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Research Brief

Genotyping *Echinococcus granulosus* from dogs from Western Iran

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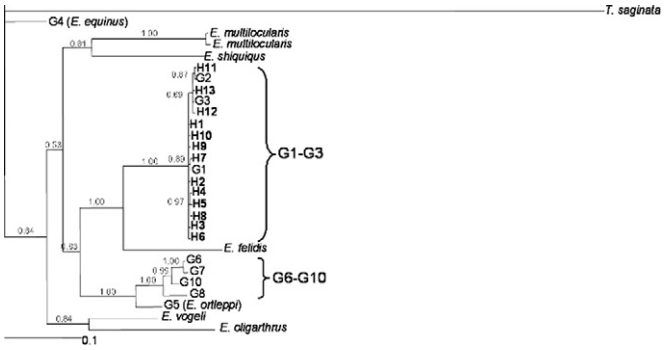
HIGHLIGHTS

- This study established the first record of *E. granulosus* G2 genotype in Iran.
- This study presents the first global report of this genotype in dogs as definitive host.
- The presence of G1 genotype of *E. granulosus* as dominant genotype in dogs is emphasized.

GRAPHICAL ABSTRACT

Genetic relationships of *Echinococcus granulosus* dog isolates from western Iran and reference sequences for *E. granulosus sensu lato* and other species of *Echinococcus* from previous studies as well as *Taenia saginata* as the outgroup.



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ABSTRACT

Cystic echinococcosis is a zoonotic infection caused by the dog tapeworm, *Echinococcus granulosus*. In the present study, adults of *E. granulosus* (n = 20) were collected from 71 dogs from Western Iran and were genetically characterized using DNA sequencing of the partial mitochondrial cytochrome *c* oxidase subunit 1 (*cox*1) and NADH dehydrogenase 1 (*nad*1). Consensus sequences were obtained for *cox*1 (366) and *nad*1 (471) genes. Phylogenetic analysis of concatenated *nad*1 and *cox*1 nucleotide sequence data was performed using Bayesian Inference approach. Overall, the dog isolates indicated nine different sequences in *cox*1 and seven in *nad*1 genes. Three genotypes (G1 [75%], G2 [10%] and G3 [15%]) were identified from the isolates. The G2 sequences indicated 100% homology with reference G2 sequence in both *cox*1 (Genbank accession number M84662) and *nad*1 (AJ237633) genes. G3 sequences showed 100% homology with G3 reference sequence in *nad*1 (AJ237633), but displayed two different cox1 profiles, each having 99% homology with reference G3 sequence (M84663). In the phylogenetic tree all of the isolates were grouped into a distinct cluster corresponding to the G1–G3 complex with relevant reference sequences. The presence of G1 genotype (sheep strain) of *E. granulosus* sensu stricto as dominant genotype in dogs is emphasized. To the best of our knowledge, this study established the first record of *E. granulosus* sensu stricto, G2 genotype in Iran.

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1. Introduction

Echinococcus granulosus, the causative agent of cystic echinococcosis, is an important cause of morbidity and mortality in humans worldwide, particularly in sheep-raising countries (Dakkak, 2010). Carnivores, especially dogs, play a role for definitive hosts and harbor adult parasites in their intestine, while herbivores serve as intermediate host and can harbor the larval stage or hydatid cyst in any internal organ, particularly lung and liver (WHO/OIE Manual, 2002; Eckert and Deplazes, 2004).

In order to develop preventive and control strategies for echinococcosis, a better knowledge of transmission cycle of *E. granulosus* complex is necessary. A significant genetic variation has been detected within *E. granulosus* complex from different species of intermediate hosts in different geographical areas and several strains have been characterized (Thompson, 2008). To date, ten different genotypes (G1–G10) have been described for *E. granulosus* complex, based on the analyses of mitochondrial and nuclear genetic data (Bowles et al., 1992, 1994; Scott et al., 1997; Lavikainen et al., 2003; Thompson, 2008; Saarma et al., 2009). Recently, a taxonomic revision of the genus has been made mainly on the basis of mitochondrial data, in which *E. granulosus* complex splitted into four distinct species as follows: *E. granulosus sensu stricto* (G1–G3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6–G10) (Nakao et al., 2007; Moks et al., 2008; Knapp et al., 2011). Also *E. felidis* is closely related to *E. granulosus sensu stricto*. and is grouped within *E. granulosus* complex (Huttner and Romig, 2009). However, based on more complex data, including nuclear sequences and the epidemiological criteria, it was recommended by Thompson (2008) and Saarma et al. (2009) that genotypes G6–G10 could be broken into two different species namely *E. canadensis*, the cervid genotypes (G8 and G10), and *E. intermedius,* the camel/pig genotypes (G6/G7).

Since, there is a high risk of hydatid infection during experiments on dogs, usually genetic characterizations are performed on larval stages in the intermediate hosts. However, genetic identification of adult worms is required as well to provide a better understanding of existing cycles and genotypes in endemic areas. There are only few studies on genetic characterization of *E. granulosus* complex in dogs around the world (Abbasi et al., 2003; Stefanic et al., 2004; Mathis and Deplazes, 2006; Hüttner et al., 2008). Widespread recovery of adult *E. granulosus* has been reported from dogs, jackals and wolves throughout Iran (Sadjjadi, 2006). In a comprehensive study conducted in 13 provinces of Iran, the prevalence of *E. granulosus* in sheepdogs was 27.2% (Eslami and Hosseini, 1998). In Iran, previous molecular studies on *E. granulosus* complex have been performed on larval stages of *E. granulosus* isolated from human or different livestock species including sheep, goat, cattle, buffalo and camels, revealing the existence of various genotypes (G1, G3 and G6) in the country (Zhang et al., 1998; Harandi et al., 2002; Ahmadi and Dalimi, 2006; Rostami Nejad et al., 2008; Kia et al., 2010; Sharbatkhori et al., 2010; Parsa et al., 2011; Sharifyazdi et al., 2011).

Lorestan province in Western Iran could be one of hotspots for echinococcosis in the country as many people live as nomads and human and dogs are always in close contact. Very limited information is available for *E. granulosus* complex in Lorestan province (Rostami Nejad et al., 2008; Parsa et al., 2011), mainly from intermediate hosts of the parasite. The aim of this study was to genetically characterize *E. granulosus* isolates from dogs from Lorestan province using the partial sequence data of mitochondrial cytochrome *c* oxidase 1 (*cox*1) and NADH dehydrogenase 1 (*nad*1) genes to gain a better understanding of the parasite's life cycle in the studied area.

2. Materials and methods

2.1. Source of isolates and DNA extraction

Based on a ethical approval from the Municipality committee, from April to November 2011, 71 stray dogs, from western province of Lorestan, were humanely euthanized. Following necropsy the intestines of dogs were examined for adult worms of *E. granulosus*. The worms removed from each infected dog transferred into a separate tube and washed three times with normal saline and stored in 70% ethanol until further examination. Before extracting genomic DNA, the worms were thoroughly washed in distilled water to remove ethanol. Genomic DNA was extracted using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

2.2. Mitochondrial PCR amplification

A partial sequence for each of *cox*1 and *nad*1 mitochondrial genes was amplified separately from individual genomic DNA isolates using the primer sets JB3/JB4.5 (Bowles et al., 1992) and JB11/JB12 (Bowles and McManus, 1993) for *cox*1 and *nad*1 genes, respectively. PCRs were performed in a final volume of 50 μl containing 4 μl (50–100 ng) genomic DNA, 25 pmol of each primer, 3.5 mM MgCl2, 250 μM of each dNTP's and 2 units *Taq* polymerase. Amplifications were conducted under following PCR conditions: 94 °C for 5 min as an initial denaturation, 94 °C/30s, 50 °C/45s, 72 °C/35s for 35 cycles and a final extension at 72 °C for 10 min. For each set of PCRs negative (no-DNA) controls were included. Six microliter aliquots of PCR products were electrophoresed on agarose gel (1.5%, W/V) and stained with ethidium bromide (0.5 μg/ml). A 100 bp ladder (Fermentas, Vilnius, Lithuania) was used as a DNA size marker. The gels were visualized by UV transilluminator (UVitec, Cambridge, UK).

2.3. Sequencing and phylogenetic analysis

All *cox*1 and *nad*1 PCR products were subjected to automated sequencing by the Illumina Genome Analysis System, employing the same primers used in the primary PCR. The electropherogram of each sequence was checked by eye, and the sequences were compared with each other using the software BioEdit (Hall, 1999). The representative sequences for both *cox*1 and *nad*1 genes were submitted to GenBank (accession numbers JN604097 to JN604112). In order to compare the *cox*1 and *nad*1 sequences determined herein with those of reference sequences representing 10 currently known genotypes (G1–G10) of *E. granulosus* (*sensu lato*), sequences for the *cox*1 and *nad*1 genes were obtained from the public database i.e., GenBank (http://www.ncbi.nlm.nih.gov/). Upon pairwise comparison, the amounts of sequence difference (D), were obtained using the method as previously described by Chilton et al. (1995).

For phylogenetic analysis, the dataset of the concatenated *cox* + *nad*1 sequences representing all haplotypes detected was compiled, together with key reference sequences (comprising concatenated *cox*1 + *nad*1 sequences from previous studies (Bowles et al., 1992; Bowles and McManus, 1993, 1994; Gasser et al., 1999; Hüttner et al., 2008; Lavikainen et al., 2008; Nakao et al., 2007) and representing all currently recognized *Echinococcus* species and *E. granulosus* 'genotypes', and employing Taenia saginata as the outgroup; see Table 1). In every case, each pair of concatenated sequences represented the same isolate (i.e., both the *cox*1 and *nad*1 sequences were derived from the same isolate). A phylogenetic tree was constructed by employing Bayesian Inference (BI) method using the program MrBayes v.3.1.2 (http://

mrbayes.csit.fsu.edu/index.php). Posterior probabilities (pp) were adjusted for 2,000,000 generations (ngen: 2,000,000; burnin: 20 000) employing the Monte Carlo Markov Chain procedure and four simultaneous tree-building chains (nchains: 4), with every 100th tree saved (samplefreq: 100). Evolutionary distance was obtained using the General Time Reversible evolutionary model (nset: 6), arranging for a γ-shaped variation in mutation rates between codons (rates: γ). The Treeview X v.0.5.0 software (Page, 1996) was used to display the trees. All GenBank accession numbers for the sequences inferred from this study and for the reference genotypes/species used in phylogenetic analysis are shown in Table 1.

3. Results and discussion

Among 71 stray dogs examined, twenty (28.2%) were found infected with *E. granulosus* (9 males and 11 females). For all of *E. granulosus* isolates, fragments of about 450 and 500 bp were successfully PCR-amplified within *cox*1 and *nad*1 genes, respectively. For all amplicons, consensus sequences of 366 and 471 nucleotides were obtained for *cox*1 and *nad*1 genes, respectively. Alignments of the sequences determined herein with those of know genotypes of *E. granulosus* revealed the existence of genotype (G) 1 (sheep strain, 75% of isolates), G2 (Tasmanian sheep strain, 10%) and G3 (buffalo strain, 15%) in the studied area. Nine sequences were found in *cox*1 gene (designated as Lorc1 to Lorc9; GenBank accession numbers JN604097 to JN604105); whereas, seven were found in *nad*1gene (designated as Lorn1 to Lorn7; JN604106 to JN604112).Based on pairwise comparison, the differences among all of the different sequence profiles of *cox*1 ($n = 9$) and *nad*1 ($n = 7$) ranged 0.2–1% and 0.2–0.6%, respectively. A concatenated *cox*1 and *nad*1 sequences of all the isolates produced 13 haplotypes (H1 to H13). A consensus phylogenetic tree of the concatenated *cox*1 and *nad*1 sequences of this study along with reference genotypes is shown in Fig. 1. All of the sequences determined herein grouped into a distinct cluster corresponding to the G1–G3 complex (pp = 1.00) with relevant reference sequences.

Several molecular studies based on ribosomal and mitochondrial data have identified the presence of two distinct genotypes including the common sheep strain (G1) and the camel strain (G6) of *E. granulosus* complex in Iran (Harandi et al., 2002; Sharbatkhori et al., 2009, 2010, 2011; Kia et al., 2010; Parsa et al., 2011; Shahnazi et al., 2011; Sharifyazdi et al., 2011). Recently, Sharbatkhori et al. (2009, 2011), for the first time, characterized the G3 genotype (buffalo strain) from Iranian camels using sequence analysis of mitochondrial *cox*1 and *nad*1 genes (Sharbatkhori et al., 2009, 2011). Later this genotype was also reported from buffalos (Amin Pour et al., 2011), sheep, cattle and again in camels (Hajialilo et al., 2012; Sharifyazdi et al., 2011) in Iran.

In Lorestan province, hydatid surgeries constitute about 0.02–0.15% of all surgical operations (Rostami Nejad et al., 2007) and the prevalence of cystic echinococcosis has been reported ranging from 20% to 30.9% in dogs (Eslami and Hosseini, 1998; Dalimi et al., 2002). In the present study, the infection rate among dogs was 28.2% which is in concordance with previous studies (Dalimi et al., 2002; Eslami and Hosseini, 1998). In spite of high prevalence of the disease, only a few studies have employed molecular tools for the characterisation of *E. granulosus* in the study area (Parsa et al., 2011; Rostami Nejad et al., 2008). The present study presents the first report on the molecular characterisation of *E. granulosus* isolates from its definitive hosts (dog) using mitochondrial loci in Lorestan province, Iran.

In the present study genetic characterization of twenty dog isolates of *E. granulosus* employing mitochondrial *cox*1 and *nad*1 sequences revealed that the G1–G3 complex (*E. granulosus sensu*

Table 1
Echinococcus granulosus haplotypes from dogs in Lorestan Province, Iran and origins of sequences used for concatenation (*cox*1 + *nad*1) and subsequent phylogenetic analyses (see Fig. 1).

	Profile *cox*1 (accession number)	Profile *nad*1 (accession number)	References
E. granulosus haplotypes isolated from dogs in Iran			
H1	Lorc1 (JN604097)	Lorn2 (JN604107)	This study
H2	Lorc1 (JN604097)	Lorn3 (JN604108)	This study
H3	Lorc1 (JN604097)	Lorn4 (JN604109)	This study
H4	Lorc1 (JN604097)	Lorn5 (JN604110)	This study
H5	Lorc1 (JN604097)	Lorn6 (JN604111)	This study
H6	Lorc2 (JN604098)	Lorn2 (JN604107)	This study
H7	Lorc3 (JN604099)	Lorn1 (JN604106)	This study
H8	Lorc4 (JN604100)	Lorn5 (JN604110)	This study
H9	Lorc5 (JN604101)	Lorn2 (JN604107)	This study
H10	Lorc6 (JN604102)	Lorn2 (JN604107)	This study
H11	Lorc7 (JN604103)	Lorn7 (JN604112)	This study
H12	Lorc8 (JN604104)	Lorn7 (JN604112)	This study
H13	Lorc9 (JN604105)	Lorn7 (JN604112)	This study
Echinococcus genotypes/ species			
G1	Not available	AJ237632	Bowles and McManus (1993)
G2	M84662	AJ237633	Bowles et al. (1992) and Bowles and McManus (1993)
G3	M84663	AJ237634	Bowles et al. (1992) and Bowles and McManus (1993)
G4	M84664	AJ237635	Bowles et al. 1992; Bowles and McManus 1993
G5	M84665	AJ237636	Bowles et al. (1992) and Bowles and McManus (1993)
G6	M84666	AJ237637	Bowles et al. (1992) and Bowles and McManus (1993)
G7	M84667	AJ237638	Bowles et al. (1992) and Bowles and McManus (1993)
G8	AB235848	AB235848	Nakao et al. (2007)
G10	AF525457	AF525297	Lavikainen et al. (2003)
E. felidis	EF558356	EF558357	Hüttner et al. (2008)
E. multilocularis	M84668	AJ237639	Bowles et al. (1992) and Bowles and McManus (1993)
E. multilocularis	M84669	AJ237640	Bowles et al. (1992) and Bowles and McManus (1993)
E. vogeli	AB208064	AB208064	Bowles et al. (1992) and Bowles and McManus (1993)
E. oligarthrus	M84670	AJ237641	Bowles et al. (1992) and Bowles and McManus (1993)
E. shiquiqus	M84671	AJ237642	Nakao et al. (2007)
Outgroup			
T. saginata	Not available	AJ239106	Bowles and McManus (1994) and Gasser et al. (1999)

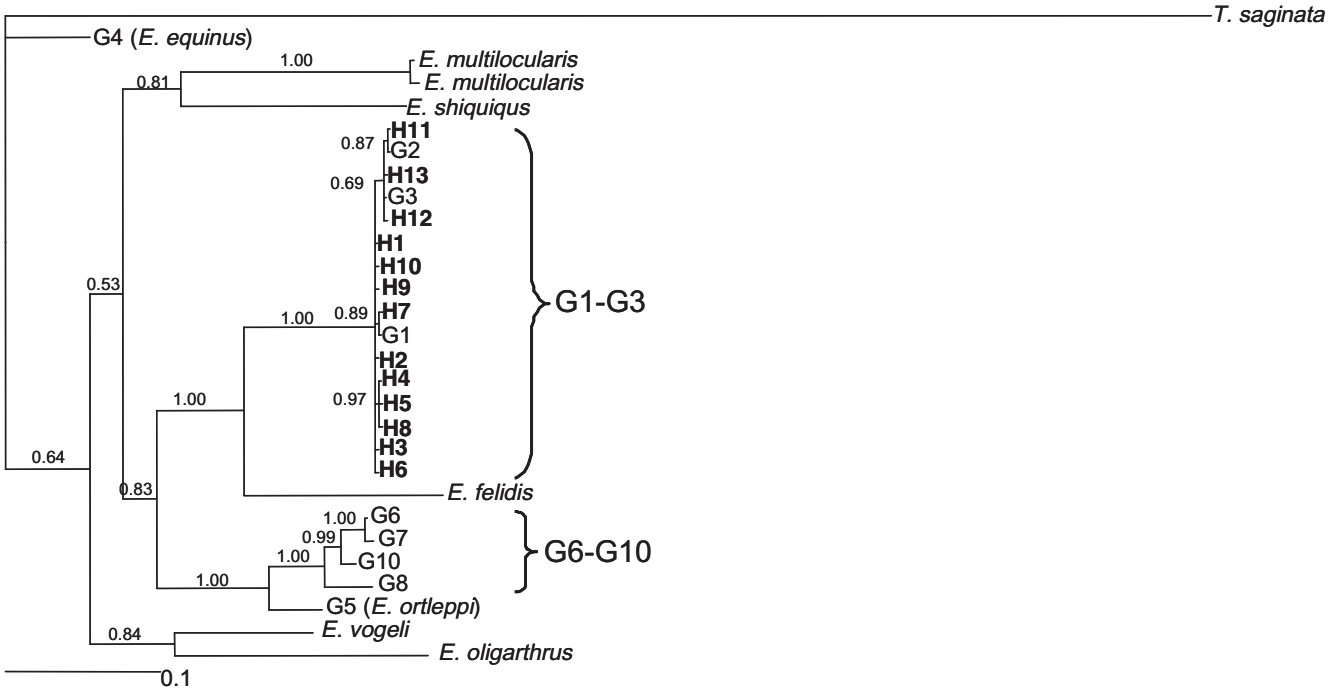

Fig. 1. Genetic relationships of *Echinococcus granulosus* dog isolates from western Iran and reference sequences for *E. granulosus sensu lato* and other species of *Echinococcus* from previous studies as well as *Taenia saginata* as the outgroup. The relationships were inferred based on phylogenetic analysis of concatenated *cox*1 + *nad*1 sequence data (H1–H13 in Bold type) using Bayesian inference. All haplotypes represent genotypes G1–G3 (G1–G3 complex, *E. granulosus sensu stricto*). The accession numbers and source of sequences are shown in Table 1. Nodal support is given as a pp value.

stricto) were present in Lorestan Province, Western Iran. A pervious study conducted in this region using a specific 12SrRNA PCR showed that all sheep and goat isolates belonged to G1 genotype (Rostami Nejad et al., 2008). Another study, using ITS1-RFLP, indicated the presence of G1 in isolates originated from sheep, goats and cattle (Parsa et al., 2011). However G1–G3 genotypes are indistinguishable by ITS1 PCR-RFLP. In this study G1 was the most prevalent genotype of the isolates. These findings suggest that sheep–dog cycle is the dominant cycle of CE in the area. G1 is the most frequent genotype identified in livestock and human throughout the world (Breyer et al., 2004; Capuano et al., 2007; Casulli et al., 2008; Moro and Schantz, 2009; Sánchez et al., 2010) although in some countries of north Africa such as Sudan and Mauritania, G6 is the most common genotype in sheep, cattle, camels and human (Bardonnet et al., 2002; Omer et al., 2010).

All isolates in the present study, designated as haplotypes 1 to 13 (H1–H13) formed a strongly supported clade (pp = 1.00), together with reference sequences representing *E. granulosus* genotypes G1–G3 (*E. granulosus sensu stricto*) to the exclusion of *E. felidis* (pp = 1.00). These findings provide further support for considering G1–G3 "complex" as a separate species and do not confirm the hypothesis that G2 is a separate species (Abushhewa et al., 2010; Hüttner et al., 2008; Lavikainen et al., 2003; Saarma et al., 2009; Vural et al., 2008).

The present study records the occurrence of G2 and G3 genotypes in dogs as definitive host. By sequencing partial *cox*1 (366 bp) and *nad*1 (471 bp) genes, Bowles et al. (1992) first reported G2 genotype as Tasmanian sheep strain of *E. granulosus* complex. The percentage of nucleotides differences in pairwise comparison of G1 and G2 is 0.8% for both partial *cox*1 and *nad*1 genes (Bowles et al., 1992; Bowles and McManus, 1993). To date, G2 genotype has been identified in human and various animals such as sheep, cattle, buffalo and camel from South America, Europe, Africa and Asia. It seems that this genotype has a wider spectrum of intermediate hosts and dispersed to a wide range of geographical areas from its original location in Tasmania. It is not clear if G2 genotype is distributed around the world by global livestock trade or via dogs as companion animals.

The presence of *E. granulosus* complex has been reported in faecal samples of wild canids in Northeast of Iran but no genotype data have been provided for the isolates (Beiromvand et al., 2011). The existence of all three genotypes of *E. granulosus sensu stricto* in dogs and the absence of G6 genotype in this study justify more research on the nature of interactions of different genotypes in dogs and other definitive hosts. More investigations are also needed to elucidate transmission dynamics of G2 and G3 genotypes in the region.

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