al.*, 2007; Lavikainen *et al.*, 2008; Jia *et al.*, 2010), although comprehensive molecular studies on *T. hydatigena* have yet to be conducted. Molecular characterization, intraspecific variation and reconstruction of phylogeny in Iranian isolates of *T. hydatigena* have not been demonstrated so far, and further studies are warranted to provide comprehensive knowledge of this taeniid member. The purpose of the present study was to determine the CO1 and 12S rRNA gene sequences and larval rostellar hook morphometry in a relatively large number of *T. hydatigena* isolates from Iran and to compare their genetic relatedness using phylogenetic analysis.

Materials and methods

Morphometrics

Up to 100 larval stages of *T. hydatigena* were obtained from sheep during routine veterinary inspection in 19 abattoirs located in the provinces of Tehran, Alborz and Kerman from August to December 2010. All samples were transferred to the School of Medicine, Kerman University of Medical Sciences, washed three times with normal saline and stored at −20°C until used. A biometric analysis was undertaken on the size of rostellar hooks. Each of seven large and small hooks per scolex were measured as previously described by Harandi *et al.* (2002). All the measurements were made by one person (S.R.).

Molecular analysis

A part of each individual scolex was cut into small pieces using a razor blade and was homogenized with an equal volume of distilled water. Each sample was frozen and thawed six times in liquid nitrogen, and DNA extraction was performed using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions. In brief, each sample was incubated overnight in 200 µl tissue lysis buffer and 20 µg/ml proteinase K at 56°C. DNA extracted from the metacestodes was stored at −20°C until use.

Two target sequences of mitochondrial DNA coding for cytochrome *c* oxidase subunit 1 (CO1) and 12S rRNA genes were amplified by polymerase chain reaction (PCR). The PCR amplification was performed in a final volume of 50 µl, containing 250 mM of each deoxynucleotide triphosphate (dNTP), 3.5 mM $MgCl_2$, 2 units *Taq* DNA polymerase, 25 pmol of each primer and 4 µl (50–100 ng/ml) of the DNA template.

Two primers, JB3 (forward): 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ and JB4.5 (reverse): 5′-TAAAGAAAGAACATAATGAAAATG-3′, were used to amplify a part of the CO1 gene (Bowles *et al.*, 1992) under the following conditions: 5 min at 94°C as an initial hot start step, followed by 35 cycles of 30 s at 94°C, 45 s at 50°C, 35 s at 72°C, and a final extension step of 10 min at 72°C.

A taeniid-specific primer pair was designed for the amplification of the 12S rRNA gene using Primer-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). PCR was

performed using 12SRF (5′- AGGGGATAGGACACAGT-GCCAGC- 3′) as the forward and 12SRR(5′- CGGTGTG-TACAT GAGCTAAAC- 3′) as the reverse primer under the following conditions: 5 min at 94°C as an initial hot start step, followed by 35 cycles of 30 s at 94°C, 45 s at 57°C, 35 s at 72°C, and a final extension step of 10 min at 72°C. The primers amplified a part of the mitochondrial 12S rRNA gene. A negative control (no DNA) was included in each experiment. The amplification reactions were conducted in a thermal cycler (Corbett Research, Sydney, Australia), and the amplicons were electrophoresed in a 1% (w/v) agarose gel containing ethidium bromide.

All the amplicons were sequenced on an ABI 3730XL capillary machine (Macrogen Inc., South Korea). Sequence data were adjusted manually and complete alignment was carried out using the softwares BioEdit (Hall, 1999) and ClustalW (Thompson *et al*., 1997). Then nucleotide sequences of CO1 and 12S rRNA genes of the isolates obtained from the present study were deposited in the National Center for Biotechnology Information (NCBI) GenBank.

Data analysis

One-way ANOVA was used to determine the statistical significance of differences among the rostellar hook length in different haplotypes. Cluster analysis was applied to classify the subjects into homogeneous subgroups, chi-square test to compare the frequency of different CO1 and 12S rRNA gene haplotypes in clusters, and ANOVA was applied to compare the mean of large and small hook lengths in the isolates. The random effects model was applied to estimate how much of a variation of the hook length was attributed to genetic differences between the subjects.

Two phylogenetic analyses of the sequence data were inferred by Bayesian inference on each of the *CO1* and 12S rDNA sequences, using the program MrBayes v. 3.1.2 (http://mrbayes.csit.fsu.edu/index.php). Evolutionary distance was calculated using the general time reversible evolutionary model. Posterior probabilities (pp) were designed for 2,000,000 generations (ngen: 2,000,000). The TreeviewX v. 0.5.0 program (Page, 1996) was used to depict the resulting trees. Each tree was run using sequences obtained in this study as well as reference sequences available for *T. hydatigena* and other *Taenia* species in GenBank. *Echinococcus granulosus* G1 genotype (NC008075) was used as an outgroup. To determine the total numbers of mutations and segregation sites, CO1 and 12S rRNA gene sequence alignments, including 100 taxa for 390 bp (for *CO1*) and 427 bp (for 12S rRNA) fragments, were analysed using DnaSP v. 5 (Librado & Rozas, 2009).

Results

The linear measurement of larval hooks was conducted for all the 100 isolates, with the mean of the total length of the large and small hooks documented as 203.4 μm and 135.9 μm, respectively. PCR amplification was successfully performed on all of the isolates ($n = 100$), for both the CO1 and 12S rRNA genes, and the amplified fragment size was approximately 400 bp and 500 bp for the CO1 and 12S rRNA genes, respectively. Sequence analyses of CO1 and 12S rRNA genes showed 40 and 39 representative profiles (haplotypes), respectively, designated as IRTHCO1–IRTHCO40 (accession nos JQ710588–JQ710627) for *CO1* and IRTHSR1–IRTHSR39 (accession nos JQ717210–JQ717248) for 12S rRNA. For the CO1 gene, most of the isolates (24%) belonged to the IRTHCO1 (JQ710588) haplotype. Based on the 12S rRNA sequence data, 45% of the isolates were categorized as IRTHSR1 (JQ717210).

A total of 40 and 46 mutations occur in the CO1 and 12S rRNA genes, in 38 and 43 segregation sites, respectively. The level of pairwise nucleotide variation between individual haplotypes of the CO1 gene is determined to be 0.3–3.4% while the overall nucleotide variation among all 40 haplotypes was 9.7%. The biggest pairwise difference was observed between IRTHCO18 and IRTHCO28. For the 12S rRNA sequence data, the level of pairwise nucleotide variation was found to range from 0.2 to 2.1% and the overall nucleotide variation was determined as 10.1% among 39 haplotypes. IRTHSR30 and IRTHSR39 displayed the highest nucleotide difference among the isolates. Figures 1 and 2 show the phylograms based on CO1 and 12S rRNA gene sequences using the Bayesian inference method.

On comparing the means of the total length of small and large rostellar hooks, a significant difference was observed between the various haplotypes of the CO1 and 12S rRNA genes ($P < 0.05$). Interclass Correlation Coefficients (ICC), obtained by the random effect model analysis, indicated that a portion of the hook length variations are attributable to the genetic differences among the individual isolates in *CO1* (28% for both, the total large and small hook length) and 12S rRNA (39% for the total large and 25% for the total small hook length).

Discussion

Several previous studies conducted on the basis of morphological and biochemical factors reinforced the accuracy of genetic diversity within the *Taenia* species (Hustead & Williams, 1977; Mills *et al*., 1983; Gasser *et al*., 1999). The present study showed relatively high intraspecific variation within the Iranian *T. hydatigena* isolates; pairwise nucleotide differences in the CO1 and 12S rRNA genes were 0.3–3.4% and 0.2–2.1%, respectively, although our phylogenetic analysis did not support the existence of defined genetic variants within the isolates. A study report from India investigated the differences in the biochemical parameters of *T. hydatigena* from sheep and goats, and the results indicated the presence of presumably two different strains (Abidi *et al*., 1989). Very few studies around the world and none in Iran have focused on the molecular characterization of *C. tenuicollis,* so the present study investigated the variability in the CO1 and 12S rRNA genes as well as the larval rostellar hook morphometric characters of 100 sheep isolates of *T. hydatigena* in Iran.

Taenia hydatigena is a cosmopolitan parasite that affects herbivores in many parts of the world. A study in an abattoir in Ethiopia estimated an annual loss of US$65,000

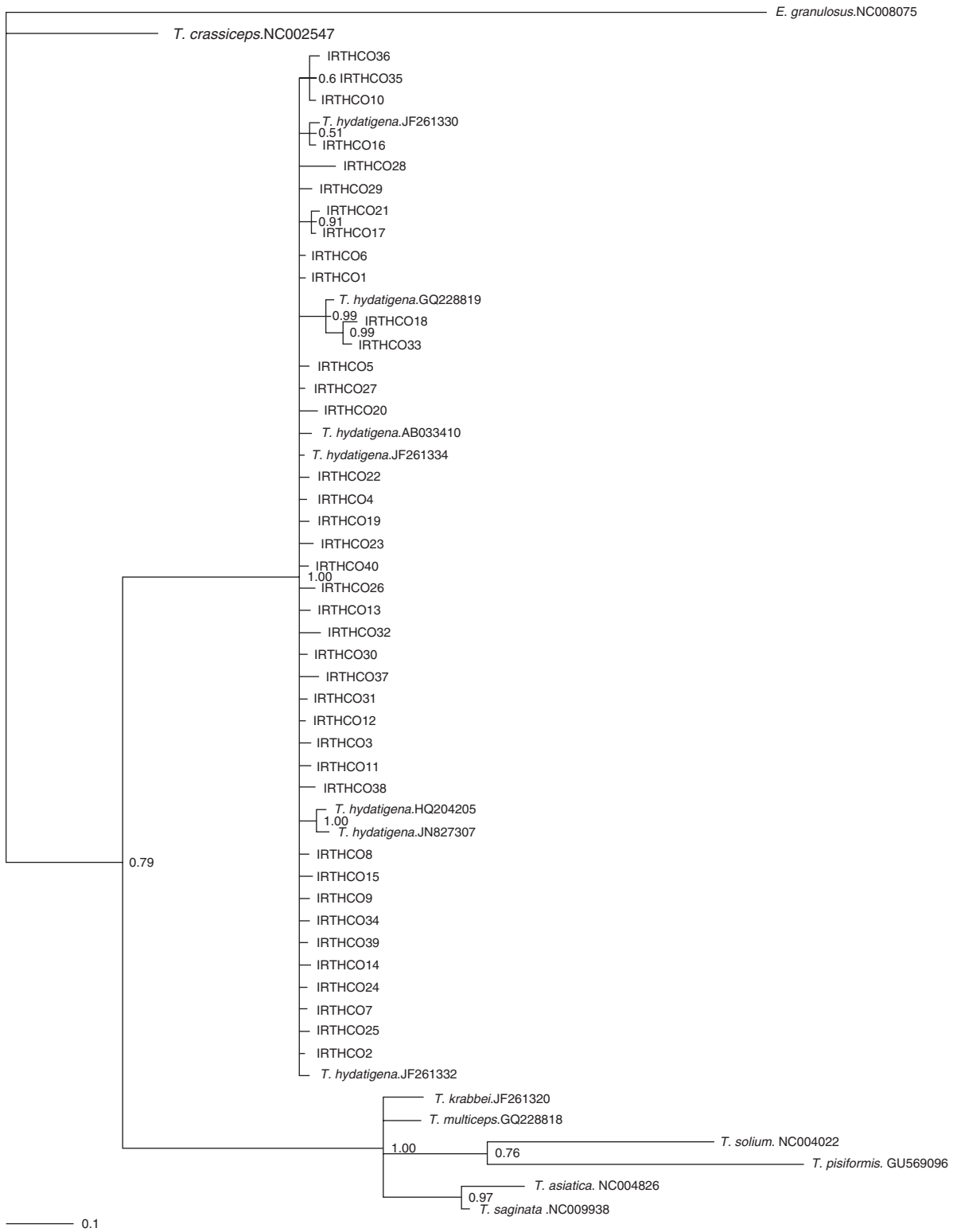

Fig. 1. Phylogenetic analyses of *Taenia hydatigena* isolates to show partial *CO1* (390 bp) sequences; distance (% substitution per site) is shown by a scale bar and nodal support by a posterior probability (pp) value.

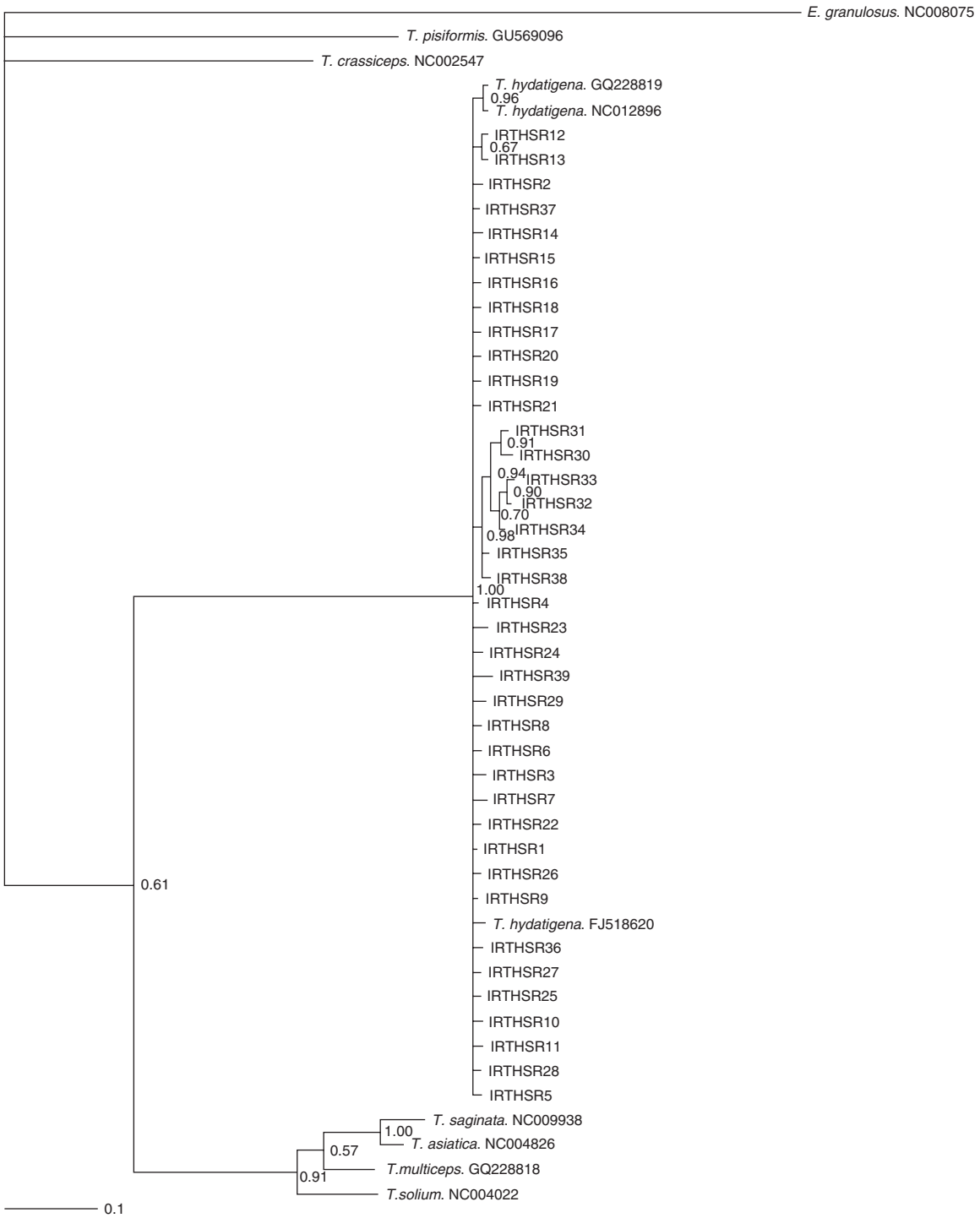

Fig. 2. Phylogenetic analyses of *Taenia hydatigena* isolates to show partial 12S rRNA (427 bp) sequences; distance (% substitution per site) is shown by a scale bar and nodal support by a posterior probability (pp) value.

caused by *T. hydatigena* infection among the livestock (Wondimu *et al.,* 2011). Understanding the genetic identity of the parasite will be crucial for the control of such parasitic infections.

We used mitochondrial DNA (mtDNA) sequence data to examine the intraspecific variation of *T. hydatigena*. mtDNA is widely used in molecular and phylogenetic studies of eukaryotic organisms due to its low or

absent recombination, maternal inheritance, conserved structure, lack of introns, higher rate of mutation and relatively high evolutionary rate (Moritz *et al.*, 1987; Avise, 2000). The CO1 and 12S rRNA genes are among the most popular mtDNA genes for studying phylogeny, inter- and intra-specific variation and evolutionary biology of helminth parasites (Gasser *et al.*, 1999; von Nickisch-Rosenegk *et al.*, 1999).

Our results showed a relatively high degree of genetic variation both in the CO1 and the 12S rRNA genes of *T. hydatigena*. Table 1 presents the pairwise comparison of the nucleotide differences between the taeniid species, including *T. hydatigena* isolates from the present study. The level of nucleotide variation in *CO1* and 12S rRNA between *T. hydatigena* haplotype 1 isolates from the present study and six other *Taenia* species was found to be 3.0–16.1% and 2.8–22.5%, respectively. This concurs with the expected level of nucleotide variations in *CO1* in the genus *Taenia*, which was estimated to be 6.3–15.8% from a study conducted by McManus & Bowles (1994).

Taenia hydatigena isolates from eight regions in Wales, Ukraine and Poland were genetically characterized for the NADH dehydrogenase subunit 1 (ND1) gene, with an intraspecific variation of 0.4–5.5% (Kedra *et al.*, 2001). Maximum genetic differences were observed between the Welsh and Ukrainian *T. hydatigena* isolates (5.5%); however, less geographically distant isolates in Poland showed lower variations, ranging from 0.4 to 3.4%. The results of the present study indicated 0.3–3.4% nucleotide differences in *CO1*. Considering the more conservative nature of *CO1* compared with other mitochondrial genes (Campbell *et al.*, 2006), the genetic diversity within the Iranian isolates of the parasite appears to be greater than that of the Polish isolates. This is quite possible because of the higher prevalence and transmission rate of cysticercosis tenuicollis, larger geographical area and more dispersed population of livestock in Iran.

The present study showed relatively high intraspecific variation within the *T. hydatigena* isolates (9.7 and 10.1% overall nucleotide variation in CO1 and 12S rRNA genes, respectively). This is in agreement with the variations found in other cestode parasites. Okamoto *et al.* (1995) showed a 9.5% *CO1* variation within the isolates from another taeniid species, *Taenia taeniaeformis*, from rodent intermediate hosts. In *Hymenolepis diminuta,* an overall variation of 6.4% was found in two isolates from Hokkaido and the United States (Okamoto *et al.*, 1997). The differences in the level of variation indicated that the 12S rRNA is less conservative than the CO1 gene, at both the inter- and intra-specific level.

Phylogenetic analysis of the CO1 and 12S rRNA genes produced trees with similar topologies for *T. hydatigena* isolates (figs 1 and 2); however, the general topology of the *CO1* and 12S rRNA trees are different for other *Taenia* species. In both the trees, all the Iranian *T. hydatigena* isolates were clustered in one clade, along with isolates from China, Turkey and Scandinavia. *Taenia hydatigena* is one of the most ubiquitous taeniid species among sheep and dogs, and the parasite appears to have been distributed across the globe through human migration and animal transportation over a long period of time. An intensive transmission of the parasite among a range of intermediate host species could increase the chance of genetic variability within different populations of the parasite in the world. There are reports of significant differences in morphological (small hook length) and biochemical characters within the *T. hydatigena* populations (Abidi *et al.*, 1989; Radfar *et al.*, 2005), although additional studies are required in other endemic regions prior to defining any genetic variant within this species.

Mean large and small hook sizes of the isolates of the present study were in agreement with the hook lengths reported by Verster (1969) and Loos-Frank (2000). Significant hook length differences were found among various *CO1* and 12S rRNA haplotypes. Furthermore, results of the random effect model analysis showed that the variability within the *CO1* and 12S rRNA sequences has a significant effect on the rostellar hook length variations in *C. tenuicollis*. It appears that the rostellar hook length is associated with mitochondrial sequence variability. In other taeniid species, the association of the mitochondrial gene variation and larval rostellar hook length has not been investigated.

There is very little information on the genetic nature of the parasite from other intermediate hosts, including goat, pig and cattle (Utuk & Piskin, 2012). Further studies on the molecular and morphological characterization of the parasite are clearly required from other intermediate

Table 1. Levels of nucleotide differences (%) between *Taenia hydatigena* haplotype 1 of the present study and six taeniid species for the mitochondrial cytochrome *c* oxidase (390 bp, upper diagonal) and 12S rRNA (427 bp, lower diagonal) genes.

	*Taenia hydatigena**	*Taenia hydatigena*	*Taenia saginata*	*Taenia solium*	*Taenia asiatica*	*Taenia multiceps*	*Taenia crassiceps*	*Taenia pisiformis*	*Echinococcus granulosus*
*T. hydatigena**	–	1.3	12.3	12.8	13.3	12.0	11.0	16.1	15.9
T. hydatigena	0.05	–	11.5	12.6	13.0	11.8	10.5	15.9	15.4
T. saginata	14.3	14.5	–	11.5	3.0	6.1	10.8	11.0	18.0
T. solium	14.5	14.7	5.9	–	13.0	10.0	14.3	12.8	17.4
T. asiatica	13.6	13.8	2.8	5.6	–	8.2	11.8	12.6	19.5
T. multiceps	14.3	14.5	5.6	5.4	4.7	–	12.0	11.5	20.0
T. crassiceps	16.6	21.3	14.0	12.9	15.0	16.2	–	14.1	15.6
T. pisiformis	16.9	22.5	16.1	15.7	16.9	16.2	14.7	–	19.7
E. granulosus	22.0	26.2	19.7	18.5	20.4	19.7	20.1	21.5	–

* Reference isolate accession numbers are as follows: *T. hydatigena* (present study) JQ710588 (CO1) and JQ717210 (12S rRNA), *T. hydatigena* (reference isolate) GQ228819, *T. saginata* NC009938, *T. solium* NC004022, *T. asiatica* NC004826, *T. multiceps* GQ228818, *T. crassiceps* NC002547, *T. pisiformis* GU569096, *E. granulosus sensu stricto* NC008075.

hosts from different parts of the world. The present study provided mitochondrial data on sheep isolates of *T. hydatigena* in Iran. More in-depth studies on nuclear genes are essential to provide a comprehensive picture on the extent and significance of genetic variation within different *T. hydatigena* populations.

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Conflict of interest

None.

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