

In-silico analysis of mt-CO1 gene of *Taenia hydatigena* sheep isolates

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Summary

Taenia hydatigena is a tapeworm that affects herbivores in different regions of the world. *Cysticercus tenuicollis* (larvae of *T. hydatigena*), is prevalent in ruminants and pigs. In the current study, phylogenetic analysis of the published mt-CO1 gene of *C. tenuicollis* sheep isolates was analyzed using in-silico method and vertical and horizontal transmission at the global level by using a meta-analysis approach. A total of 82 mt-CO1 nucleotide sequences (339 bp) of *C. tenuicollis* sheep isolates from the NCBI database (Italy -Sardinia-, Iran, Palestine, Iraq, Finland, India and China) were used to investigate haplotype and genetic relationships. Tajima's D (-2,2984) value was negative for the mt-CO1 sequences signifying the population expansion and/or purifying selection. The highly negative Fu's Fs (-60,528) values determined for the sequences reflecting the existence of uncommon haplotypes. The mt-CO1 of *C. tenuicollis* haplotype network had 47 haplotypes arranged within a star-like configuration with a main haplotype, which encompassed 25.6 % of the total isolates. In the mt-CO1 haplotype network analyzed, there were 80.5 % unique single haplotype and highest ratio was observed in *C. tenuicollis* from sheep originating from Iran, followed by Sardinia, Palestine and Finland. If the current condition continues, genetic differences in *T. hydatigena* will be able to rise, and possible new strains and/or genotypes that may influence the host adaptation and life cycle of the parasite may emerge.

Keywords: *Taenia hydatigena*; *Cysticercus tenuicollis*; sheep; CO1; in-silico analysis

Introduction

Taenia hydatigena is a cosmopolitan cestode parasite and adults reside in the small intestine of dogs and the other carnivores (such as foxes, jackals, wolves and hyenas). *Cysticercus tenuicollis*, which is the metacestode, lives mainly on sheep, goats, cattle, pigs, deer and horses as well (Taylor *et al.*, 2007). The eggs released from the gravid proglottids are passed out with the feces of the final host resulting spread the infection after being injected by the intermediate hosts during grazing. Large pasture areas,

infected shepherd and stray dogs and illegal slaughtering are important factors in the spread of *C. tenuicollis* (Harandi *et al.*, 2011; Varcasia *et al.*, 2011). The oncospheres, which are released from the eggs taken, first migrates to the liver, and then some of it adheres to the liver, but most of them come to the surface of the omentum and mesentery. However, there are few studies reporting that *C. tenuicollis* is rarely localized in the lungs, kidneys, brain, ovaries, uterus, cervix and vagina (Utuk & Piskin, 2012; Scala *et al.*, 2015). *Cysticercus tenuicollis* consists of a thin-walled cyst filled with a serous fluid and an invaginated scolex (Chemben-

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sofu *et al.*, 2017). The majority of diseases are asymptomatic and usually do not attract attention to the slaughtered animals. Clinical findings vary according to the severity of the infection (Chembensofu *et al.*, 2017). There is some evidence that cysticercus larvae, especially those located in the liver, are as harmful as in the peritoneal cavity (Kaufmann, 2013). It has been reported that larvae migrating in severe infections, especially in young animals, can cause eosinophilia in the liver parenchyma and severe destruction of the liver parenchyma with heavy inflammation, which can sometimes be fatal. In addition to creating an animal health problem, cysticercosis is important in terms of causing economic losses for the meat industry (Scala *et al.*, 2016). In Italy, economic losses due to cysticercosis were 0.40 € per kilogram, 315,622.2 €/year in liver destruction, 18,035.5 €/year in offal, and approximately 333,657 € in total (Scala *et al.*, 2015).

The prevalence and economic importance of *C. tenuicollis* were reported in some European countries (Scala *et al.*, 2015). The prevalence of *C. tenuicollis* in sheep was 15.17 % in India (Nimbalkar *et al.*, 2011), 0.2 % in Tanzania (Mellau *et al.*, 2010), 16.93 % in Egypt (Sultan *et al.*, 2010), 13.03 % in Nigeria (Saulawa *et al.*, 2011), 79 % in Ethiopia (Sissay *et al.*, 2008) and 12.87 % in Iran (Rostami *et al.*, 2015). In Turkey, the prevalence rate of *C. tenuicollis* in sheep ranged between 12.3 % and 65.67 % (Deger *et al.*, 2001; Aydin, 2003; Deger & Bicek, 2005; Kara *et al.*, 2009; Adwan *et al.*, 2018).

For the molecular characterization of *T. hydatigena* isolates, mostly mitochondrial cytochrome c oxidase 1 (CO1) gene has been preferred. It is due to the mt-CO1 gene can better and more reliably identify the genetic relationship among the isolates (Boufana *et al.*, 2015a, b).

This study aimed to evaluate the phylogenetic analysis of *T. hydatigena*'s sheep isolates (*C. tenuicollis*) by in-silico method using the mt-CO1 gene sequences retrieved from National Center for Biotechnology Information, USA, (NCBI) (www.ncbi.nlm.nih.gov).

Materials and Methods

Data Collection

In current study, the mt-CO1 gene sequences of *T. hydatigena*'s sheep isolates which were submitted to The National Center for Biotechnology Information, USA, (NCBI) (www.ncbi.nlm.nih.gov) until October 24th, 2019 were retrieved and dataset were created. As a result of the sequence data search in NCBI database, a total of 86 sequence data was obtained.

Data and Phylogenetic Analysis

The mt-CO1 sequences were individually downloaded in FASTA format from NCBI database and uploaded to the MEGA X program (Kumar *et al.*, 2018). By aligning of the different length sequences, a consensus reference sequence (accession number GQ228819) of *T. hydatigena* was selected. All sequences were aligned to the consensus sequence, then 339 bp length sequences were selected, which did not have indel problems. A total of 82 sequences selected by this strategy and were trimmed at both ends and the length of the sequences were equalized. Later on, these sequences were analyzed in the MEGA X program by the Akaike Information Criterion (AIC) and The Bayesian Information Criterion, and the most suitable base change models were obtained. Following, the phylogenetic tree was constructed by using the Maximum Likelihood (ML) method was obtained with the Hasegawa-Kishino-Yano model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1727)). Statistical support for specific clades was obtained via 1000 bootstrap replicates. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

Haplotype Analysis

All the sequence data was uploaded on DnaSP 6 (Rozas *et al.*, 2017). The population diversity indices (numbers of haplotype

Table 1. Access numbers of the mt-CO1 gene region of *C. tenuicollis* isolates used in the study.

Geographical region	Number of isolates	Accession Numbers	Genbank Submit Date
China	2	NC012896 and GQ228819	2010 and 2016
Iran	40	JQ710588 - JQ710599 JQ710600 - JQ710627	2012
Palestine	20	KM032284 - KM032299 KM032300 - KM032303	2014
Italy (Sardinia)	14	KT372517 - KT372518 KT372520 - KT372531	2015
Finland	1	EU544551	2016
India	1	DQ995656	2016
Iraq	4	MH113919 - MH113922	2018
Total	82		

Table 2. Diversity and neutrality indices for *Taenia hydatigena* isolates from sheep originating from various geographical regions using nucleotide data of mt-CO1 (339 bp).

Geographical region (n)	Polymorphic sites	hn	hd±SD	nd ± SD	Tajima's D	p value	Fu's Fs	p value
Palestine (20)	10	9	0.705 ± 0.111	0.004 ± 0.0011	-1.610	0.093*	-4.546	0.008
Iran (40)	36	35	0.992 ± 0.008	0.011 ± 0.001	-2.017	0.031*	-44.411	0.000
Sardinia (14)	12	10	0.934 ± 0.051	0.007 ± 0.0013	-1.501	0.125*	-5.775	0.003

N: number of isolates, hn: number of haplotypes; hd: haplotype diversity; nd: nucleotide diversity; SD: standard deviation

(h), haplotype (Hd) and nucleotide diversities (π), the neutrality indices (Tajima's D (Tajima, 1989), and Fu's statistics (Fu, 1997)) were calculated using DnaSP 6 (Rozas *et al.*, 2017). DnaSP 6 was used to generate using a series of output formats, such as NEXUS, which allows the user to add additional information for the next analysis. Geographical, phenotypic or other traits associated with sequences were also be included in NEXUS (Maddison *et al.*, 1997) files in a Traits block. Following networks were created using the minimum spanning networks (MSN) method, which includes all the edges visible in the minimum span tree of arrays by means of PopART-1.7 software (Bandelt *et al.*, 1999) (<http://popart.otago.ac.nz>).

Ethical Approval and/or Informed Consent

In the current study no informed consent was required from the open resources of gene sequences.

Results

In this study, a data set of mt-CO1 sequences of *C. tenuicollis* sheep isolates from NCBI database was used for bioinformatic analysis (Table 1).

Cysticercus tenuicollis populations: for geographical regions diversity and neutrality indices

The diversity and neutrality indices for mt-CO1 sequences of *C. tenuicollis* sheep isolates from Italy (Sardinian), Iran and Palestine are shown in Table 2. *Cysticercus tenuicollis* in Iranian sheep showed the highest haplotype diversity within the sheep isolates. Tajima's D and Fu's Fs were significantly negative for nearly all the *T. hydatigena* isolates in the localities. Fu's Fs for Iranian isolates was at least 11 and 8 fold that seen in Palestinian and Sardinian

an isolates, respectively, which is a reflection of the presence of unique singleton haplotype characteristics of the Iranian isolates of *C. tenuicollis*. In addition, the identification of 35 haplotypes in 40 isolates of Iranian origin supports this finding. When the haplotype analysis was done between Iran, Palestine and Italy (Sardinia) isolates; 36 polymorphic regions containing 44.4 % (16/36) parsimony informative sites were detected for mt-CO1 sequences in Iranian isolates. The mt-CO1 network consisted of 35 haplotypes, out of them 88.6 % (31/35) were geographically unique haplotypes. Although it is not a central main haplotype, the haplotype network that combines 3 nucleotide sequences that make up a maximum of 7.5 % (3/40) has been identified. Apart from that, it was observed with 1 – 11 mutation points separated from other haplotypes. In Palestinian isolates; 10 polymorphic regions containing 30 % (3/10) parsimony informative sites were determined for mt-CO1 sequences. It has been observed that 77.8 % (7/9) of the data network consists of 9 geographically unique haplotypes and 55 % (11/20) of total isolates are located in a main haplotype point. In Italy (Sardinia) isolates, for the mt-CO1 sequences, 12 polymorphic regions containing 25 % (3/12) parsimony informative sites were identified. 80 % (8/10) of the haplotypes consisted of 10 geographically unique haplotypes. The existence of two main haplotypes was determined at the main haplotype point, and it was observed that 21.4 % (3/14) of the total isolates were in this main haplotype network.

Cysticercus tenuicollis populations: for geographical regions diversity and neutrality indices

A total of 82 mt-CO1 nucleotide sequences (339 bp) of *C. tenuicollis* sheep isolates from the NCBI database from Italy (Sardinia), Iran, Palestine, Iraq, Finland, India and China were used to determine haplotype and genetic relationships. In total, we identified 44 polymorphic sites for mt-CO1 sequences, out of which

Table 3. Diversity and neutrality indices for *Cysticercus tenuicollis* isolates from Sardinia, Iran, Palestine, Iraq, Finland, India and China using nucleotide data of mt-CO1 gene (339 bp).

No of isolates (n)	Polymorphic sites	hn	hd±SD	π d ± SD	Tajima's D	p value	Fu's Fs	p value
Total (82)	44	47	0.926 ± 0.023	0.008 ± 0.0009	-2.2984	0.009*	-60.528	0.000

n: Number of isolates, hn: number of haplotypes; hd: haplotype diversity; π d: nucleotide diversity; SD: standard deviation

Table 4. Grouping haplotypes of *Cysticercus tenuicollis* mt-CO1 sequences and accession numbers of isolates forming groups.

Haplotype name	No of isolates	Accession Numbers
Hap01	21	KM032303-Palestine, KM032302-Palestine, KM032300-Palestine, KM032299-Palestine, KM032298-Palestine, KM032289-Palestine, KM032288-Palestine, KM032287-Palestine, KM032286-Palestine, KM032285-Palestine, KM032284-Palestine, JQ710614-Iran, JQ710599-Iran, JQ710588-Iran, KT372530-Italy, KT372529-Italy, KT372522-Italy, MH113922-Iraq, MH113921-Iraq, MH113920-Iraq, MH113919-Iraq
Hap02	2	JQ710627-Iran, JQ710594-Iran
Hap03	1	JQ710626-Iran
Hap04	1	JQ710625-Iran
Hap05	1	JQ710624-Iran
Hap06	1	JQ710623-Iran
Hap07	1	JQ710622-Iran
Hap08	1	JQ710621-Iran
Hap09	1	JQ710620-Iran
Hap10	1	JQ710619-Iran
Hap11	1	JQ710618-Iran
Hap12	2	JQ710617-Iran, KM032294-Palestine
Hap13	1	JQ710616-Iran
Hap14	1	JQ710615-Iran
Hap15	1	JQ710613-Iran
Hap16	8	JQ710612-Iran, JQ710593-Iran, KT372528-Italy, KT372525-Italy, KT372524-Italy, KM032292-Palestine, KM032291-Palestine, DQ995656-India
Hap17	1	JQ710611-Iran
Hap18	1	JQ710610-Iran
Hap19	1	JQ710609-Iran
Hap20	1	JQ710608-Iran
Hap21	1	JQ710607-Iran
Hap22	1	JQ710606-Iran
Hap23	1	JQ710605-Iran
Hap24	2	JQ710604-Iran, KM032296-Palestine
Hap25	1	JQ710603-Iran
Hap26	1	JQ710602-Iran
Hap27	1	JQ710601-Iran
Hap28	2	JQ710600-Iran, JQ710591-Iran
Hap29	1	JQ710598-Iran
Hap30	1	JQ710597-Iran
Hap31	1	JQ710596-Iran
Hap32	1	JQ710595-Iran
Hap33	2	JQ710592-Iran, KT372520-Italy
Hap34	1	JQ710590-Iran
Hap35	3	JQ710589-Iran, KT372518-Italy, KM032301-Palestine
Hap36	1	KT372531-Italy

Hap37	1	KT372527-Italy
Hap38	1	KT372526-Italy
Hap39	1	KT372523-Italy
Hap40	1	KT372521-Italy
Hap41	1	KT372517-Italy
Hap42	1	KM032297-Palestine
Hap43	1	KM032295-Palestine
Hap44	1	KM032293-Palestine
Hap45	1	KM032290-Palestine
Hap46	1	EU544551-Finland
Hap47	2	NC012896-China, GQ228819-China

40.9 % (18/44) were parsimony informative. Large haplotype and weak nucleotide diversities were detected in mt-CO1 as shown in the Table 3. There were no conserved DNA regions in accordance with the studied sequences. Tajima's D was negative for the mt-CO1 partial sequences signifying population expansion and/or purifying selection. The highly negative Fu's F_s values detected for the sequences indicated the presence of unusual haplotypes expected from a recent population enlargement. The existence of unmatched single haplotypes for the mt-CO1 (38/47) sequences of *C. tenuicollis* was coherent with the structure of the haplotype networks.

Haplotype Networks

The mt-CO1 of *C. tenuicollis* haplotype network had 47 haplotypes arranged within a star-like configuration with a main haplotype, separated from other haplotypes by 1 – 11 mutational steps and which encompassed 25.6 % (Hap01: 21/82) of the total isolates (Table 4).

This principle haplotype contained 55 % (11/20) of the Palestine isolates, 100 % (4/4) of the Iraq isolates, 7.5 % (3/40) of the Iran isolates, 21.4 % (3/14) of the Italians (Sardinia) isolates of sheep. Again, the second main haplotype was found to represent 9.7 %

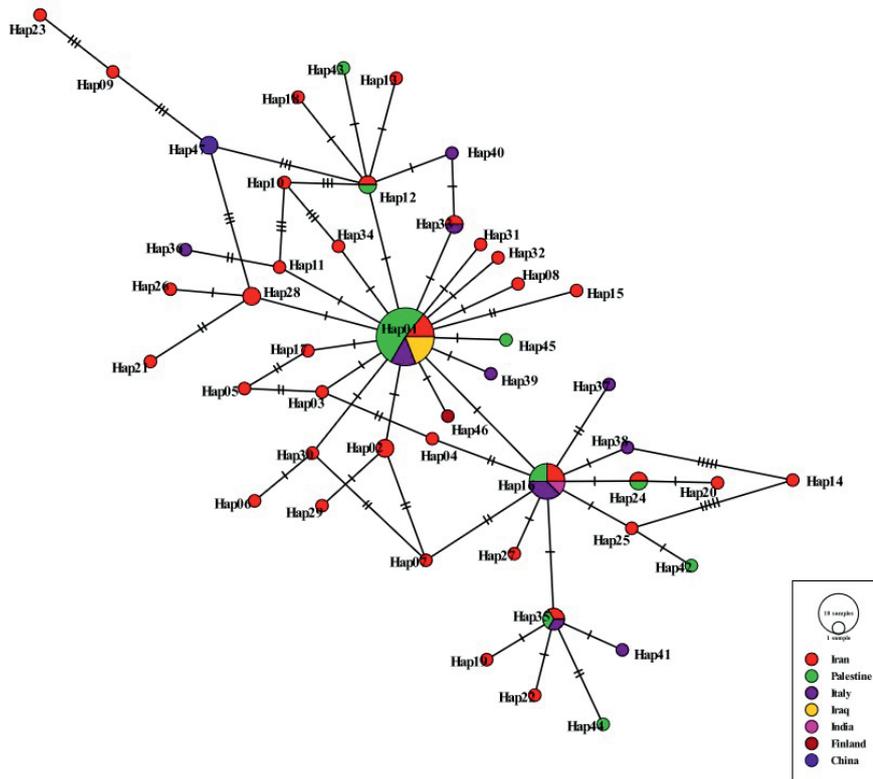


Fig. 1. The appearance of mt-CO1 (339 bp) haplotypes of sheep isolates of *Cysticercus tenuicollis*. Geographical distribution of the haplotypes is indicated by different colors. The size of the circles is related to the haplotype frequency. Number of mutations distinguishing the haplotypes is shown by hatch marks.

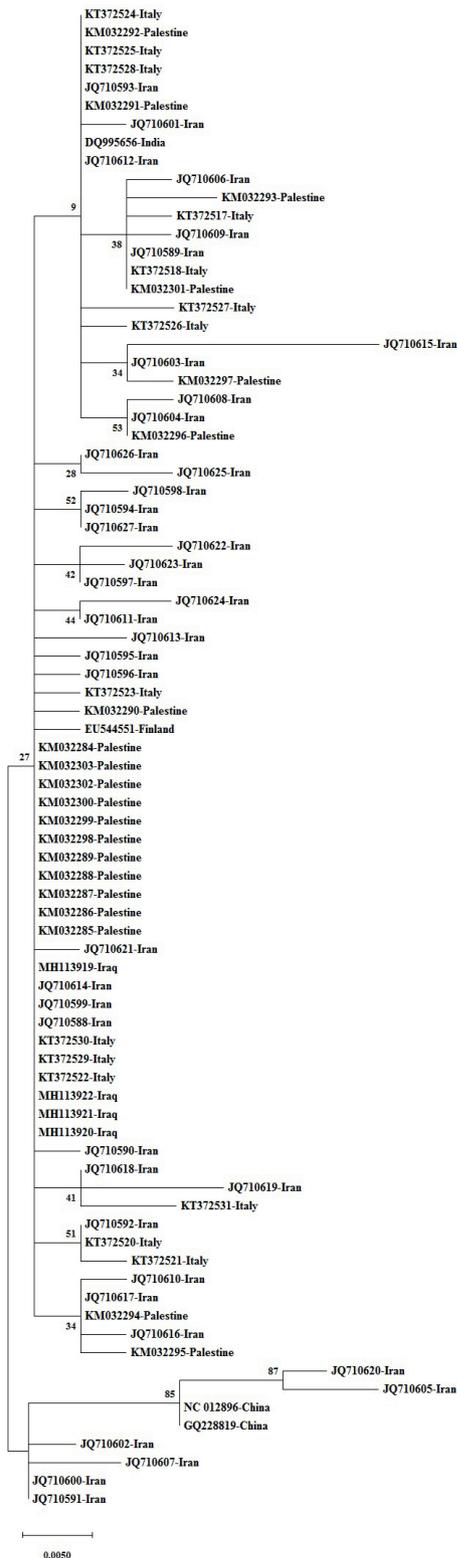


Fig. 2. Phylogenetic tree view of sheep isolates using mt-CO1 gene (339 bp) sequences and reference sequences. MEGA X was used to construct a Maximum Likelihood tree based on the HKY+G model. The reliability of the tree was assessed by 1000 bootstrap replications.

(Hap16: 8/82) of the total isolates. This haplotype placed 100 % (1/1) of India, 21.4 % (3/14) of Italy (Sardinia), 10 % (2/20) of Palestine and 5 % (2/40) of Iran isolates. In the mt-CO1 haplotype network analyzed in the study, there were 80.5 % (38/47) unique single haplotype and this ratio was highest observed for *C. tenuicollis* from sheep originating from Iran (n=27) isolates, followed by Sardinia (n=6), Palestine (n=4) and Finland (n=1) (Fig. 1).

Phylogenetic tree

The genetic tree view created as a result of the alignment of the sequences was shown in Figure 2. As a result, JQ710615 (Hap14) and JQ710605 (Hap23) were designated as two most distant isolates in two clusters owing to 11 nucleotide differences. Besides, JQ710615 caused the second furthest branching to JQ710620 (Hap09) due to 10 nucleotide differences. Basically, the isolates in the Hap28 group and the isolates in the Hap01 group formed two separate groups. Moreover, it was observed that the isolates in the Hap16 group were the closest to the Hap01 group.

Discussion

Taenia hydatigena is prevalent in herbivores in different regions of the globe. A slaughterhouse based study in Ethiopia estimated annual economic losses of US\$ 65.000 due to *T. hydatigena* infection in the livestock (Wondimu *et al.*, 2011). In past few years, it has become prominent that further precedence should be given to *C. tenuicollis* therefore its veterinary and economic losses in the meat industry, particularly in undeveloped countries (Nourani *et al.*, 2010; Samuel & Zewde, 2010; Scala *et al.*, 2015). Understanding the genetic identification of the parasite will be very important to control of like this parasitic infection. This is the first study about in silico analysis sheep isolates of *C. tenuicollis* mt-CO1 gene region.

The mt-CO1 gene is among the most popular mitochondrial DNA genes, used to study the phylogeny, specific strain variation, and historical biology of helminth parasites (Liu *et al.*, 2012; Jia *et al.*, 2012; Biswal *et al.*, 2014; Liu *et al.*, 2014). The mt-CO1 gene of taeniid cestodes has been indicated to be a promising candidate for the classification of intra- and extra specific variants (Bowles *et al.*, 1992). One of the major advantage is that mitochondrial DNA is present in more than one copy per cell, it has a high diagnostic value, especially when samples were broken. In addition, the mt-DNA has a high mutation rate and undergoes little or no recombination (i.e. mutations accumulate progressively) (Saarma *et al.*, 2009). Therefore, in current study the mt-CO1 gene sequence was used to analyze the selected isolates for in-silico analysis.

Taenia hydatigena is one of the most ubiquitous taeniid species between sheep and dogs, and the parasite appears to be dispersed around the world for a long time through human migration and animal transport. Monitoring the vertical and horizontal spread of meta-analytical studies are important in terms of evaluating the effectiveness of protection and control measures. In this study,

phylogenetic relationships between sheep isolates of *C. tenuicollis* mt-CO1 gene isolated from different geographies were examined in-silico and the vertical spread of the highly conserved mt-CO1 gene sequences over the years was shown, on the other hand, horizontal spread has been revealed geographically.

Neutrality tests such as Tajima D, Fu's F_s were used to test the neutrality of values such as nucleotide variability and population expansion (Korneliussen *et al.*, 2013). While Tajima D focuses on old mutations that can reflect population events for a long time, Fu's F_s value is mainly sensitive to the last mutation.

It has been determined that the main haplotype reported for the mt-CO1 gene region of *C. tenuicollis* is common. Indeed, the finding of in-silico analysis performed revealed that the sequences in only four countries such as Palestine (n=11), Iraq (n=4), Iran (n=4) and Italy (n=3) were among the main haplotype of the sequences reported from seven countries. It shows that there was no genetic difference between the *C. tenuicollis* isolates detected in sheep in the Sardinia region of Italy, where an island with an isolated geographical location, and the isolates from Iran, Iraq and Palestine. Indeed it was remarkable that 3 of the 14 isolates examined in Sardinia were included in the main haplotype.

Total haplotype diversity and total nucleotide diversity were relatively low for Palestinian isolates having values of 0.705 and 0.0045, respectively. These results shows that Palestinian *C. tenuicollis* haplotypes were not genetically different. In this case, it is not surprising that 11 out of 21 isolates that constitute the main haplotype belong to sheep isolates of Palestinian origin. With the feature of taeniid populations being hermaphrodite, self-fertilization can maintain intermediate gene flow, leading to inraspecific phenotype monotony (Boufana *et al.*, 2015b; Nakao *et al.*, 2003). This feature is consistent with the low haplotype variation observed in Palestine for *T. hydatigena* metacestodes derived from sheep. The genetic variation in the mt-CO1 gene observed in the Palestinian isolate was lower than the values reported among other countries, as seen in table 2 (Boufana *et al.*, 2015a; Rostami *et al.*, 2015). These results might be due to low prevalence and spread of *C. tenuicollis*, the smaller Palestine territory and current management system (Adwan *et al.*, 2018).

The highest haplotype diversity in the current study was identified in Iranian sheep isolates, followed by Sardinian and Palestinian isolates. 35 haplotypes were identified in 40 Iranian isolates. The formation of unique (single) haplotypes (27 out of 38) in Iranian sheep isolates were an indicator of this situation. These findings probably indicate that sheep was first domesticated in the middle east countries and that *C. tenuicollis* first appeared in this geographically and then spread to Europe and other continents (Kinkar *et al.*, 2018). It was quite interesting to identify the two furthest haplotypes from the same country (Iran: JQ710605 (Hap23)-JQ710615 (Hap14)). Although, there were 6 years between the submission dates of Chinese isolates, it has been found that there was no haplotype difference between the Chinese isolates. Moreover, the low number of samples prevents us to make definitive

comments on this issue. It might be possible that the Chinese isolates form a separate haplotype because of the four nucleotide difference between the isolates that make up the main haplotype. Finland isolates with accession number EU544551 (Hap46) also formed a separate haplotype due to the single nucleotide difference from the main haplotype. Indian isolate (DQ995656) was located in Hap16 (with Sardinia, (n=3), Iran, (n=2), Palestine, (n=2) and India (n=1)), which is the second main haplotype with a single nucleotide difference from the main haplotype.

In conclusion, the highest haplotype diversity was detected in Iranian sheep and 88.6 % of the haplotypes were geographically unique. Besides, 44 polymorphic sites were identified and large haplotype and weak nucleotide diversities were detected in mt-CO1 gene. There were 80.5 % (38/47) unique single haplotype and this ratio was highest observed for *C. tenuicollis* from sheep originating from Iran isolates, followed by Sardinia, Palestine and Finland. If the current evolving conditions continue, genetic differences in *T. hydatigena* sheep isolates may be increased, and new strains and/or genotypes may be emerged. Finally, the complete mitochondrial genome sequence may be useful for understanding of the molecular ecology and population genetics of *T. hydatigena*.

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Conflict of interest

The authors declare that there is no conflict of interests.

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