Heterogenity of *Echinococcus canadensis* genotype 6 - the main causative agent of cystic echinococcosis in Birjand, Eastern Iran

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ABSTRACT

Little is known about the genotypes of *Echinococcus* spp. and their life cycles in eastern Iran. We analysed the partial sequences of the *nad1* and *cox1* genes from 17 isolates from hydatid cyst-infected patients (n=9), camels (n=5) and sheep (n=3) in Birjand, eastern Iran. A new primer pair was also used to amplify the long fragment (1180 bp) of the *cox1* gene. All camel and eight human isolates were G6 strains of *Echinococcus canadensis* while one human isolate and the three sheep isolates were G1 genotypes (sheep strain) of *E. granulosus* sensu stricto (s.s.). *Nad1* and *cox1* sequence analyses showed high G6 genetic homogeneity, similar to previously reported G6 strains from southeast and central Iran, Sudan and Mauritania. Low nucleotide and haplotype diversity similar to G6 strains from Russia (Altai republic) and Kazakhstan was also found, consistent with a bottleneck effect. In this study, G6 was the most common *Echinococcus* genotype. Genetic homogeneity of east, southeast and central Iranian G6 and its low genetic diversity may be due limited mobility and contact between humans and camels from other regions because of large, inhospitable deserts.

Keywords: Phylogenetic structure, cystic echinococcosis, *cox1*, *nad1*, *Echinococcus canadensis* genotype 6, Eastern Iran

1. Introduction

Echinococcosis, a cosmopolitan disease that encompasses cystic echinococcosis (CE), also known as hydatid disease, and alveolar echinococcosis (AE), is caused by the larval stages of the genus *Echinococcus*. AE is a disease of cooler climates whilst CE has a global distribution and is one of the most important zoonotic parasitic diseases (Zhang et al., 2014). CE's life cycle is maintained by intermediate hosts, including sheep, cows, camels, goats, pigs, horses, and donkeys. The definitive hosts are dogs, other Canidae and Felidae; hydatid eggs passed in faeces lead to the infection of man and other intermediate hosts (Nakao et al., 2013a). Iran has endemic and hyperendemic foci of cystic echinococoosis; important intermediate hosts include sheep, goats, cattle, buffalo, and camels (Sadjjadi, 2006).

Echinococcus granulosus shows high intra-species variation and such strains can be differentiated from each other on the basis of morphological, physiological and biochemical characteristics (Eckert and Thompson, 1997). The development of DNA-based molecular methods has resulted in a revolution in phylogenetic classification of a wide spectrum of microorganisms, including *E. granulosus*.

Genetic variations in *E. granulosus* can be detected by several techniques, including randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) (Scott and McManus, 1994), restriction fragment length polymorphism (RFLP) (Chaâbane-Banaoues et al. 2016; Tigre et al., 2016), single strand conformational polymorphism (SSCP) (Jabbar et al., 2011) and gene sequencing. Sequencing has the greatest discriminatory power and is used routinely for

genotyping (Bowles et al., 1992; Lavikainen et al., 2003; Roinioti et al., 2016; Debeljak et al., 2016). Although various coding and non-coding genes are used for intra-species discrimination, but their resolutions power is not the same. Most recent studies have used mitochondrial markers (e.g. *cox*1 and *nad*1) which have high discriminatory power (Kinkar et al., 2016; Rojas et al., 2016).

Based on the evaluation of biological and molecular data, *E. granulosus* is now recognised as an assemblage of cryptic species with considerably different phenotypic characteristics, including morphology, development, and infectivity/pathogenicity (Romig et al., 2015). Ten *E. granulosus* genotypes (Gs) have been described, some with notable intermediate host specificity, and most cause CE in man. Some genotypes are considered separate species e.g. G1-G3, *E. granulosus sensu stricto*, G4, *E. equines*, G5, *E. ortleppi*, G6–G8 and G10, *E. canadensis* (Nakao et al., 2013a). Of these, G1, G6 and, recently, G2 and G3 genotypes (using *cox*1 and *nad*1 markers) have been found in human and animal reservoirs in Iran, including sheep, goats, camels and buffalos (Zhang et al., 1998; Sharbatkhori et al., 2009; Farhadi et al., 2015; Rostami et al., 2015). There has never been a study that has genotyped the *Echinococcus* species responsible for hydatid disease in man and animals in eastern Iran. We undertook this genetic study using human, sheep and camel hydatid cysts and compared the haplotypes with reliable sequence types retrieved from GenBank to investigate the heterogeneity and population structure of aetiological agents of CE in this region.

2. Materials and methods

2.1. Study area and samples

South Khorasan Province (151,913 km^2) is located in east Iran and has a predominantly desert climate. South Khorasan has large camel and sheep populations, 29,400 and 1 million, respectively (Ebadzadeh et al., 2016).

Nine pathologically-confirmed, paraffin-embedded CE samples and/or haematoxylin–eosin (HE)-stained sections from CE operated patients between 2006 and 2015 were included in this study. The diagnoses were confirmed by pathologists from the Department of Pathology, Birjand University of Medical Sciences, Birjand, the capital of South Khorasan province. Eight hydatid cysts were collected from five dromedary camels (*Camelus dromedarius*) and three sheep (*Ovis aries*); only one cyst was collected from each animal.

2.2. DNA extraction and Polymerase chain reaction (PCR) amplifications

The paraffin-embedded tissue samples were rechecked for the presence of hydatid cysts and their paraffin blocks were cut into sections with a thickness of 5–10 µm. Material from the H & E stained slides was scraped using a sterile scalpel. The tissue sections and scrapings were deparaffinised using xylene and rehydrated using 100%, 90%, 80% and 70% ethanol (Schneider et al., 2008). The rehydrated samples were kept at ambient temperature for 2 to 3 h and then

DNA was extracted using a DNA isolation kit (Takapouzist, Iran). For the camel and sheep samples, the germinal layers and protoscoleces were used for DNA extraction using the Dynabio Tissue DNA Extraction Mini Kit (Takapouzist, Iran), according to the manufacturer's instructions. PCR amplification of the mitochondrial NADH dehydrogenase 1 (*nad*1) gene (397 bp long fragment) was performed by using MS1 (5'-CGTAGGTATGTTGGTTTGTTTGGT-3') and MS2 (5'-CCATAATCAAATGGCGTACGAT-3') primers (Sharbatkhori et al., 2009).

For the amplification of the mitochondrial cytochrome oxidase 1 (*cox*1) gene, two primer sets were selected. The primers JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′) (Bowles et al., 1992), were used to amplify a 420 bp short sized fragment of *cox1* gene.

The primer pairs CO1F6 (5′-TTTGTYTCTTTRGATCATAAGCG-3′) and Cox1_schist_3′(5′-TAATGCATM GGAAAA AAACA-3′) (Lockyer et al., 2003; Morgan et al., 2003) were used to amplify a 1180 base pairs (bp) long fragment of the mitochondrial *cox1* gene in *Echinococcus* spp for the first time. Amplification was carried out in a final volume of 25 µL containing 250 µM each of dNTP, 20 pmol of each primer, 1.5 units of high yield Taq DNA polymerase (Jena Bioscience, Germany), 2.5 µL of PCR buffer containing MgCl2 (Bioneer, Korea), and 3 µL of DNA. The reaction was cycled using a step-down programme, including a per-denaturing step at 94 °C for 6 min followed by 94 ºC for 30 s, 51–47 °C (step down 1 °C for each three cycle) for 30 s, 72 ºC for 2 min (15 cycles); 94 °C for 30 s, 46 ºC for 30 s, 72 °C for 2 min (20 cycles) and a single final extension at 72 ºC for 5 min. PCRs were performed in a Mastercycler gradient thermal cycler (Eppendorf, Germany). Negative and positive controls were used in each set of PCRs. The PCR products were electrophoresed on agarose gel (1.7%), stained with ethidium

bromide and visualized by a transilluminator. A 100 bp ladder (Jena Bioscience, Germany) was used as the DNA size standard.

2.3. DNA sequencing

The PCR products were sent to Bioneer Company (South Korea) and sequenced using the Applied Biosystems automated DNA sequencer (3730 XL). Sequencing was performed in both directions using the same PCR primers by the BigDye Terminator v3.1 cycle sequencing kit. The sequences of *nad*1 and *cox*1 genes were deposited in the GenBank database under the accession numbers KP751432 to KP751442, KR337812 to KR337818, and KP751426 to KP751431.

2.4. Phylogenetic analysis

Blast software (http://www.ncbi.nlm.nih.gov) was used to compare our obtained *cox*1 and *nad*1 sequences with those deposited in GenBank to preliminary identify the genotype of the isolated hydtid cysts. Phylogenetic trees were generated by using our sequences from Birjand, sequences from other areas of Iran, and sequences from other countries retrieved from GenBank (Tables 1 and 2). Alignment was performed using ClustalW and the aligned sequences manually refined in BioEdit software (version 7.2.5) (Hall, 2011); maximum likelihood (ML) trees were inferred by MEGA 6 software (Tamura et al., 2013). Nodal support was assessed by bootstrapping with 1000 replicates. Population diversity (haplotype diversity and nucleotide diversity) and neutrality indices were calculated by DnaSP software version 5.10 (Rozas et al., 2010) based on the long *cox*1 gene for *E. canadensis*, G6 isolates from east Iran (current study), Mongolia (Ito et al., 2014), Kazakhstan, Russia (Altai republic and far east) (Nakao et al., 2013b; Nakao et al., 2007)

and Ethiopia (Nakao et al., 2007) were used in generating the ML tree. We tried to use all the valid long *cox1* sequences that were in GenBank.

3. Results

DNA extraction and amplification of *nad*1 and short *cox*1 loci were successful for all samples. Long size *cox*1 fragments were amplified in samples preserved in ethanol while the inhibitory effect of formalin fixation prevented the amplification of DNA in paraffinised samples obtained from human tissue blocks. Sequence analysis of *nad*1 and short size *cox*1 genes showed dominance of the G6 genotype in samples obtained from human subjects; eight and one of the nine isolates were G6 and G1 genotypes, respectively. All camel and all sheep isolates were identified as G6 and G1 genotypes, respectively.

Fig. 1 presents the geographical distributions of hydatid cyst genotypes by province in Iran and Fig. 2 the proportions of G6 human cases. All east Iranian G6 isolates had identical *nad*1 genes and belonged to a new sequence type in comparison to sequences retrieved from GenBank. Based on the tree topology, the most homogeneity was found among G6 *nad*1 sequence types from east Iran, neighbouring regions in central (Isfahan) and southeast (Kerman) Iran, Sudan and Mauritania. G6 genotype isolates from the west (Khuzestan province), north (Tehran) and northeast (Mashhad and Golestan provinces) Iran and those from Egypt, Algeria and Mongolia showed more heterogeneous sequences. Isolates from Mongolia, Egypt and Iran displayed the most genetic heterogeneity, respectively.

The 17 isolates from Birjand were also genotyped by amplification and sequence analysis of the short size *cox*1 fragments and the results were consistent with those characterized by *nad*1 sequencing. *Cox*1 was amplified by JB3 and JB 4.5 primers and produced a 420 bp size band. The ML tree topology of the short size *cox*1 region showed that all G6 sequences from humans and camels in east Iran were very similar to isolates from Mongolia, Russia, Ethiopia, Sudan and Brazil. However, these isolates diverged from those from other regions of Iran.

Amplification of long *cox*1 fragments (1183 bp) was only successful in the non-paraffinised samples that included hydatid cysts obtained from camels and sheep. In contrast to the short *cox*1 fragments, two different sequence types were found in the long *cox*1 sequences of G6 isolates. Sequence type Cox1-EIran-1 was found in three camels (KP751428- KP751430) while sequence type Cox1-EIran-2 was found in two camels (KP751426 and KP751427). There were very few long *cox*1 sequences in GenBank to compare with our east Iran sequence types. Cox1-EIran-1 was identical to G6 isolates from Mongolia (human, AB893259), Russia (wolf, AB777909, and human, AB688142) and Ethiopia (camel, AB777922) while Cox1-EIran-2 showed one nucleotide difference (T≥C 607) with these isolates. ML tree topology showed heterogeneous structures of G6 genotypes in Mongolia and Ethiopia whist isolates from Kazakhstan and Russia showed more intra-country homogeneity. Most isolates from east Iran (Cox1-EIran-1) and the only isolate from southwest Iran had similar positions and Cox1-EIran-2 isolates were situated close by (Fig. 4). Sequence analysis of long *cox1* fragments (amplified by Cox1_schist_3′ and CO1F6 primers) revealed 138 polymorphism points within and between different hydatid cyst genotypes (G1, G3, G5-G8 and G10); by contrast, the short size *cox*1 sequencing showed only 54 polymorphism points (Fig. 5).

The haplotype, nucleotide diversities and neutrality indices of Tajima D and Fu's Fs p were calculated for the long *cox*1 sequences of G6 isolates from Mongolia, Kazakhstan, Russia, Ethiopia and east Iran that were used for generating the phylogenetic tree. Five isolates from our study were included in the calculation from east Iran whilst all others were obtained from published work: Ethiopia (n=2 isolates), Russia (Altai republic and far east areas)/Kazakhstan (n=6) and Mongolia (n=26) (Ito et al. 2014; Nakao et al. 2013b; Nakao et al. 2007) and included in the analysis. Haplotype diversity was high for the Ethiopian (1) and Mongolian (0.775) G6 genotypes but low for Russian/Kazakhstan (0.00045) and east Iranian (0.00051) G6 genotypes. Tajima d and Fu's Fs p indices results were positive for the east Iranian and Russian/Kazakhstan isolates and negative for the Mongolian population but none of these values was statistically significant (all P values were > 0.05). The Neutrality test could not be performed for the Ethiopian samples because the number of isolates that were analysed for the long *cox*1 sequence was too small (Table 3).

4. Discussion

Our study from east Iran has identified a predominance of the G6 genotype in humans and G6 and G1 genotypes in the camel and sheep samples, respectively. Amplification and sequence analysis of the long *cox*1 fragments revealed an extra 84 polymorphism points among the seven genotypes (G1, G3, G5-G8 and G10) of the hydatid cysts that were not found on the short size *cox*1. Two G6 sequence types, Cox1-EIran-1 and Cox1-EIran-2, were found among the G6 isolates with a higher frequency for the former. Homogeneity of isolates was found in east Iran,

Russia and Kazakhstan whereas isolates from Mongolia and Ethiopia showed heterogeneous populations. *Nad*1 sequence analysis of G6 isolates showed monophyletic population structures from east, central, northeast and southeast parts of Iran and Sudan and Mauritania whereas G6 sequence types from west (Khuzestan province) and north (Tehran) Iran and those from Egypt, Algeria and Mongolia showed more heterogeneity.

Few phylogenetic studies have compared G6 isolates from different endemic regions (Nakao et al., 2013b). Based on the analysis of the long *cox*1 gene, east Iranian, Russian and Kazakhstan populations showed intra-country homogeneity that may be explained partly by geographical factors. Vast deserts surround the east Iranian focus, limiting the movement of people, camels and other livestock to other regions. This could also explain partly the low polymorphism seen in Kazakhstan, the Asian (Altai region) and far eastern areas of Russia where there are vast plains. The low genetic expansion of ancestors may also partly explained this bottle neck effect model.

High heterogeneity exists in Mongolian G6 isolates where a high degree of haplotype diversity was also found (Ito et al., 2014). High haplotype diversity in conjunction with low nucleotide diversity in the Mongolian G6 isolates suggests the recent population expansion of this genotype in this region; negative but non-significant results of Tajima D and Fu's Fs p supports the notion of recent population expansion. High haplotype diversity and low nucleotide diversity was also found in Ethiopian G6 isolates but the small sample size limits the interpretation of these data; moreover, neutrality tests could not be calculated for the Ethiopian samples because only two isolates of G6 were available.

*Nad*1 sequence analysis showed heterogeneity of G6 isolates in Mongolia, but suggested a homogenous structure for G6 isolates from east, southeast and central Iran but greater

heterogeneity for isolates from north, northeast and west Iran. East, southeast and central Iran are surrounded by the large Kavir-e-loot and Kavir-e-namak deserts that could have limited the geographical expansion of CE isolates, resulting in a high degree of genetic homogeneity. Supporting this hypothesis are genetic studies which have demonstrated high homogeneity of east, southeast and central Iran *Leishmania tropica* in comparison to other foci, suggesting vast deserts as environmental factor effects on the monophyletic population in Iran (Ghatee et al., 2014; Karamian et al., 2016; Fakhar et al., 2016) where aridity confined the distribution of *Leishmaniasis infantum* (Ghatee et al., 2013). Moreover, the similarity of G6 isolates from arid regions of Iran with those from Sudan (Omer et al., 2010) and Mauritania (Millard et al., 2007), both Saharan countries, adds further weight for a role of dry environments limiting the transmission of CE and/or in the selection of specific haplotypes and so limiting genetic heterogeneity.

Globally, G1 is the most frequent genotype in CE cases, followed by G6 and G7 (Cassuli et al., 2009; Romig and Wassermann, 2015). The G1 genotype is considered the most common cause of CE in the main endemic foci of Iran (Rostami et al., 2015; Spotin et al., 2016) but G6 is emerging as an important genotype. In 2002, G6 was responsible for 9.1% of hospitalised CE cases and 40.8% of pathology specimens from 125 CE patients were reported from central and southeast Iran (Rostami et al., 2015). In our study, G6 was found in 8 (88%) of 9 human cases.

The higher prevalence of G1 genotype in different countries may be related to the greater number and diversity of its intermediate hosts, allowing an increased opportunity for G1 parasite transmission (Rojas et al., 2014; Romig et al., 2015). Accordingly, the high rate of G6 in countries such as Sudan and Egypt may be explained by the predominance of G6 infected

intermediate hosts like camels and goats and the relative lack of suitable intermediate hosts for transmitting the G1 “sheep” strain (Amer et al., 2015). Although South Khorasan province (east focus of CE) is one of the most important centres for camel breeding, the number of sheep is higher, estimated at ~ 1 million (Ebadzadeh et al., 2016). Therefore, the greater number of sheep cannot explain the dominance of the G6 genotype in this region. Although there are no data on the environmental resistance of eggs of different CE genotypes scattered by Canidae, arid and hot environments like deserts may favour the survival of G6 eggs in east Iran and countries like Sudan, Mauritania and Egypt. Moreover, the extensive drought affected regions in Iran over recent decades (Rostami et al., 2015) may be linked to the apparent increase in G6 CE.

Current taxonomy of *Echinococcus* spp. is based on the sequence analysis of the short size fragment of the *nad*1 and *cox*1 genes. However, some genotypes like G6 and G7 cannot be discriminated clearly by these genetic markers. Thus, some researchers advocate using non-maternal nuclear genes for the genotyping CE isolates (Gudewar et al., 2009). The whole sequencing of mitochondrial genes or amplification of different genes may overcome this challenge but this is a very costly technique that is not widely available (Nakao et al., 2007). Designing primers, which could amplify a longer fragment of single genes may lead to more informative data on the nucleotide sequences of the parasite. However, to date, the CO1F6 and Cox1_schist_3′ primers have only been used to amplify the *cox*1 gene of avian *Schistosoma* (Fakhar et al., 2016). Because of the similarity between the genomes of Cestodes (e.g. *Echinococcus*) and Trematodes (e.g. *Schistosoma*) and a previous successful pilot study using these primers by our team, we decided to use them to amplify the longer fragments of *cox*1. This

demonstrated a notable number of nucleotide variations in the sequences that had not been amplified by the routinely used JB3 and JB4.5 primers.

In conclusion, the *E. canadensis* G6 genotype is the main causative agent of CE in east Iran and has high genetic homogeneity, low polymorphism and low haplotype diversity, consistent with a bottle neck effect. We hypothesise that the harsh desert environment of eastern Iran restricts the movements of man and camels to other regions resulting in "genetic isolation." Using primers amplifying the long fragment of *cox*1 could produce more reliable results and is an area of future research. Whole genome sequencing or multi locus sequence analysis could improve greatly our knowledge of the structures and trends in expansion of different species and genotypes but their cost limits the use in developing countries.

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References

Amer, S., Helal, I.B., Kamau, E., Feng, Y., Xiao, L., 2015. Molecular Characterization of *Echinococcus granulosus* Sensu Lato from Farm Animals in Egypt. PloS one. 10, e0118509.

Bowles, J., Blair, D., McManus, D.P., 1992. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol. Biochem. Parasitol. 54, 165–173.

Chaâbane-Banaoues, R., Oudni-M'rad, M., M'rad, S., Amani, H., Mezhoud, H., Babba, H. 2016. A novel PCR-RFLP assay for molecular characterization of *Echinococcus granulosus* sensu lato and closely related species in developing countries. Parasitol. Res. 115, 3817-3824.

Debeljak, Z., Boufana, B., Interisano, M., Vidanovic, D., Kulisic, Z., Casulli, A., 2016. First insights into the genetic diversity of *Echinococcus granulosus* sensu stricto (s.s.) in Serbia. Vet. Parasitol. 223, 57-62.

Ebadzadeh, H., Ahmadi, K., Mohammadnia-Afrozi, S., Abbas-Taghani, R., Moradi-Eslami, A., Abbasi, M., Yari, S., 2016. Agricultural Statistics, Vol 2. Ministry of Agriculture, Tehran, 384 pp. [in Persian]

Eckert, J., Thompson, R.C.A., 1997. Intraspecific variation of *Echinococcus granulosus* and related species with emphasis on their infectivity to humans. Acta. Trop. 64, 19-34.

Fakhar, M., Ghobaditara, M., Brant, S.V., Karamian, M., Gohardehi, S., Bastani, R., 2016. Phylogenetic Analysis of nasal avian schistosomes (*Trichobilharzia*) from aquatic birds in Mazandaran Province, northern Iran. Parasitol. Int. 65, 151-158.

Fakhar, M., Pazoki Ghohe, H., Rasooli, S.A., Karamian, M., Mohib, A.S., Ziaei Hezarjaribi, H., Pagheh, A.S., Ghatee, M.A., 2016. Genetic diversity of *Leishmania tropica* strains isolated from clinical forms of cutaneous leishmaniasis in rural districts of Herat province, Western Afghanistan, based on ITS1-rDNA. Infect. Genet. Evol. 41,120-127.

Farhadi, M., Fazaeli, A., Haniloo, A., 2015. Genetic characterization of livestock and human hydatid cyst

isolates from northwest Iran, using the mitochondrial cox1 gene sequence. Parasitol. Res. 114, 4363-4370.

Ghatee, M.A., Sharifi, I., Haghdoost, A.A., Kanannejad, Z., Taabody, Z., Hatam, G., Abdollahipanah, A., 2013. Spatial correlations of population and ecological factors with distribution of visceral leishmaniasis cases in southwestern Iran. J. Vector. Borne. Dis. 50,179-187.

Ghatee, M.A., Sharifi, I., Kuhls ,K., Kanannejad, Z., Harandi, M.F., de Almeida, M.E., Hatam, G., Mirhendi, H., 2014. Heterogeneity of the internal transcribed spacer region in *Leishmania tropica* isolates from southern Iran. Exp. Parasitol. 144, 44-51.

Gudewar, J., Pan, D., Bera, A.K., Das, S.K., Konar, A., Rao, J.R., Tiwari, A.K., Bhattacharya, D., 2009. Molecular characterization of *Echinococcus granulosus* of Indian animal isolates on the basis of nuclear and mitochondrial genotype. Mol. Biol. Rep. 36, 1381-1385.

Hall, T., 2011. BioEdit: an important software for molecular biology. GERF. Bull. Biosci. 2, 60-61.

Jabbar, A., Narankhajid, M., Nolan, M.J., Jex, A.R,, Campbell, B.E,, Gasser, R.B., 2011. A first insight into the genotypes of *Echinococcus granulosus* from humans in Mongolia. Mol. Cell. Probes. 25, 49-54

Ito, A., Dorjsuren, T., Davaasuren, A., Yanagida, T., Sako, Y., Nakaya, K., Nakao, M., Bat-Ochir, O.E,, Ayushkhuu, T., Bazarragchaa, N., Gonchigsengee, N., Li, T., Agvaandaram, G., Davaajav, A., Boldbaatar, C., Chuluunbaatar, G., 2014. Cystic echinococcoses in Mongolia: molecular identification, serology and risk factors. PLoS. Negl. Trop. Dis. 8, e2937.

Karamian, M., Kuhls, K., Hemmati, M., Ghatee, M.A., 2016. Phylogenetic structure of *Leishmania tropica* in the new endemic focus Birjand in East Iran in comparison to other Iranian endemic regions. Acta. Trop. 158, 68-76.

Kinkar, L., Laurimäe, T., Simsek, S., Balkaya, I., Casulli, A., Manfredi, M.T., Ponce-Gordo, F., Varcasia,

A., Lavikainen, A., González, L.M., Rehbein, S., VAN DER Giessen, J., Sprong, H., Saarma, U., 2016. High-resolution phylogeography of zoonotic tapeworm *Echinococcus granulosus* sensu stricto genotype G1 with an emphasis on its distribution in Turkey, Italy and Spain. Parasitology. 143, 1790-1801.

Lavikainen, A., Lehtinen, M.J., Meri, T., Hirvelä-Koski, V., Meri, S., 2003. Molecular genetic characterization of the Fennoscandian cervid strain, a new genotypic group (G10) of *Echinococcus granulosus*. Parasitology 127:207-215

Lockyer, A.E., Olson, P.D,, Østergaard, P., Rollinson, D., Johnston, D.A., Attwood, S.W., Southgate, V.R., Horak, P., Snyder, S.D., Le, T.H., Agatsuma, T., McManus, D.P., Carmichael, A.C., Naem, S., Littlewood, D.T., 2003. The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. Parasitology. 126, 203-224.

Maillard, S., Benchikh-Elfegoun, M.C., Knapp, J., Bart, J.M., Koskei, P., Gottstein, B., Piarroux, R., 2007. Taxonomic position and geographical distribution of the common sheep G1 and camel G6 strains of *Echinococcus granulosus* in three African countries. Parasitol. Res. 100, 495-503.

Morgan, J.A., DeJong, R.J., Kazibwe, F., Mkoji, G.M. and Loker, E.S., 2003. A newly-identified lineage of Schistosoma. Int. J. Parasitol. 33, 977-985.

Nakao, M., Lavikainen, A., Yanagida, T., Ito, A., 2013a. Phylogenetic systematics of the genus *Echinococcus* (Cestoda: Taeniidae). Int. J. Parasitol. 43, 1017-1029.

Nakao, M., Yanagida, T., Konyaev, S., Lavikainen, A., Odnokurtsev, V.A., Zaikov, V.A., Ito, A., 2013b. Mitochondrial phylogeny of the genus *Echinococcus* (Cestoda: Taeniidae) with emphasis on relationships among *Echinococcus canadensis* genotypes. Parasitology. 140, 1625-1636.

Nakao, M., McManus, D.P., Schantz, P.M., Craig, P.S., Ito, A., 2007. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. Parasitology. 134, 713-722.

Roinioti, E., Papathanassopoulou, A., Theodoropoulou, I., Simsek, S., Theodoropoulos, G., 2016.

Molecular identification of *Echinococcus granulosus* isolates from ruminants in Greece. Vet. Parasitol. 226, 138-44.

Rojas, C.A., Ebi, D., Gauci, C.G., Scheerlinck, J.P., Wassermann, M., Jenkins, D.J., Lightowlers, M.W., Romig, T., 2016. Microdiversity of *Echinococcus granulosus* sensu stricto in Australia. Parasitology. 143, 1026-1033.

Rojas, C.A., Romig, T., Lightowlers, M.W., 2014. *Echinococcus granulosus* sensu lato genotypes infecting humans–review of current knowledge. Int. J. Parasitol. 44, 9-18.

Romig, T., Ebi, D., Wassermann, M., 2015. Taxonomy and molecular epidemiology of *Echinococcus granulosus* sensu lato. Vet. Parasitol. 213, 76-84.

Rostami, S., Torbaghan, S.S., Dabiri, S., Babaei, Z., Mohammadi, M.A., Sharbatkhori, M., Fasihi Harandi, M., 2015. Genetic characterization of *Echinococcus granulosus* from a large number of formalin-fixed, paraffin-embedded tissue samples of human isolates in Iran. Am. J. Trop. Med. Hyg. 92, 588-594.

Rozas, J., Librado, P., Sanchez-DelBarrio, J.C., Messeguer, X., Rozas, R., 2010. DnaSP Version 5 Help Contents [Help File]. Available with the program at http://www. ub. edu/dnasp.

Sadjjadi, S.M., 2006. Present situation of echinococcosis in the Middle East and Arabic North Africa. Parasitol. Int. 55, s197-s202.

Schneider, R., Gollackner, B., Edel, B., Schmid, K., Wrba, F., Tucek, G., Walochnik, J., Auer H., 2008. Development of a new PCR protocol for the detection of species and genotypes (strains) of *Echinococcus* in formalin-fixed, paraffin-embedded tissues. Int. J. Parasitol. 38, 1065-1071.

Scott, J.C., McManus, D.P., 1994. The random amplification of polymorphic DNA can discriminate species and strains of *Echinococcus*. Trop. Med. Parasitol. 45, 1-4.

Sharbatkhori, M., Mirhendi, H., Jex, A.R., Pangasa, A., Campbell, B.E., Kia, E.B., Eshraghian, M.R.,

Harandi, M.F., Gasser, R.B., 2009. Genetic categorization of *Echinococcus granulosus* from humans and herbivorous hosts in Iran using an integrated mutation scanning- phylogenetic approach. Electrophoresis. 30, 2648-2655.

Spotin, A., Mahami-Oskouei, M., Harandi, M.F., Baratchian, M., Bordbar, A., Ahmadpour, E., Ebrahimi, S., 2016. Genetic variability of *Echinococcus granulosus* complex in various geographical populations of Iran inferred by mitochondrial DNA sequences. Acta. Trop. 165, 10-16.

Tigre, W., Deresa, B., Haile, A., Gabriël, S., Victor, B., Pelt, J.V., Devleesschauwer, B., Vercruysse, J., Dorny, P., 2016. Molecular characterization of *Echinococcus granulosus* s.l. cysts from cattle, camels, goats and pigs in Ethiopia. Vet. Parasitol. 215, 17-21.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725-2729.

Zhang, L., Eslami, A., Hosseini, S.H., McManus, D.P., 1998. Indication of the presence of two distinct strains of *Echinococcus granulosus* in Iran by mitochondrial DNA markers. Am. J. Trop. Med. Hyg. 59, 171-174.

Zhang, T., Yang, D., Zeng, Z., Zhao, W., Liu, A., Piao, D., Jiang, T., Cao, J., Shen, Y., Liu, H., Zhang, W., 2014. Genetic characterization of human- derived hydatid cysts of *Echinococcus granulosus* sensu lato in Heilongjiang Province and the first report of G7 genotype of *E. canadensis* in humans in China. PloS one. 9, e109059.

Table 1

GenBank accession numbers of *cox1* of *Echinococcus canadensis* genotype G6/7 used in phylogenetic analysis in the present study.

Accession Numbers	Location	*cox1* haplotypes	Hosts (parasite stages)	References
AB208063	Kazakhstan	EcG6	Camel (L)	Nakao *et al.* 2007
NC_011121	Kazakhstan	n. d.	Camel (L)	Nakao *et al.* 2007
AB688142	Russia:Altai	EcRUS1	Human (L)	Konyaev *et al.* 2012
AB777909	Russia	EcRUS2	Wolf (A)	Nakao *et al.* 2013
AB777922	Ethiopia	EcETH1	Camel (L)	Nakao *et al.* 2013
AB777923	Ethiopia	EcETH2	Camel (L)	Nakao *et al.* 2013
AB813182	Mongolia	EcMGL1	Wolf (A)	Yanagida *et al.* 2013
AB813183	Mongolia	EcMGL2	Wolf (A)	Yanagida *et al.* 2013
AB893252	Mongolia	EcMGL5	Human (L)	Ito *et al*. 2014
AB893253	Mongolia	EcMGL2	Human (L)	Ito *et al*. 2014
AB893254	Mongolia	EcMGL6	Human (L)	Ito *et al*. 2014
AB893255	Mongolia	EcMGL7	Human (L)	Ito *et al*. 2014
AB893256	Mongolia	EcMGL8	Human (L)	Ito *et al*. 2014
AB893257	Mongolia	EcMGL9	Human (L)	Ito *et al*. 2014
AB893258	Mongolia	EcMGL10	Human (L)	Ito *et al*. 2014
AB893259	Mongolia	EcMGL11	Human (L)	Ito *et al*. 2014
AB893260	Mongolia	EcMGL12	Human (L)	Ito *et al*. 2014
AB893261	Mongolia	EcMGL13	Human (L)	Ito *et al*. 2014
AB893262	Mongolia	EcMGL14	Human (L)	Ito *et al*. 2014
AB893263	Mongolia	EcMGL15	Human (L)	Ito *et al*. 2014
KP751430	Iran:Birjand	Cox1-EIran-1	Camel (L)	This study
KP751426	Iran:Birjand	Cox1-EIran-2	Camel (L)	This study
KR337822	Iran:Gachsaran	Cox1-EIran-1	Goat (L)	Karamian *et al*. 2015

L-larva

A-adult

Table 2

GenBank accession numbers of *nad1* of *Echinococcus canadensis* genotype G6/7 used in phylogenetic analysis in the present study.

Accession Numbers	Location	Hosts (parasite stages)	References
AJ237637	Unknown	Camel (L)	Bowles & McManus 1993
AF408689	Algeria	Camel (L)	Bardonnet *et al.* 2003
HM563036	Iran:Kerman	Sheep (L)	Fasihi Harandi *et al.* 2010
HM563037	Iran:Kerman	Human (L)	Fasihi Harandi *et al.* 2010
HM853658	Iran:Isfahan	Camel (L)	Sharbatkhori *et al.* 2011
HM853659	Iran:Isfahan	Camel (L)	Sharbatkhori *et al.* 2011
HM853660	Iran:Isfahan	Camel (L)	Sharbatkhori *et al.* 2011
HM853661	Iran:Isfahan	Camel (L)	Sharbatkhori *et al.* 2011
HQ231401	Mongolia	Human (L)	Jabbar *et al.* 2010
HQ231402	Mongolia	Human (L)	Jabbar *et al.* 2010
HQ231403	Mongolia	Human (L)	Jabbar *et al.* 2010
HQ585934	Iran:Mashhad	Camel (L)	Sharifiyazdi *et al.* 2011
JN637176	Sudan	Camel (L)	Ahmed *et al.* 2013
JN560698	Iran:Tehran	Human (L)	Sadjjadi *et al.* 2013
JN562748	Iran:Tehran	Human (L)	Sadjjadi *et al.* 2013
JN562750	Iran:Tehran	Human (L)	Sadjjadi *et al.* 2013
JN621321	Iran:Khuzestan	Human (L)	Sadjjadi *et al.* 2013
JN621322	Iran:Khuzestan	Human (L)	Sadjjadi *et al.* 2013
KM513638	Iran:Golestan	Camel (L)	Sharbatkhori *et al.* 2016
AB921100	Egypt	Camel (L)	Amer *et al.* 2015
AB921111	Egypt	Camel (L)	Amer *et al.* 2015
AB921117	Egypt	Camel (L)	Amer *et al.* 2015
AB921118	Egypt	Camel (L)	Amer *et al.* 2015
AB921119	Egypt	Camel (L)	Amer *et al.* 2015
AB921120	Egypt	Camel (L)	Amer *et al.* 2015
AB921121	Egypt	Camel (L)	Amer *et al.* 2015
KP751432	Iran:Birjand	Human (L)	This study
KR349048	Algeria	Human (L)	Zait *et al.* 2016
KT316343	Algeria	Camel (L)	Zait *et al.* 2016
KT363811	Mauritania	Camel (L)	Chaâbane-Banaoues et al. 2015

L-larva

A-adult