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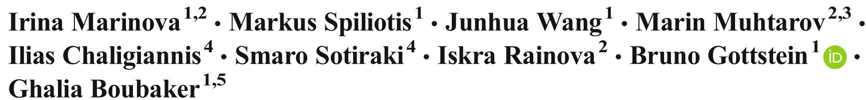
ORIGINAL PAPER

Molecular characterization of *Echinococcus granulosus* isolates from Bulgarian human cystic echinococcosis patients



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Abstract Although cystic echinococcosis (CE) is highly endemic in Bulgaria, there is still scarce information about species and/or genotypes of the *Echinococcus granulosus* complex that infect humans. Our study tackled the genetic diversity of *E. granulosus* complex in a cohort of 30 Bulgarian CE patients. Ten animal *E. granulosus* isolates from neighboring Greece were additionally included. Specimens were comparatively analyzed for partial sequences of five mitochondrial (mt) (*cox I*, *nad I*, *rrnS*, *rrnL*, and *atp6*) and three nuclear (nc) genes (*act II*, *hbx 2*, and *ef-1α*) using a PCR-sequencing approach. All 30 Bulgarian isolates were identified as *E. granulosus* sensu stricto (s.s.) and were showing identical sequences for each of the three examined partial nc gene markers. Based upon concatenated sequences from partial mtDNA markers, we detected 10 haplotypes: 6 haplotypes (H1-H6) clustering with *E. granulosus* s.s. (G1) and 4 haplotypes (H9-H13) grouping with *E. granulosus* s.s. (G3), with H1 and H10 being the most frequent in Bulgarian patients. The haplotypes H1, H4, and H11 were also present in Greek hydatid cyst samples of animal origin. In conclusion, *E. granulosus* s.s. (G1 and G3 genotypes) is the only causative agent found so far to cause human CE in Bulgaria. However, further studies including larger sample sizes and other additional geographic regions in Bulgaria will have to be performed to confirm our results.

Keywords *Echinococcus* spp. · PCR · Genotyping · Speciation · Nuclear markers · Mitochondrial markers

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Introduction

Cystic echinococcosis (CE) is a chronic helminthic zoonosis of worldwide distribution. Disease (CE) is caused by the development of larval stages of tapeworms (cestodes) belonging to the genus *Echinococcus* (family Taeniidae). CE affects a wide variety of livestock species which act as intermediate hosts (IH), while humans actually represent aberrant IH (Eckert et al. 2004). Within the genus *Echinococcus*, two major species are of medical importance, including *E. granulosus* sensu lato (s.l.) and *E. multilocularis* that cause CE and alveolar echinococcosis (AE), respectively (Eckert et al. 2004). In contrast to *E. multilocularis* (Haag et al. 1997; Snabel et al. 2006), *E. granulosus* s.l. exhibits a high genotypic diversity with 10 genotypes (G1-G10) that were molecularly distinguished so far, predominantly based on genetic polymorphism of mitochondrial (mt) genes (Bowles et al. 1992a, 1994; Bowles and McManus 1993; Scott et al. 1997; Lavikainen et al. 2003). The taxonomy and relationships among various species of the genus *Echinococcus* were widely reviewed in recent times, and accordingly, *E. granulosus* s.l. was regarded as an oversimplified species (Thompson and McManus 2002; Jenkins et al. 2005; Nakao et al. 2007; Hüttner et al. 2008; Saarma et al. 2009; Knapp et al. 2011; Nakao et al. 2013).

Thus to date, the *E. granulosus* complex consists of five species: *E. granulosus* sensu stricto (s.s.) (clustering genotypes G1, G2, and G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (grouping genotypes G6-G8 and G10), and *E. felidis* (G9) (Thompson and McManus 2002; Jenkins et al. 2005; Nakao et al. 2007; Hüttner et al. 2008; Saarma et al. 2009; Knapp et al. 2011; Nakao et al. 2013). The complex phylogenetic relationships among genotypes of the *E. canadensis* (G6-G8, G10) cluster were revealed by a discrepancy between nuclear (nc) and mt phylogenies. Consequently, this genetic and ecological complexity of *E. canadensis* (G6-G8, G10) has led to an open debate about dividing it into two or more species (Thompson et al. 2006; Tompson 2008; Moks et al. 2008; Saarma et al. 2009; Lymbery et al. 2015a, b; Nakao et al. 2015).

E. granulosus s.s. accounts for most of the global burden of CE in humans and livestock (Alvarez et al. 2014), and it harbors the largest and the most diverse number of mtDNA haplotypes, which could be explained by many factors such as a large population size, a natural wide IH spectrum, environmental heterogeneity, and/or a clonal reproduction during the larval stage. In general, reported mt haplotypes gathered by similarity either around G1 or G3, while G2 seems to be a micro-variant of G1 or often of G3 (Snabel et al. 2009; Alvarez et al. 2014). Nevertheless, because of the unclarity on inter-genotypic genetic distances between genotypes G1, G2, and G3 of the *E. granulosus* s.s., the genotype terminology has been questioned for its biological and genetic validity (Busi et al. 2007; Snabel et al. 2009; Beato et al. 2010; Nakao et al. 2013; Alvarez et al. 2014). Subsequently, to date, two different systems are used to categorize parasite specimens; researchers refer either directly to the species level: *E. granulosus* s.s. (with various lineages) (Piccoli et al. 2013; Mutwiri et al. 2013; Boufana et al. 2015; Bakal et al. 2015; Wang et al. 2015) or to genotype (with various haplotypes).

Human CE is considered highly endemic in Bulgaria, with an annual incidence of 5.71 cases per 100,000 inhabitants for the period between 2003 and 2012 (Euro Surveillance team 2013; Rainova et al. 2014; Jordanova et al. 2015). In rural areas of Bulgaria such as Sliven, Yambol, Razgrad, Targovishte etc., prevalence may reach up to 7.5–16 cases per 100,000 inhabitants.

So far, there is limited data on the molecular typing of species and genotyping of the *E. granulosus* complex circulating in Bulgaria (Breyer et al. 2004; Casulli et al. 2012; Boubaker et al. 2013), and accordingly, only *E. granulosus* s.s. (G1/G2/G3) was found in humans (Casulli et al. 2012) and animals (Breyer et al. 2004; Casulli et al. 2012; Boubaker et al. 2013). In contrast, *E. granulosus* s.s (G1) and *E. canadensis* (G7) were identified in both human and livestock from countries adjacent to Bulgaria, such as Greece (Varcasia et al. 2007), Romania (Bart et al. 2006; Casulli et al. 2012; Piccoli et al. 2013), Turkey (Snabel et al. 2009), and Serbia (Maillard et al. 2009; Bobic et al. 2012). *E. canadensis* (G7) has been also isolated from Macedonian CE patients (Schneider et al. 2010). Thus, preliminary findings showing a non-existence of *E. canadensis* (G7) in Bulgarian territory, based upon a restricted number of samples, definitively require further investigations for confirmation or else.

Overall, in the present study, we intended (i) to molecularly identify species and genotypes of the *E. granulosus* complex infecting human Bulgarian CE patients and (ii) to investigate their genetic variation respective to nc and mt targets.

In addition, we included Greek *E. granulosus* isolates with the aim to briefly search for matching haplotypes circulating in this neighboring countries.

Materials and methods

Ethical statement

Parasite samples of human origin were derived from an existing collection of *Echinococcus* DNA of the NRL "Diagnostics of Parasitic Diseases" at the National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria, designed and approved to be used for basic research studies. Cysts were obtained during the routine diagnostic activities of the NRL. The derived samples used in the present study uniquely contained DNA of parasitic origin; thus, no human DNA was included in this study. The parasite samples were fully anonymized respective to the patients' private data, thus requiring no further ethical consideration.

In this study, we also included 10 *E. granulosus* samples originating from Greek animal cysts that were collected as a part of the governmental meat inspection program of public slaughterhouses. Samples were obtained from the Veterinary Research Institute Hellenic Agricultural Organization-Demeter (former NAGREF), NAGREF Campus, Thermi, Thessaloniki, Greece (Chaligiannis et al. 2015). All protoscoleces and/or cyst walls (germinal and laminar layer) collected were preserved in 70% ethanol and stored at −20 °C for a later parasitological and molecular processing and identification.

Bulgarian human CE patients

Thirty CE patients (20 females and 10 males) originating from different regions of Bulgaria were included in this study. Cyst collection (25 hepatic and 5 pulmonary) took place during conventional surgical interventions, which occurred between 2004 and 2014. According to WHO-IWGE classification (Pawlowski et al. 2001), hepatic cysts were categorized as follows: 19 cysts were CE1 (76%), 2 cysts as CE2 (8%), and 4 cysts as CE3 (16%). The median age of the patients was 43 ± 24 years (min. 10 years, max. 79 years).

Animal hydatid cysts

Ten hydatid cysts isolated from Greek animals (3 sheep, 3 buffaloes, 2 goats, and 2 wild boars) were additionally included with the aim to briefly search for common haplotypes circulating in this neighboring countries.

Molecular analyses

DNA extraction

Hydatid cysts from Bulgarian patients were stored in 96% ethanol prior to DNA extraction. Depending on the fertility or infertility of the cysts, genomic DNA (gDNA) was extracted either from protoscoleces or from cyst walls (germinative layers), respectively. The gDNA extraction was carried out using a commercial DNAeasy tissue kit (QIAGEN, Germany). Concerning animal samples, gDNAs were extracted from protoscoleces and/or germinal layers using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) (Chaligiannis et al. 2015). Subsequently, all isolated gDNAs were checked with NanoDrop 1000 (Thermo scientific) for qualitative and quantitative appropriateness. The gDNA samples were stored at −20 °C until use.

Mitochondrial and nuclear gene targets

In total, eight DNA targets in the nc and mt genomes were separately amplified by polymerase chain reaction (PCR). Mitochondrial targets were partial gene sequences coding for cytochrome c oxidase I (*cox I*) (Bart et al. 2006), NADH dehydrogenase I (*nad I*) (Bart et al. 2006), the small ribosomal RNA (*rrn*S), the large ribosomal RNA (*rrn*L) (Boubaker et al. 2016), and ATP synthase subunit 6 (*atp 6*). The two primer pairs *rrn*S-F/*rrn*S-R and *atp 6*-F/*atp 6*-R were developed in this study.

Nuclear gene markers were covering both coding and/or non-coding sequences, including actin II (*actII*) (da Silva et al. 1993), homeodomain protein (*hbx2*) (Haag et al. 1997), and elongation factor 1α (*ef-1α*) (Saarma et al. 2009).

The sequences of the primer pairs used are shown in Table 1.

PCR conditions

PCRs were performed in a 40 μl volume containing 4 μl DNA (80–200 ng DNA), 100 μM dNTPs, 0.5 μM of each primer, and 0.4 U Taq polymerasein 1× PCR Buffer (all Promega, Switzerland). The amplification was carried out in a thermocycler (Biometra T3000 Thermocycler, Labrepco) under the following conditions: an initial start at 94 °C for 3 min followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at optimal temperature for each primer pair as listed in Table 1, extention at 72 °C for 90 s for *ef-1α* and 30 s for the other primers, and a final extention for 5 min at 72 °C. The electrophoretic resolution of amplificates was performed on 2% agarose gel stained with ethidium bromide, and the result was visualized under UV light (Syngen, Synoptic group). Sequences with single-point mutations were additionally amplified with High Fidelity Phusion polymerase (Promega, USA) and sequenced as described below for further confirmation of the results.

Sequencing and phylogenetic analyses

PCR products were purified using High Pure PCR Product Purification Kit (Roche, Switzerland). The automated sequencing was performed with Big Dye kit (Invitrogen) and the Applied Biosystems/Hitachi 3730 DNA analyzer. All amplicons were sequenced in both directions with the corresponding forward and reverse primer (Table 1). The obtained sequences were analyzed using Bioedit version 7.1 software (Hall 1999). The alignments were performed using CrustalW program and sequences were checked manually. The sequences were blasted against available sequences from GenBank database for identification [http://www.ncbi.nlm.nih.gov/]. Phylogenetic analyses were performed with the MEGA 6 software (http: www.megasoftware.net) (Tamura et al. 2013). For the reconstruction of the two phylogenetic trees from the concatenated nc and mt genes, neighbor-joining (NJ) method was applied (Saitou and Nei 1987) with bootstrap test of 1000 replications (Felsenstein 1985) and Kimura 2-parameter model for the estimation of transitions and transversions (Kimura 1980). *Taenia solium* was used as an outgroup control sequence. Haplotype network was constructed using TCS 1.21 (Clement et al. 2000). Diversity and neutrality indices and divergence (Da) were calculated with the DNASP software (Rozas and Rozas 1995) and MEGA 6. The divergence time (T) between haplotypes was calculated as T = Da/2 μ, where Da is the divergence between haplotypes and 2 μ is the divergence rate of mtDNA. The divergence rate of mtDNA was accepted to be 2–4% per 1 million years (Cann et al. 1987).

Statistical methods

Statistical analyses were performed using SPSS16.0 for Windows. In the study, the following tests were applied: Fisher exact test, Shapiro-Wilks test for normality, Kruskal-Wallis test, and Chi-square test for goodness and fit. Values of $P < 0.05$ were considered significant.

Accession numbers

The accession numbers for the known complete mt genome sequences of *E. granulosus* s.*l*. used in this study were as follows: references: G1 (AF297617.1), G3 (KJ559023.1), G4 (AF346403.1), G5 (AB235846.1), G6 (AB208063.1), G7 (AB235847.1), G8 (AB235848.1), G10 (AB745463.1).

Table 1 List and characteristics of primers used in the study

Gene	Primer name	Sequence (5″-3″).	Tm (°C)	Ref
mt targets				
cox 1	EgCOI1	TTTTTTGGCCATCCTGAGGTTTAT	56	(Bart et al. 2006)
	EgCOI2	TAACGACATAACATAATGAAAATG		
nad 1	EgNDI1	AGTCTCGTAAGGGCCCTAACA	56	(Bart et al. 2006)
	EgNDI2	CCCGCTGACCAACTCTCTTTC		
rrnS	rrnS-F	GTTTATCAGTACGAAAGGACAG	56	This study
	rrnS-R	ACACCCTTATTAATGTAACACA		
rrnL	rrnL-F	TTATTTGCCTTTTGCATCA	58	(Boubaker et al. 2016)
	rrnL-R	AAAAGATCCTAGGGTCTTTCCGT		
atp6	atp6-F	TTTGGTCGTGTGTCATATTAC	56	This study
	atp6-R	CTAATGATCGACTGAAAAATC		
nc targets				
actII	actII-F	GTCTTCCCCTCTATCGTGGG	63	(da Silva et al. 1993)
	actII-R	CTAATGAAATTAGTGCTTTGTGCGC		
hbx2	hbx2-F	TTCTCCTCTAGCCAGGTCCA	63	(Haag et al. 1997)
	hbx2-R	TATAGCGCCGATTCTGGAAC		
ef-1α	ef-1α-F	TCATTGTTATCGGTCACGTC	63	(Saarma et al. 2009)
	ef-1α-R	CTTCTGGGCAGATTTTGTG		

Tm annealing temperature

The complete mt genome of *T. solium* was retrieved under this number: NC_004022.1. Accession numbers of references nc targets were as follows: *act II* (AF528500.1 and AF003749.1), *hbx 2* (FJ997243.1 and AY129568.1), and *ef-1α* (FN568380.1 and FN568384.1). The nucleotide sequences of the *E. granulosus* s.*l*. mt and n genes identified in this study have been deposited in GenBank under the following accession numbers: with accession numbers KR070964-KR070992 (mt-DNA) and KR070993-KR070994 and KR138703-KR138706 (n-DNA).

Results

Amplification and sequencing were successful for all studied samples and targets, resulting thus in 320 readable sequences, consisting of the following: 334 nucleotides for *cox I*, 475 for *nad I*, 406 for *rrn*S, 605 for *rrn*L, 390 for *atp 6*, 201 for *hbx 2*, 280 for *act II*, and 1200 for *ef-1α*.

Genetic polymorphism among Bulgarian *E. granulosus* complex human isolates based on mitochondrial markers

All Bulgarian human cysts were identified as *E. granulosus* s.s. with a total of 21 identified haplotypes among our datasets including five mt gene markers: *cox I* (2 haplotypes; C1 and C3), *nad I* (7 haplotypes; N1-N7), *rrnS* (3 haplotypes; S1-S3), *rrnL* (4 haplotypes; L1-L4), and *atp 6* (5 haplotypes; ATP1-ATP5) (Table 2). We found six novel sequences which did not match any sequence in the GenBank database, and they were derived from the following: *nad I* (2 haplotypes; KR070986 and KR070988), *rrn*S (1 haplotype KR070971), *rrn*L (1 haplotype KR070974), and *atp6* (2 haplotypes KR070979 and KR070982).

Eleven out of the 21 haplotypes were also detected in the Greek samples (Table 2).

The alignments from the five partial mt gene sequences were then concatenated to give a single multiple sequence alignment covering 2210 bp and resulting in 10 haplotypes (H1-H6; H9-H12) among Bulgarian *E. granulosus* s.s. population. Six haplotypes (H1-H6) were identified in 16 human isolates and clustered with *E. granulosus* s.s. (G1). Four haplotypes (H9-H12), found in 14 isolates, grouped with *E. granulosus* s.s. (G3) (Table 2 and Fig. 1).

Three haplotypes (H1 (G1), H4 (G1), and H11 (G3)) were shared by samples from Bulgaria and Greece.

One haplotype (H14) was strictly identified in 3 Greek cysts (2 goats and 1 wild boar) and was categorized as *E. canadensis* (G7) (Table 2 and Fig. 1).

In the concatenated sequences, a set of 9 single nucleotide polymorphisms (SNPs) differentiated all *E. granulosus* s.s. (G1) isolates from *E. granulosus* s.s. (G3). Two additional SNPs were detected in 12 samples belonging to haplotypes H10-H13 (also in the reference for G3) and differentiate G1 and G3 genotypes of the *E. granulosus* s.s. (Table 3).

A subset of 286 and 283 SNPs differentiate *E. canadensis* (G7) (H14) from *E. granulosus* s.s. genotypes G1 (H1) and G3 (H3), respectively.

Table 2 Concatenated mitochondrial haplotypes detected in this study

Haplotype No.	*cox I*[a,b]	*nad I*	*rrnS*	*rrnL*	*atp 6*	No. [c] of BS	No. of GS
H1	KR070964 (C1)	KR070984 (N1)	KR070969 (S1)	KR070973 (L1)	KR070978 (ATP1)	11	2
H2	KR070964 (C1)	KR070986 (N3)	KR070969 (S1)	KR070973 (L1)	KR070978 (ATP1)	1	-
H3	KR070964 (C1)	KR070988 (N5)	KR070969 (S1)	KR070974 (L2)	KR070978 (ATP1)	1	-
H4	KR070964 (C1)	KR070985 (N2)	KR070969 (S1)	KR070973 (L1)	KR070978 (ATP1)	1	1
H5	KR070964 (C1)	KR070989 (N6)	KR070969 (S1)	KR070973 (L1)	KR070978 (ATP1)	1	-
H6	KR070964 (C1)	KR070987 (N4)	KR070969 (S1)	KR070973 (L1)	KR070979 (ATP2)	1	-
H7	KR070965 (C2)	KR070984 (N1)	KR070969 (S1)	KR070973 (L1)	KR070978 (ATP1)	-	1
H8	KR070964 (C1)	KR070991 (N8)	KR070969 (S1)	KR070973 (L1)	KR070978 (ATP1)	-	1
H9	KR070966 (C3)	KR070990 (N7)	KR070970 (S2)	KR070976 (L4)	KR070981 (ATP4)	4	-
H10	KR070966 (C3)	KR070990 (N7)	KR070970 (S2)	KR070975 (L3)	KR070980 (ATP3)	8	-
H11	KR070966 (C3)	KR070990 (N7)	KR070971 (S3)	KR070975 (L3)	KR070980 (ATP3)	1	1
H12	KR070966 (C3)	KR070990 (N7)	KR070970 (S2)	KR070975 (L3)	KR070982 (ATP5)	1	-
H13	KR070967 (C4)	KR070990 (N7)	KR070970 (S2)	KR070975 (L3)	KR070980 (ATP3)	-	1
H14	KR070968 (C5)	KR070992 (N9)	KR070972 (S4)	KR070977 (L5)	KR070983 (ATP6)	-	3

[a] Accession number in GenBank of each individual gene markers

[b] Designation of the haplotype in the individual genes is given in parenthesis

[c] Number of Bulgarian (BS) and Greek samples (GS) with each haplotype

The network of mt haplotypes based on the concatenated sequences was drawn to evaluate relationships among the different *E. granulosus* s.s. haplotypes (Fig. 2). Thus, haplotype H1 was inferred as the hypothetical ancestral haplotype. H2-H8 differed from H1 in 1 to 2 mutational steps. H8 presented as an intermediate ancestral haplotype to H2. H9-H13 differed from H1 by 9 to 12 mutational steps. H9 appeared as an intermediate ancestral haplotype to H10, and H10 appeared as an ancestral haplotype to H11-H13, which differed from each other by single-point mutations (Fig. 2).

The divergence between haplotypes H1 and H9 was Da = 0.00407 (0.41%), between H1 and H10 was Da = 0.0054 (0.54%), and between H9 and H10 was Da = 0.0009 (0.09%). The divergence time between H1 and H9 was estimated at 100,000–200,000 years of evolution, between H1 and H10 at 135,000–270,000 years of evolution, and between H9 and H10 at 35,000–70,000 years of evolution. Data for the diversity and neutrality indices of *E. granulosus* s.s.in the Bulgarian and Greek samples from the 5 mt targets are shown in Table 4.

Genetic polymorphism among Bulgarian *E. granulosus* Complex human isolates based on nuclear markers

For each of the three investigated nc markers (*act II*, *hbx*, and *ef-1α*), all Bulgarian human isolates were showing identical sequences and formed one cluster with *E. granulosus* s.s. (G1/G2/G3) (Table 5 and Fig. 3).

The concatenated sequences of the partial nc genes consisted of 1521 readable nucleotides and they formed one single *E. granulosus* s.s. (G1/G2/G3) haplotype (N1) for all Bulgarian human cysts. This later haplotype (N1) was also present in Greek samples, where additionally one more haplotype (N2) was detected, referring to *E. canadensis* (G6-G8, G10) (Table 5). Within the present study sample collection,

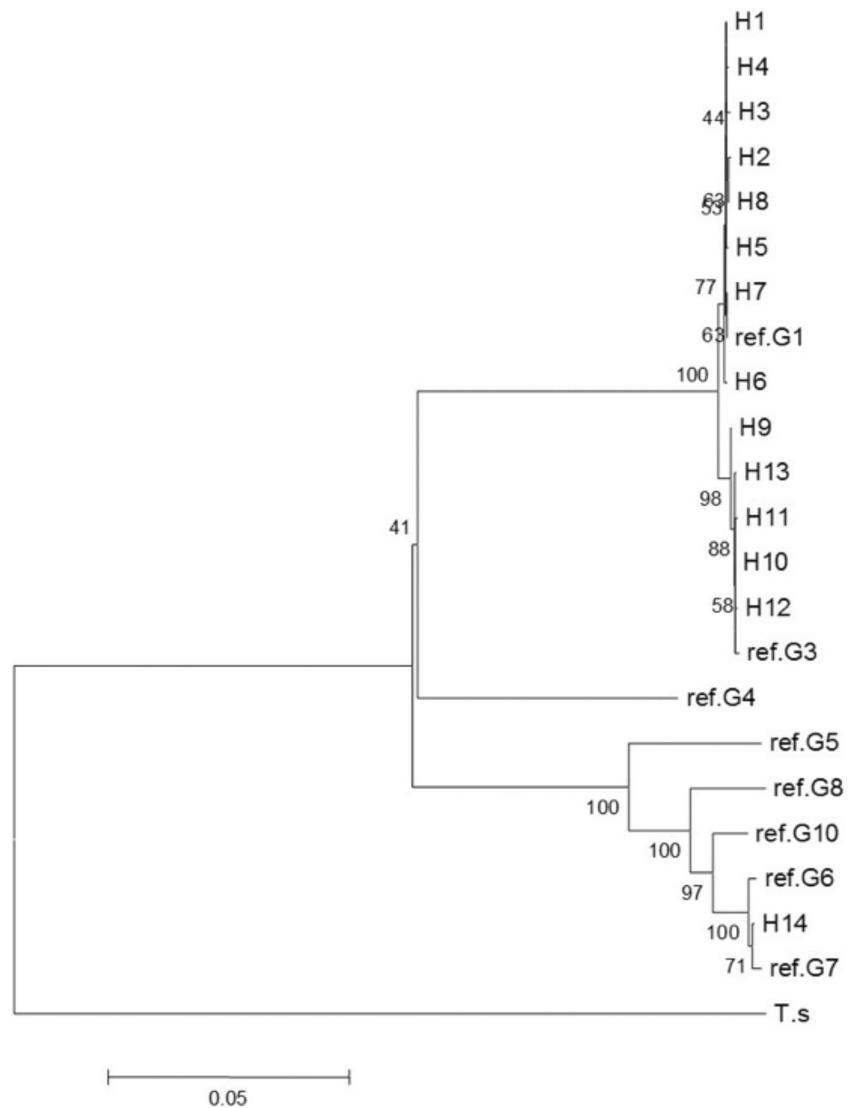

Fig. 1 Dendrogram of concatenated sequences (haplotypes H1-H14) of Bulgarian and Greek samples and references for genotypes G1-G8 and G10. The neighbor-joining method with Kimura 2-parameter method and bootstrap test (1000 replicates) was applied. *Taenia solium* (*T. s*) was used as outgroup. The numbers next to the branches represent the percentage of replicate trees, in which the associated taxa cluster together in the bootstrap test

we report a total of 32 SNPs distinguishing between N1 (*E. granulosus* s.s.) and N2 (*E. canadensis)*.

The sequences in the present study are deposited in GenBank under following accession numbers KR070993-KR070994 (*ef-1*α), KR138703-KR138704 (*act II*), and KR138705-KR138706 (*hbx*).

Geographical distribution and correlation of *E. granulosus* s.s. haplotypes with human CE in Bulgaria

The analysis of mt and nc genome sequences revealed that the Bulgarian samples belonged to *E. granulosus* s.s. Based on mt genomes, 16 isolates clustered with the reference for genotype G1 and 14 with genotype G3. In order to compare the samples, we divided them into these two clusters *E. granulosus* s.s. (G1) and *E. granulosus* s.s. (G3). The geographical distribution revealed that they were evenly spread across the territory of Bulgaria, and in some regions, where more samples were collected, they occurred sympatrically, although in different patients. Comparison according to the gender of the patients, age, and location of the cyst did not show statistically significant difference between the two groups (Fisher exact test, $P > 0.05$). The median values of the cyst size of G1 (6.6 cm ±3.5 SD) and G3 (5.5 cm ±3.8 SD) showed again a lack of statistically significant difference (Shapiro-Wilks test for normality $P < 0.01$; Kruskal-Wallis test 1.906, $P > 0.05$). According to the ultrasound classification of the liver cysts, the predominant form in both groups was CE1 (84% in G1 and 75% in G3), the results being statistically significant (Chi-square test—14.965 and 9.5 for G1 and G3, respectively, $P < 0.05$).

Discussion

All 30 Bulgarian CE patients included in this study, who underwent surgery for removal of hepatic or pulmonary CE

Table 3 Nucleotide differences among mitochondrial haplotypes of *E. granulosus* s.s.

mt-gene	atp 6					nad I									*cox I*			*rrnS*			*rrnL*			
Position	**3628***	**3671***	3814	**3851***	3859	5234	5277	5297	5348	5384	5552	5582	5609	5663	7569	7614	**7760***	**8528***	8792	9034	9579	9596	9605	9749
Haplotype																								
Ref G1	**C**	**T**	T	**G**	T	A	A	C	T	T	T	T	T	A	C	T	**T**	**A**	C	A	A	A	G	A
Ref G3	•	•	C	**A**	C	G	G	·	·	·	·	·	·	G	T	C	**C**	•	T	G	G	G	A	·
H1 (13 isolates)	•	•	·	•	·	·	·	·	·	·	·	·	·	·	·	·	•	•	·	·	·	·	·	·
H2 (1 isolate)	•	•	·	•	·	·	·	·	·	·	·	C	C	·	·	·	•	•	·	·	·	·	·	·
H3 (1 isolate)	•	•	·	•	·	·	·	·	C	·	·	·	·	·	·	·	•	**G**	·	·	·	·	·	·
H4 (2 isolates)	•	•	·	•	·	·	·	·	·	C	·	·	·	·	·	·	•	•	·	·	·	·	·	·
H5 (1 isolate)	•	•	·	•	·	·	·	·	·	·	C	·	·	·	·	·	•	•	·	·	·	·	·	·
H6 (1 isolate)	**T**	•	·	•	·	·	·	T	·	·	·	·	·	·	·	·	•	•	·	·	·	·	·	·
H7 (1 isolate)	•	•	·	•	·	·	·	·	·	·	·	·	·	·	·	T	•	•	·	·	·	·	·	·
H8 (1isolate)	•	•	·	•	·	·	·	·	·	·	·	·	C	·	·	·	•	•	·	·	·	·	·	·
H9 (4 isolates)	•	•	·	**A**	·	G	G	·	·	·	·	·	·	G	T	·	**C**	•	T	·	G	·	A	·
H10 (8 isolates)	•	•	C	**A**	·	G	G	·	·	·	·	·	·	G	T	·	**C**	•	T	G	G	·	A	·
H11 (2 isolates)	•	•	C	**A**	·	G	G	·	·	·	·	·	·	G	T	·	**C**	•	T	G	G	·	A	G
H12 (1 isolate)	•	**C**	C	**A**	·	G	G	·	·	·	·	·	·	G	T	·	**C**	•	T	G	G	·	A	·
H13 (1 isolate)	•	•	C	**A**	·	G	G	·	·	·	·	·	·	G	T	T	**C**	•	T	G	G	·	A	·

Only positions with differences between isolates are shown. Identical bases are represented with dots. Numbering is after the positions of the bases in the references AF297617.1 (complete mt genome of G1) and KJ559023.1 (complete mt genome of G3)

*Synonymous substitutions

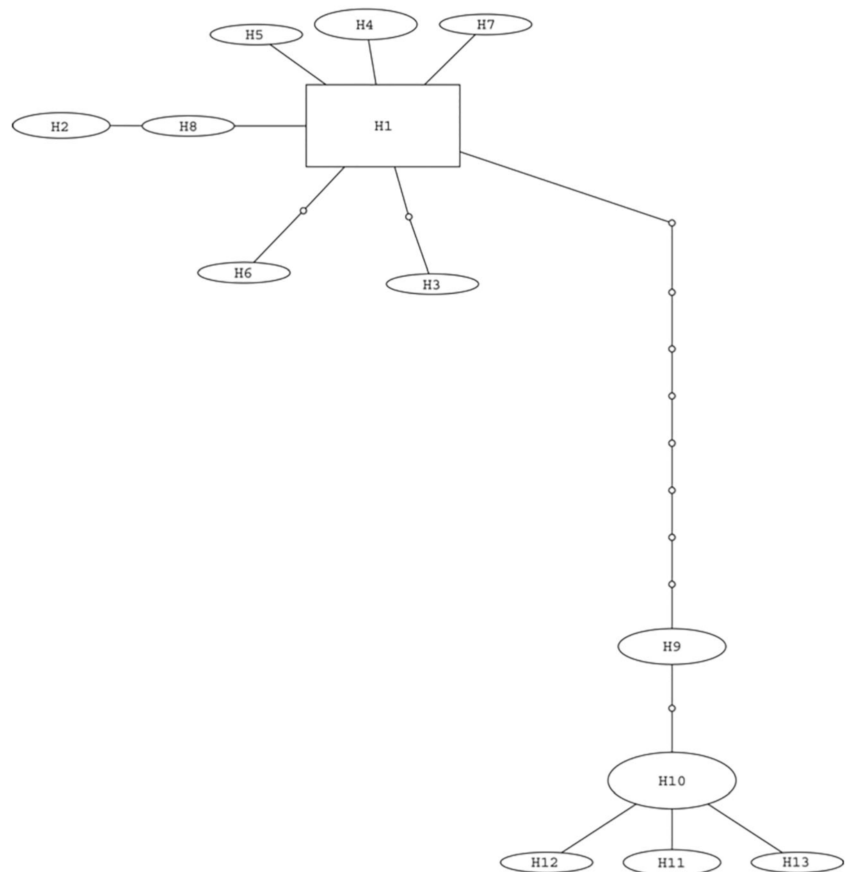

Fig. 2 Haplotype network of concatenated sequences (H1-H13) of Bulgarian and Greek *E. granulosus* s.s. samples, composed from the mitochondrial genes. The *rectangle* represents the hypothetical ancestral haplotype (the outgroup) and the *ovals* are haplotypes arising from it. Their size is equivalent to the number of haplotypes. *Branches* represent point mutations and the *dots* on them are hypothetical missing haplotypes

between 2004 and 2014, were found to be infected with either the *E. granulosus* s.s. G1 or G3 genotype. These findings were in agreement with previous reports that had already documented the presence of G1 and G3 in humans (Casulli et al. 2012) and livestock (Breyer et al. 2004; Boubaker et al. 2013) in Bulgaria. Although CE with G2 had also been previously recorded in Bulgaria (Casulli et al. 2012), G2 was not found in our study. Overall, G2 is principally not well characterized yet, and especially, its complete mt genome is still missing. Genotype G2 is often described as a micro-variant of G1 or of G3 (Snabel et al. 2009; Alvarez et al. 2014).

In our study, we found an amazingly high rate (47%) of human CE cases to be associated with *E. granulosus* s.s. (G3); high rates of human infection with G3 were previously reported from Italy (Latium: 87%) (Busi et al. 2007), India (North India: 53.1%) (Sharma et al. 2013a), and Iran (Ardabil Province: 22%) (Pezeshki et al. 2013); additionally, G3 is infecting humans in

Table 4 Diversity and neutrality indexes of *E. granulosus* s.s. in Bulgarian ($n = 30$) and Greek ($n = 7$) samples in the studied genetic markers

Gene	Country	Haplotypes	S	Haplotype diversity	Nucleotide diversity	D	Fs
cox I	BG	2	2	0.515 ± 0.027	0.003 ± 0.001	2.130	3.459
	Gr	4	3	0.714 ± 0.181	0.004 ± 0.003	1.220	−0.780
nad I	BG	7	9	0.664 ± 0.059	0.004 ± 0.002	−0.447	−0.537
	Gr	4	5	0.810 ± 0.130	0.004 ± 0.003	−0.099	−0.132
rrnS	BG	3	3	0.545 ± 0.042	0.003 ± 0.001	1.054	1.868
	Gr	3	3	0.524 ± 0.209	0.003 ± 0.002	0.050	0.406
rrnL	BG	4	3	0.664 ± 0.050	0.002 ± 0.001	0.890	0.512
	Gr	2	2	0.476 ± 0.171	0.002 ± 0.001	0.687	1.702
atp 6	BG	5	4	0.662 ± 0.061	0.003 ± 0.001	0.250	−0.360
	Gr	2	2	0.476 ± 0.171	0.002 ± 0.002	0.687	1.702

BG Bulgaria *Gr* Greece *S* number of segregating sites *D* Tajima's statistics *Fs* Fu's statistics

Table 5 Nuclear haplotypes resulted from concatenated sequences

Haplotype	Accession number and (haplotype name)			Genetic identidy	No.	
	Act II	Hbx 2	Ef-1α		BS	GS
N1	KR138703 (AC1)	KR138705 (HB1)	KR070993 (EF1)	*E. granulosus* s.s. (G1/G2/G3)	30	7
N2	KR138704 (AC2)	KR138706 (HB2)	KR070994 (EF2)	*E. canadensis* (G6-G8, G10)	-	3

No. number of samples *BS* Bulgarian samples *GS* Greek samples

Turkey (Simsek et al. 2011), Tunisia (M'rad et al. 2010), Algeria (Zait et al. 2016), and China (Yan et al. 2016).

Molecular data on animal and human cysts showed that, over the world, the G3 genotype, known as "buffalo strain," is expanding in geographic extent as well as in IH range's diversity; larval stage of G3 can be hosted by sheep (Busi et al. 2007), cattle (Busi et al. 2007), camels (Sharbatkhori et al. 2011), Nilelechwe (Busi et al. 2007), goats (Calderini et al. 2012), and pigs (Pednekar et al. 2009). The *E. granulosus* s.s. (G3) is now circulating in almost all continents: Asia (Sharma et al. 2013a, b; Wang et al. 2015), Africa (M'rad et al. 2010; Zait et al. 2016), South America (Cucher et al. 2016), Europe (Busi et al. 2007; Casuli et al. 2012), and Australia (Guo et al. 2011). Thus, even though that CE infection in humans and animals is more associated with *E. granulosus* s.s. (G1), the real contribution of G3 to the global burden of CE could have been underestimated. In fact, the genetic differentiation potential between G1 and G3 is low and can only be reliably tracked upon mtDNA analyses. Genotypic characterization of *E. granulosus* s.s. specimens widely depends upon the resolving power of the genetic markers used; indeed, in many reports, human cysts were characterized referring to the species name *E. granulosus* s.s., without determining the corresponding genotype (Piccoli et al. 2013; Mutwiri et al. 2013; Bakal et al. 2015; Boufana et al. 2015; Wang et al. 2015).

Besides the identification of the *E. granulosus* s.s. G1 (50%) and G3 (20%) genotypes in the Greek samples, *E. canadensis* (G7) isolates were revealed in two goats and in a wild boar.

As in the five countries bordering Bulgaria, namely Greece, Romania, Turkey, Serbia, and Republic of Macedonia, both *E. granulosus* s.s. (G1/G2/G3) and *E. canadensis* (G7) were found in both humans and livestock, our findings were expected (Bart et al. 2006; Varcasia et al. 2007; Maillard et al. 2009; Snabel et al. 2009; Schneider et al. 2010; Casulli et al. 2012; Bobic et al. 2012; Piccoli et al. 2013). However, the lack of *E. canadensis* (G7) detectability in our Bulgarian study group has to be interpreted with caution, since the number of respective samples examined (cysts from 25 livers and 5 lungs) is a limiting factor for excluding the occurrence of *E. canadensis* (G7), and we may need a much larger study group to allow a statistically significant exclusion of G7 as an infection organism in the Bulgarian human population (Breyer et al. 2004; Casulli et al. 2012). This relativation of our interpretation is underlined by other similar studies, where human CE cases with hepatic and pulmonary infections due to the *E. canadensis* G7 were recorded (Schneider et al. 2010; Zhang et al. 2014; Dybicz et al. 2015). In some other areas, *E. canadensis* (G7) appeared even as the predominant cause of human CE, such as in Poland and Austria (Romig et al. 2015).

In contrast to *Echinococcus* nuclear genes where only inter-species differentiation occurs, three levels of discrimination became possible when using partial mt DNA sequences, including species, genotypes, and haplotypes. A significant discrepancy between the nuclear and mitochondrial phylogenies of the *E. granulosus* complex was previously reported by

Fig. 3 Dendrogram of concatenated sequences (haplotypes N1-N2) of Bulgarian and Greek samples with regard to the nuclear genes. The neighbor-joining method with Kimura 2-parameter method and bootstrap test (1000 replicates) was applied. *Taenia solium* (T.s) was used as an outgroup control. The numbers next to the branches represent the percentage of replicate trees in which the associated taxa cluster together in the bootstrap test. *Ts Taenia solium*, *Egr* reference for *E. granulosus* s.s., *Ecan* reference for *E. canadensis*

others (Bart et al. 2006; Saarma et al. 2009; Knapp et al. 2011; Sharbatkhori et al. 2011).

With regard to haplotype characterization, we detected 21 distinct mt DNA haplotypes in Bulgarian samples based on sequence analyses of *cox I* (C1_C2), *nad I* (ND1_ND7), *rrnS* (S1_S3), *rrnL* (L1_L4), and *atp 6* (ATP1_ATP5), with three haplotypes—ND1 (Breyer et al. 2004), C1 (Casulli et al. 2012), and C2 (Casulli et al. 2012) having already previously been described in Bulgaria.

In our study, *nad 1* was the fastest mutating mt gene in both Bulgarian and Greek populations, and this finding is in line with a recent study showing that *nad 2* is the fastest evolving mt gene in *Echinococcus* (Wang et al. 2014).

In our study, the analysis of the concatenated sequences of the five mt genes from Bulgarian and Greek isolates resulted in a total of 14 haplotypes. In the haplotype network, H1 appeared as the hypothetical ancestral haplotype of all other haplotypes reported herein. The highest divergence time in the Bulgarian populations (between H1 and H10) was estimated to be 135,000–270,000 years of evolution. This finding is actually very similar with the results published for the common ancestor of all surviving mt types in humans, which was estimated at 140,000–290,000 years (Cann et al. 1987).

Overall, although the molecular typing of the *E. granulosus* complex has begun more than 20 years ago (Bowles et al. 1992a, b), nowadays, we still do not know all details about possible differential clinical patterns of human CE across species and genotypes of this complex. It has been suggested that in humans, the *E. canadensis* (G6) larvae has higher affinity to brain than other organs (Sadjjadi et al. 2013). Similarly, in another study, patients with hepatic CE and infected with G7 appeared to harbor larger cysts than those infected with the G1 genotype (Schneider et al. 2010). In our study, we could not detect any association between distinct *E. granulosus* s.s. genotypes (G1 and G3) and clinical (cyst localization, cyst size) and demographic (age and gender) features of CE patients. Furthermore, liver cysts of both G1 and G3 were predominantly classified as CE-1 (84% G1 and 75% G3), according to the WHO-IWGE sonographic classification of echinococcal cysts (Pawlowski et al. 2001). In another study from Poland, among seven liver cysts due to the *E. canadensis* (G7), four were of CE-2 (Dybicz et al. 2015).

In summary, and to the best of our knowledge, we provide the first relatively large report series on molecular epidemiology of human CE in Bulgaria. Our findings highlight roles of *E. granulosus* s.s. (genotype G1 and G3) as the sole cause of hydatid disease in Bulgaria; nevertheless, the reason for lack of *E. canadensis* (G7) remains still open and needs further investigations. The results presented here may have implications in planning and implementing of effective strategies and public health policies towards a better control for CE in Bulgaria.

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