High-resolution phylogeography of zoonotic tapeworm Echinococcus granulosus sensu stricto genotype G1 with an emphasis on its distribution in Turkey, Italy and Spain

Kinkar, Liina

2016-11

Kinkar , L , Laurimae , T , Simsek , S , Balkaya , I , Casulli , A , Manfredi , M T , Ponce-Gordo , F , Varcasia , A , Lavikainen , A , Miguel Gonzalez , L , 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 , vol. 143 , no. 13 , pp. 1790-1801 . https://doi.org/10.1017/S003

http://hdl.handle.net/10138/228496
https://doi.org/10.1017/S0031182016001530

publishedVersion

High-resolution phylogeography of zoonotic tapeworm *Echinococcus granulosus* sensu stricto genotype G1 with an emphasis on its distribution in Turkey, Italy and Spain

LIINA KINKAR[1], TEIVI LAURIMÄE[1], SAMI SIMSEK[2], IBRAHIM BALKAYA[3], ADRIANO CASULLI[4], MARIA TERESA MANFREDI[5], FRANCISCO PONCE-GORDO[6], ANTONIO VARCASIA[7], ANTTI LAVIKAINEN[8], LUIS MIGUEL GONZÁLEZ[9], STEFFEN REHBEIN[10], JOKE VAN DER GIESSEN[11], HEIN SPRONG[11] *and* URMAS SAARMA[1]*

[1] *Department of Zoology, Institute of Ecology and Earth Sciences, University of Tartu, Vanemuise 46, 50410 Tartu, Estonia*
[2] *Department of Parasitology, Faculty of Veterinary Medicine, University of Firat, 23119, Elazig, Turkey*
[3] *Department of Parasitology, Faculty of Veterinary Medicine, University of Atatürk, Erzurum, Turkey*
[4] *European Reference Laboratory for Parasites, Department of Infectious Diseases, Intituto Superiore di Sanità, viale Regina Elena 299, 00161 Rome, Italy*
[5] *Department of Veterinary Sciences and Public Health, University of Milan via Celoria 10, 20133 Milano, Italy*
[6] *Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza Ramóny Cajal s/n, 28040 Madrid, Spain*
[7] *Laboratorio di Parassitologia, Ospedale Didattico Veterinario, Università degli Studi di Sassari, Via Vienna 2, 07100 Sassari, Italy*
[8] *Department of Bacteriology and Immunology/Immunobiology Program, Faculty of Medicine, University of Helsinki, P.O. Box 21, FIN-00014, Finland*
[9] *Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Majadahonda, 28220 Madrid, Spain*
[10] *Merial GmbH, Kathrinenhof Research Center, Walchenseestr. 8–12, 83101 Rohrdorf, Germany*
[11] *National Institute of Public Health and Environment (RIVM), Center for Zoonoses and Environmental Microbiology, Antonie van Leeuwenhoeklaan 9, P.O. Box 1, 3720 BA Bilthoven, The Netherlands*

(*Received 1 March 2016; revised 21 June 2016; accepted 26 July 2016; first published online 30 August 2016*)

SUMMARY

Echinococcus granulosus is the causative agent of cystic echinococcosis. The disease is a significant global public health concern and human infections are most commonly associated with *E. granulosus* sensu stricto (s. s.) genotype G1. The objectives of this study were to: (i) analyse the genetic variation and phylogeography of *E. granulosus* s. s. G1 in part of its main distribution range in Europe using 8274 bp of mtDNA; (ii) compare the results with those derived from previously used shorter mtDNA sequences and highlight the major differences. We sequenced a total of 91 *E. granulosus* s. s. G1 isolates from six different intermediate host species, including humans. The isolates originated from seven countries representing primarily Turkey, Italy and Spain. Few samples were also from Albania, Greece, Romania and from a patient originating from Algeria, but diagnosed in Finland. The analysed 91 sequences were divided into 83 haplotypes, revealing complex phylogeography and high genetic variation of *E. granulosus* s. s. G1 in Europe, particularly in the high-diversity domestication centre of western Asia. Comparisons with shorter mtDNA datasets revealed that 8274 bp sequences provided significantly higher phylogenetic resolution and thus more power to reveal the genetic relations between different haplotypes.

Key words: cystic echinococcosis, high genetic variability, hydatid disease, mitochondrial genome, mtDNA, sheep domestication, zoonosis, zoonotic pathogens.

INTRODUCTION

Cystic echinococcosis (CE), a zoonotic disease caused by the larval stage of the tapeworm *Echinococcus granulosus* sensu lato (s. l.), is a significant global public health concern (Eckert *et al*. 2001). CE is listed among the most severe parasitic diseases in humans, ranking second in the list of food-borne parasites globally (FAO/WHO report, 2012) and representing one of the 17 Neglected Tropical Diseases prioritised by the World Health Organisation (Daumerie *et al*. 2010). The life cycle of the parasite involves mainly dogs and wild carnivores as definitive hosts (e.g. Moks *et al*. 2006; Deplazes *et al*. 2011; Laurimaa *et al*. 2015), which harbour the adult worms in the intestine. A wide range of domestic and wild mammals, but also humans, can serve as intermediate hosts (Eckert *et al*. 2001). Proglottids containing eggs or free eggs are passed to the environment by faeces of the

* Corresponding author: Department of Zoology, Institute of Ecology and Earth Sciences, University of Tartu, Vanemuise 46, 51014 Tartu, Estonia. E-mail: Urmas.Saarma@ut.ee

doi:10.1017/S0031182016001530

definitive host and a suitable intermediate host becomes infected after oral infection with eggs. The hydatid cysts develop in the intermediate host, mainly in internal organs such as liver and lungs. The cycle is completed if a fertile hydatid cyst of an infected intermediate host is eaten by a suitable carnivore (Haag *et al*. 1999; Eckert *et al*. 2001).

Echinococcus granulosus s. l. exhibits considerable intraspecific variability in terms of genetic diversity, host range, infectivity to humans, pathogenicity, antigenicity and developing rate (Eckert *et al*. 2001). Molecular studies have identified a number of genotypes/species within the *E. granulosus* complex (Bowles *et al*. 1992, 1994; Thompson and McManus, 2002; Lavikainen *et al*. 2003; Thompson, 2008; Knapp *et al*. 2011) that are closely related to other species in the genus *Echinococcus* (Knapp *et al*. 2015). Traditionally, the complex is considered to consist of genotypes G1–G10, but the taxonomy is currently under debate (Saarma *et al*. 2009; Knapp *et al*. 2011; Nakao *et al*. 2015; Romig *et al*. 2015). It has been proposed that some of these genotypes deserve the species status: *E. granulosus* sensu stricto (s. s.; genotypes G1–G3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6–G10) (Thompson and McManus, 2002; Nakao *et al*. 2007; Knapp *et al*. 2011). Genotype G9 is not considered as valid (Kedra *et al*. 1999).

Cystic echinococcosis is a widespread problem in Europe despite efforts to control it and the parasite maintains constant prevalence in areas where extensive farming is common (Giannetto *et al*. 2004; Carmena *et al*. 2008; Garippa and Manfredi, 2009; Cardona and Carmena, 2013). The highest rates for ovine hydatidosis in Europe has been reported in Romania, Greece, Turkey and central-southern Italy (particularly the islands of Sardinia and Sicily) where the prevalence in livestock ranged from 30·2 to 75·3% (Altintas, 2003; Giannetto *et al*. 2004; Scala *et al*. 2006; Varcasia *et al*. 2006; Mitrea *et al*. 2014; Chaligiannis *et al*. 2015). The parasite spreading is promoted by slaughter-houses with poor control over waste management, home slaughtering, low public awareness of the disease, high numbers of stray dogs and low sanitation (Dakkak, 2010; Varcasia *et al*. 2011).

Echinococcus granulosus s. s. genotype G1, also known as the common sheep strain, is widely distributed in southern Europe with the highest prevalence in the Mediterranean countries (Romig *et al*. 2006; Casulli *et al*. 2012). In northern and north-eastern Europe this genotype is rare, though it has been recently found in a cat in St. Petersburg, Russian Federation (Konyaev *et al*. 2012) and in urban dogs in Tartu, Estonia (Laurimaa *et al*. 2015). The genotype has been identified also in humans (Finland, Norway), but the diagnosed patients were immigrants mainly from the Near East or African countries (A. Lavikainen, pers. comm.). In northern and north-eastern European countries such as Finland, Sweden, Estonia and Latvia, genotypes G8 and G10 dominate (Lavikainen *et al*. 2003, 2006; Moks *et al*. 2006, 2008; Marcinkute *et al*. 2015; Oksanen and Lavikainen, 2015). In the Mediterranean countries, genotype G1 has been reported in definitive hosts such as dogs or wolves in Albania, Spain, Italy, Greece and Turkey (Sobrino *et al*. 2006; Xhaxhiu *et al*. 2011) and also in a wide range of intermediate hosts: human, cattle, sheep, pig, wild boar, goat and buffalo (González *et al*. 2002; Daniel-Mwambete *et al*. 2004; Varcasia *et al*. 2006, 2007; Busi *et al*. 2007; Casulli *et al*. 2008; Martin-Hernando *et al*. 2008; Vural *et al*. 2008; Dore *et al*. 2014). In other European countries, G1 has been reported in dogs, jackals or wolves in Austria, Portugal, Kosovo, Bulgaria and Romania (Breyer *et al*. 2004; Sherifi *et al*. 2011) and in intermediate hosts such as humans, pigs, cattle or sheep (Breyer *et al*. 2004; Bart *et al*. 2006; Badaraco *et al*. 2008; Beato *et al*. 2010; Schneider *et al*. 2010). The genotype has been described also in horse in Italy (Varcasia *et al*. 2008), horse, mule and donkey in Turkey (Utuk and Simsek, 2013; Simsek and Cevik, 2014; Simsek *et al*. 2015) and in red deer in Romania (Onac *et al*. 2013). In addition to being widely spread among wild and domestic animals in Europe, genotype G1 is the most frequently implicated genotype in human infections, 88% worldwide (Alvarez Rojas *et al*. 2014), therefore deserving particularly close attention.

To date, although numerous studies have analysed the genetic diversity and population structure of *E. granulosus* s. s. (Nakao *et al*. 2010; Casulli *et al*. 2012; Yanagida *et al*. 2012; Andresiuk *et al*. 2013; Yan *et al*. 2013; Boufana *et al*. 2015; Romig *et al*. 2015), data covering large geographical areas are scarce. The largest geographical coverage in Europe is provided by Casulli *et al*. (2012) who analysed the genetic variability of *E. granulosus* s. s. in Italy, Bulgaria, Romania and Hungary. However, the analytical power has remained low in most studies (Europe and elsewhere) as the analyses have largely been based on short sequences of mitochondrial DNA, most often on a single gene, e.g. the full cytochrome c oxidase subunit 1 gene (*cox1*) (Yanagida *et al*. 2012; Romig *et al*. 2015) or partial sequence of the *cox1* or *nad1* (e.g. Casulli *et al*. 2012; Andresiuk *et al*. 2013). Analysing significantly larger portion of the mitochondrial genome could potentially yield more detailed insight into the genetic variability and phylogeography of *E. granulosus* s. s.

The objectives of the present study were to: (i) investigate the genetic diversity and phylogeography of *E. granulosus* genotype G1 in part of its distribution range in Europe, and (ii) compare the results

http://dx.doi.org/10.1017/S0031182016001530

derived from the 8274 bp of the mitochondrial genome with previously used shorter sequences (351 and 1674 bp of *cox1*) and highlight major differences.

MATERIALS AND METHODS

Parasite material

Two hundred and fifty *E. granulosus* s. s. genotypes were initially analysed, of which 106 gave positive polymerase chain reaction (PCR) with all primers (the remaining samples did not yield positive PCR most probably due to low DNA quality). Samples were obtained during routine meat inspections or from hospital cases and were ethanol-preserved at −20 °C until further use. We confirmed the identity of G1 genotypes based on phylogenetic comparison with other *E. granulosus* genotypes according to Bowles *et al*. (1992). However, genotype G3 samples ($n = 15$) could be distinguished with confidence from genotype G1 samples based on 8274 bp of mtDNA (Kinkar *et al*. unpublished data), and were excluded from the analysis. Thus, a total of 91 genotype G1 samples were analysed in this study originating from 6 intermediate host species (cattle, sheep, pig, goat, wild boar and human) in 7 European countries: Turkey ($n = 69$), Spain ($n = 10$), Italy ($n = 7$), Albania ($n = 2$), Romania ($n = 1$), Greece ($n = 1$), Finland (Algeria) ($n = 1$) (Fig. 1; Table 1). Although the relatively large number of final samples in this study originates from Turkey, considering its important geographical location near the ancient domestication centre of ruminants such as sheep and cattle, this area is likely to represent a large part of G1 genetic diversity in Europe and can therefore provide valuable insight into the phylogeography of G1.

DNA extraction, PCR amplification and sequencing

DNA was extracted from protoscoleces or cyst membranes using High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany), following the manufacturer's protocols. To analyse large portion of the mitochondrial genome, 10 novel primer pairs were designed (Table 2). PCR reactions were carried out in a total volume of 20 μL, using 1 × BD Advantage-2 PCR buffer (BD Biosciences, Franklin Lakes, NJ, USA), 0·2 mM dNTP (Fermentas, Vilnius, Lithuania), 0·25 μM of each primer, 1U Advantage-2 Polymerase mix (BD Biosciences) and 20–50 ng of purified genomic DNA. Touchdown protocol was used for PCR: initial denaturation at 95 °C for 1 min, followed by 10 cycles of 95 °C for 20 s, 55 °C for 45 s (annealing temperature progressively reduced by 0·5 °C in each cycle) and 68 °C for 2 min; followed by 25 cycles of 95 °C for 20 s, 50 °C for 45 s, 68 °C for 2 min; and finishing with a final elongation step at 68 °C for 3 min. Of the amplified PCR products 10 μL were examined on 1·2% agarose gel electrophoresis. The remaining 10 μL of the PCR products were purified with 1 unit of shrimp alkaline phosphatase/exonuclease I (Fermentas, Vilnius, Lithuania). The mixture was subsequently incubated at 37 °C for 30 min and then heated 80 °C for 15 min to inactivate the enzymes.

Sequencing was performed using the same primers as for the initial PCR amplification (Table 2) with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA), following the manufacturer's protocols. Cycling parameters were 96 °C for 1 min, followed by 25 cycles of 96 °C for 10 s, 50 °C for 15 s and 60 °C for 4 min. Sequences were resolved on the ABI 3130xl sequencer (Applied Biosystems). All sequences were deposited in GenBank and are available under accession numbers KU925351–KU925433.

Data analysis

Sequences were assembled in CodonCode v4.2.7, manually corrected in BioEdit v7.2.5 and aligned with a *E. granulosus* genotype G1 sequence available in GenBank (NC_008075) (Yang *et al*. 2005) using Clustal W. Phylogenetic networks were calculated using Network v4.612 (Bandelt *et al*. 1999) (http://www.fluxusengineering.com/, Fluxus Technology Ltd., 2004). Networks were constructed for 3 different alignments: (1) 8274 bp of mtDNA; (2) complete sequence of *cox1* gene (1674 bp, according to AB786664; Nakao *et al*. 2013); (3) reduced dataset of 351 bp – a fragment of *cox1* gene, used previously in *E. granulosus* phylogeographic analysis in Europe (according to JF513058; Casulli *et al*. 2012; note that majority of publicly available G1 sequences fall between 300–400 bp).

The total length of all amplicons was >10 kb. However, after alignment, manual correction and trimming, the final length of aligned mtDNA sequences used for further analysis was 8274 bp (the sequence lengths varied between 8269 and 8274 bp). This included 15 full length gene coding areas: cytochrome b (*cytb* 717–1784; positions according to NC_008075), NADH dehydrogenase 4L (*nd4l* 1798–2058), ATP synthase subunit 6 (*atp6* 3473–3985), NADH dehydrogenase 1 (*nad1* 5100–5993), cytochrome c oxidase subunit 1 (*cox1* 6760–8367), 9 tRNA-encoding genes (*tRNA-Gln* 3282–3343, *tRNA-Phe* 3343–3405, *tRNA-Met* 3402–3467, *tRNA-Val* 4900–4962, *tRNA-Ala* 4968–5031, *tRNA-Asp* 5032–5096, *tRNA-Asn* 6010–6075, *tRNA-Thr* 8358–8422, *tRNA-Cys* 9400–9462) and small-subunit ribosomal RNA (*ssu-rRNA* 9463–10162); and 6 gene fragments: NADH dehydrogenase subunit 4 (*nd4* 2019–2091;

http://dx.doi.org/10.1017/S0031182016001530

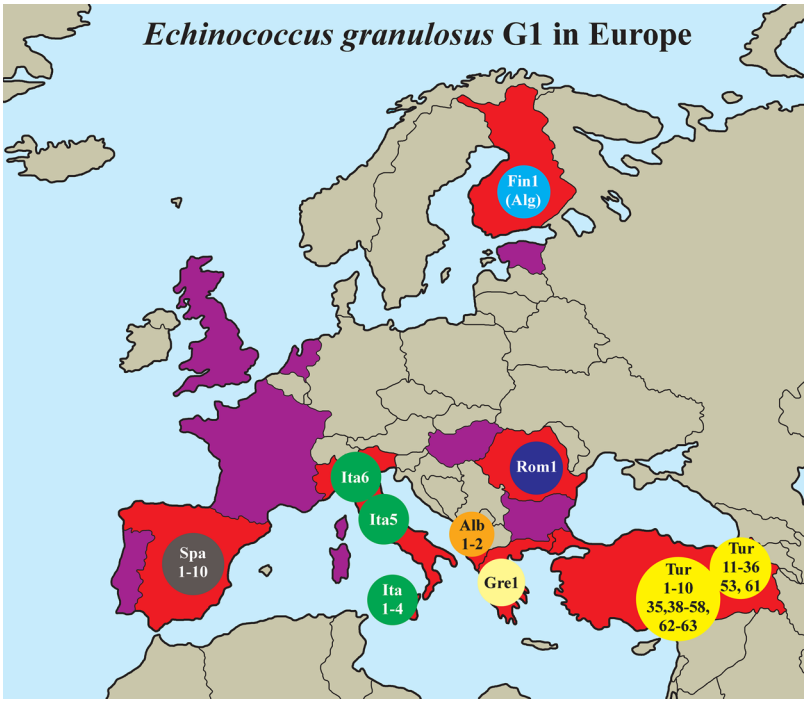

Fig. 1. Geographic locations of *Echinococcus granulosus* s. s. genotype G1 samples ($N = 91$; red) from Europe analysed in this study. Additional distribution range of G1 in Europe is represented in pink.

Table 1. Data for 91 *Echinococcus granulosus* s. s. genotype G1 isolates analysed in this study.

				Host			
Geographical origin	Cattle	Sheep	Human	Wild boar	Pig	Goat	Total
Albania		2					2
Finland (Algeria)			1				1
Greece		1					1
Italy	3	4					7
Romania	1						1
Spain		5	2	1	1	1	10
Turkey	38	31					69
Total	42	43	3	1	1	1	91

Note that the G1 isolate identified in Finland was from a patient originating from Algeria.

2518–3278), NADH dehydrogenase subunit 2 (*nd2* 3994–4176; 4356–4361; 4430–4875), cytochrome c oxidase subunit 2 (*cox2* 10182–10574), 2 tRNA encoding genes (*tRNA-His* 667–714, *tRNA-Pro* 6082–6086), and *lsu-rRNA* (8423–8495; 8789–9399).

The population diversity indices (number of haplotypes, haplotype diversity and nucleotide diversity) were calculated using DnaSP v5.10.01 (Librado and Rozas, 2009). Neutrality indices Tajima's *D* (Tajima, 1989) and Fu's Fs (Fu, 1997) and pairwise fixation index (Fst) were calculated using the population genetics package Arlequin 3·1 (Excoffier *et al*. 2005). Indices were calculated separately for total population, different localities and hosts. The minimum sample size for localities and hosts that were included in the analysis was five.

RESULTS

Variations in nucleotide sequences

A total of 8274 bp of mtDNA was successfully sequenced for 91 *E. granulosus* G1 sequences (out of 250) from seven European countries (Albania, Finland, Greece, Italy, Romania, Spain and Turkey), covering the majority of the G1 range in Europe. The geographical origin of the samples is shown in Fig. 1. Phylogenetic networks were constructed considering both indels and point mutations. Total number of variable sites was 288.

mtDNA networks

The results of this study demonstrated extremely high genetic diversity of *E. granulosus* genotype G1

http://dx.doi.org/10.1017/S0031182016001530

Table 2. Primers used for *E. granulosus* s. s. G1 mtDNA analysis; positions are according to NC_008075 in GenBank.

Primer	Primer sequence	Primer position	PCR product length
Ef1	TCGTTTTACACGCGATTGAACT	4931…4952	1271 bp
Er1	ACCTGCTATGCAGCCCTATT	6157…6176	
E2fn	GATGCTGTTAACTTCAAGAAATG	6034…6056	1053 bp
E2r2	CTCAAAGCATTCAAACGC	7054…7071	
E3fn	GTTGATTCGTGTTAATTTTTTGGAG	6874…6898	722 bp
E3rn	GAAAACATAGCAAACAACAACCC	7574…7596	
E4f2	GTGATCCTATTTTATTTCAAC	7461…7481	1516 bp
E4r	TGCTACCTTTGCACAGTCAA	8975…8994	
E5f	ATGTATGTGGCTAGAAGGTC	8672…8691	1266 bp
E5r	CAAGAGTGAAATAATAGGTGGA	9905…9926	
E6f	TAAGGGTGATGCAATTTGAG	9627…9646	1250 bp
E6r	ACAACCATCTACAGCACGAA	10853…10872	
E10f	GATTACTGTTACTGGTTTTCA	312…332	1467 bp
E10r	CAACTTAAAAACAAGCATCATCA	1757…1779	
E11f	TTTTATGCTATTCTTCGGTGTA	1522…1543	1780 bp
E11r	CAAAAACACCTCATTAAACCAC	3281…3302	
E12f	TTGTGGTGTTTTTTATGATG	2925…2943	1299 bp
E12r	CACAGACGATAACCCAGA	4207…4224	
E13f	CGGGTCTTTTATTTTGATGTTG	4006…4027	1530 bp
E13r	GATCCAAAAGCACATCGA	5515…5532	

Table 3. Diversity and neutrality indices for *E. granulosus* s. s. genotype G1 in Europe based on 8274 bp of mtDNA. The Southern European samples (South Eur) included all samples except Turkish and Finnish (Algerian).

	Diversity				Neutrality	
	n	Hn	Hd ± S.D.	π ± S.D.	*D*	Fs
Total	91	83	0·997 ± 0·002	0·00143 ± 0·00006	−2·69188**	−24·31666**
Origin						
Turkey	69	62	0·996 ± 0·004	0·00145 ± 0·00007	−2·58214**	−24·38893**
Spain	10	10	1·000 ± 0·045	0·00147 ± 0·00015	−1·83614*	−2·82179*
Italy	7	6	0·952 ± 0·096	0·00068 ± 0·00013	−1·05903	−2·73369*
South Eur	21	20	0·995 ± 0·016	0·00132 ± 0·00015	−2·28954*	−12·40475**
Host						
Cattle	42	41	0·999 ± 0·006	0·00152 ± 0·00009	−2·42935**	−24·43759**
Sheep	43	38	0·991 ± 0·009	0·00131 ± 0·00009	−2·44968**	−24·56183**

n, number of isolates examined; Hn, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity; D (*D*), Tajima's; Fs, Fu's Fs; S.D., standard deviation.
** Highly significant *P* value ($P < 0·000001$).
* Significant *P* value ($P < 0·05$).

in Europe. The analysed 91 sequences were divided into 83 haplotypes: among these, 62 were found in Turkey, 10 in Spain and 6 in Italy (Table 3). The structure of the phylogenetic network is shown in Fig. 2. The average number of mutational steps was 12 and the maximum 27 (Alb2 and Tur45). No predominant haplotype was found in the phylogenetic network, most haplotypes were singletons ($n = 76$). Five haplotypes (Tur45, Tur10, Tur35, Tur56 and Ita3) included two samples and one haplotype (Tur3) included 4 samples.

As expected, we found that numerous geographically distant samples were also genetically distant, for example Spanish and Albanian haplotypes Spa2 and Alb2 (separated by 25 mutations), also Turkish and Spanish haplotypes Tur41 and Spa1 (separated by 20 mutations) and Turkish and Italian haplotypes Tur12 and Ita6 (separated by 18 mutations). Also, numerous geographically close samples were genetically closely related, for example Turkish haplotypes Tur11 and Tur13 (separated by 1 mutation) and Italian haplotypes Ita4 and Ita2 (separated by 2 mutations).

However, numerous samples collected from geographically close localities showed remarkably high genetic diversity and distance. Turkish samples collected from Erzurum and Elazig provinces in Eastern Turkey, demonstrated high genetic variation despite the geographical proximity. For example, haplotypes Tur12 and Tur26 from

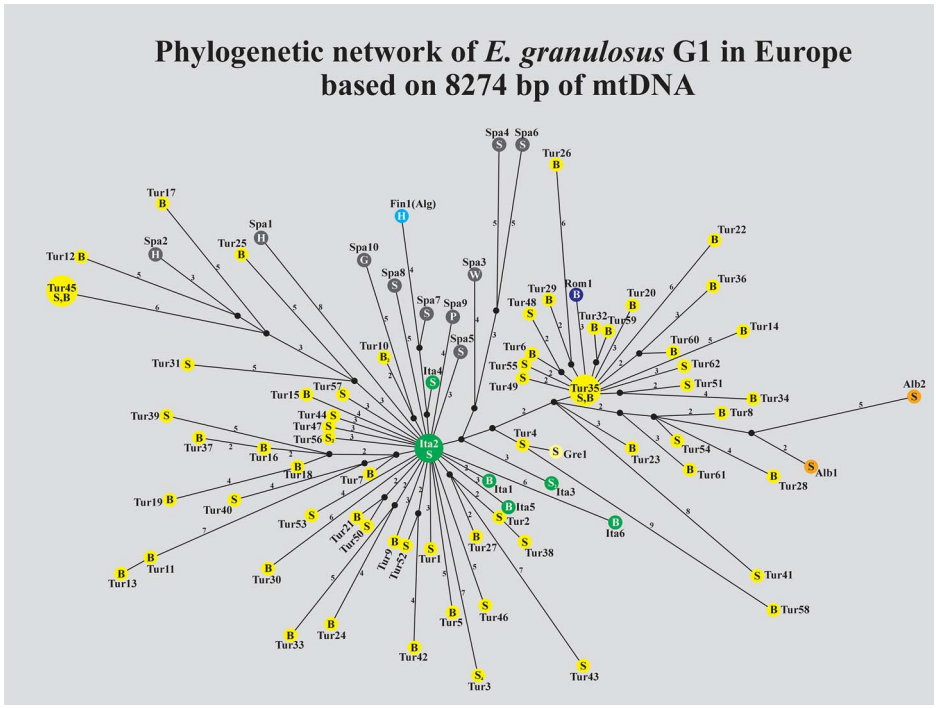

Fig. 2. Phylogenetic network of *Echinococcus granulosus* s. s. genotype G1 based on 8274 bp of mtDNA. Circles represent haplotypes. Haplotype names and colours represent different geographical origins: Tur (yellow) – Turkey, Rom (dark blue) – Romania, Fin-Alg (light blue) – Finland (a patient from Algeria), Alb (orange) – Albania, Gre (light yellow) – Greece, Spa (gray) – Spain, Ita (green) – Italy. Small black circles are median vectors (i.e. hypothetical haplotypes: haplotypes not sampled or extinct). Host species are indicated with letters (B – bovine, S – sheep, H – human, P – pig, W – wild boar, G – goat). The number inside haplotype circles indicates the frequency of the haplotype.

Erzurum were separated by 24 mutations and Tur43 and Tur58 from Elazig by 20 mutations. Spanish samples obtained from Central Spain were highly divergent as well, for example, haplotypes Spa2 and Spa4 were separated by 20 mutations.

Moreover, numerous samples from geographically distant localities were genetically closely related, i.e. several monophyletic groups comprised samples from different countries. These include Albanian and Turkish monophyletic group (Alb2, Alb1, Tur8, Tur28, Tur61, Tur54), Greek and Turkish group (Gre1, Tur58, Tur4) and Romanian and Turkish group (Rom1 and all Turkish samples derived from central haplotype Tur35). Also, two monophyletic groups comprised samples from Spain and Turkey (Spa2, Tur17, Tur25, Tur12, Tur45, Tur63 and Spa10, Tur10) and one group included one Italian (Ita4), Spanish (Spa7) and Finnish/Algerian (Fin1) sample.

No host-specific structure was detected. Cattle and sheep samples were frequently genetically closely related, for example haplotype Tur35 consists of samples from sheep and cattle. Human G1 haplotypes were not genetically closest to one another, but to those of cattle and sheep. Haplotypes obtained from wild boar, pig and goat were genetically closest to haplotype Ita2 obtained from sheep (6, 4 and 6 mutations, respectively).

In the networks based on reduced datasets of 1674 and 351 bp in length, the sequences were divided into 49 and 11 haplotypes respectively, of which two were predominant in both networks (Fig. 3). In comparison between 8274 and 1674 bp datasets, some haplotypes were positioned into different haplogroups, e.g. Spa7 and Fin1, whereas haplotypes Spa4, Spa10, Tur6, Tur9, Tur42 and Tur43 assumed different phylogenetic relations to each other (Figs 2 and 3).

Diversity and neutrality indices

Haplotype diversity was extremely high in the overall population (Hd = 0·997), whereas nucleotide diversity was rather low ($\pi = 0{\cdot}00143$) (Table 3). High haplotype diversity and low nucleotide diversity was also observed in the Italian, Spanish and Turkish subpopulations, ranging from 0·952 to 1·000 and 0·00068 to 0·00147, respectively. The Italian population showed the lowest values for both indices. High haplotype and low nucleotide diversities were also observed in cattle and sheep (Hd = 0·999, $\pi = 0{\cdot}00152$ and Hd = 0·991, $\pi = 0{\cdot}00131$, respectively). In comparison with the two shorter datasets, haplotype diversity was almost equally high for the 8274 bp and the full *cox1* gene (1674 bp; Hd = 0·920; Table S1), whereas considerably

http://dx.doi.org/10.1017/S0031182016001530

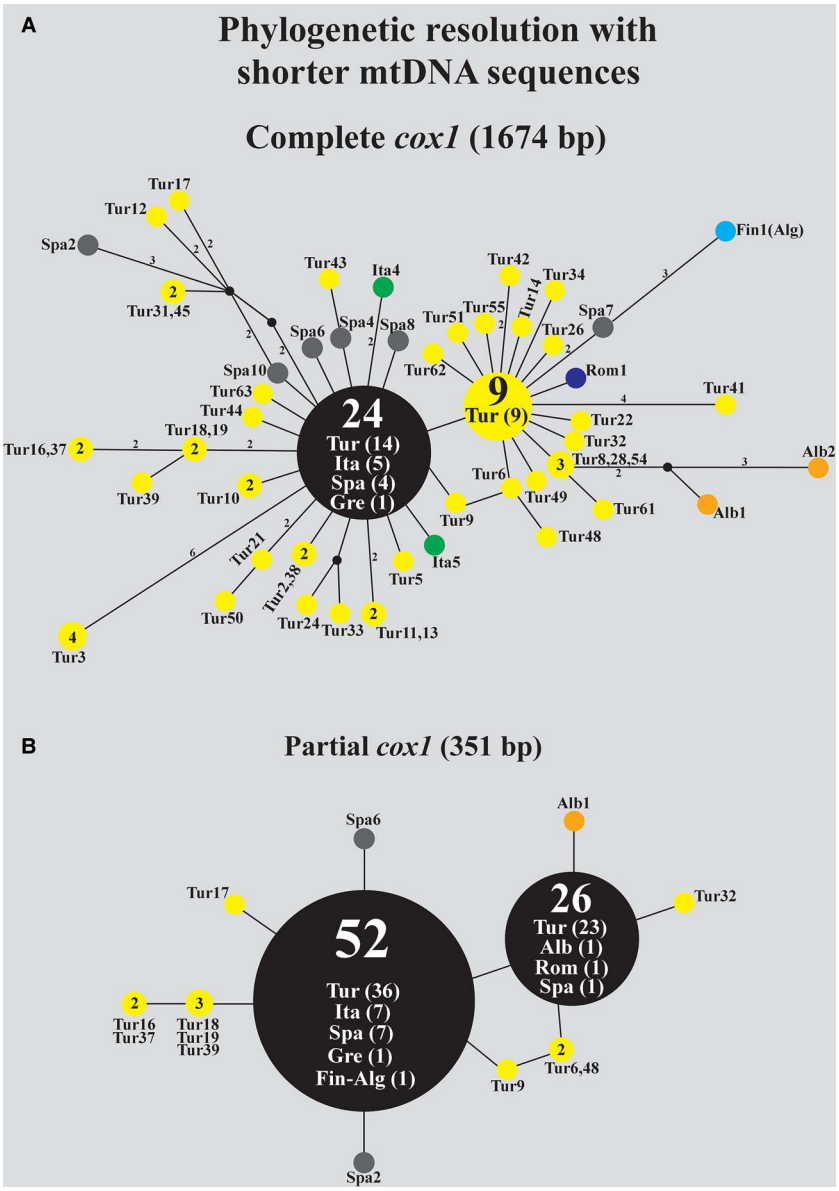

Fig. 3. Phylogeographic networks of *Echinococcus granulosus* s. s. genotype G1, using exactly the same set of samples as in Fig. 2, but shorter sequences: (A) complete sequence of *cox1* gene, 1674 bp; (B) partial sequence of *cox1* gene, 351 bp.

lower for the 351 bp dataset (Hd = 0·596; Table S2). Low nucleotide diversities were observed for both of the reduced datasets: $\pi = 0{\cdot}00196$ based on full *cox1* gene (1674 bp) and $\pi = 0{\cdot}00219$ for the partial *cox1* gene (351 bp; Tables S1 and S2).

Neutrality indices such as Tajima's D and Fu's Fs were significant for most of the analysed variants (Table 3). The highest values were detected for the overall population and for Turkish samples. Cattle and sheep populations showed also high negative values. The Tajima's D was nonsignificant for the Italian samples.

Fixation indices

Low Fst values were observed among different localities (Table S3). The Fst value for 8274 bp dataset was statistically significant only between Spain and Turkey ($F_{ST} = 0{\cdot}04130$, $P < 0{\cdot}05$). Relatively low *F*st values ($F_{ST} = 0{\cdot}01180$, $P < 0{\cdot}05$) were also recorded between cattle and sheep subpopulations.

DISCUSSION

The results of this study demonstrated extremely high haplotype diversity of *E. granulosus* s. s. genotype G1 in Europe (Fig. 2): 91 analysed samples were divided into 83 haplotypes (overall haplotype diversity 0·997). From earlier studies it is known that G1 has the highest host variability among all *E. granulosus* genotypes, capable of infecting numerous taxa, including wild and domesticated mammals and humans (Bowles *et al*. 1992; Eckert *et al*. 2001). It is likely that the high genetic variation observed in

http://dx.doi.org/10.1017/S0031182016001530

this study reflects, at least to some extent, the ability of G1 isolates to infect such a wide range of hosts. This can be regarded as a warning sign, suggesting that associations with new species may easily form if G1 distribution widens in Europe.

There was not only very high global haplotype diversity, but the diversity was high also locally. For example, haplotype diversity indices were 1·0 or close to that number in Italian, Spanish and Turkish G1 populations (Table 3), pointing to a very high degree of genetic diversity of genotype G1 across the Mediterranean countries, the main distribution area for G1 in Europe. The genetic diversity of *E. granulosus* G1 is likely to be higher at the domestication centre, while declining as the distance from the centre grows. However, the phylogenetic structure of G1 observed in this study does not follow this pattern. The Anatolia region, roughly corresponding to the Asian part of Turkey, is at the immediate vicinity of the Fertile Crescent, both considered as part of a domestication centre for the majority of livestock. Anatolia is also known as one of the earliest centres in Europe from which livestock were distributed westward along the Mediterranean coast, and only later towards north (Chessa *et al*. 2009). Sheep and cattle were among the first livestock species domesticated about 11–10 thousand years ago in the area from where they were shortly after domestication transported to the Mediterranean region by humans (Zeder, 2008). For example in sheep, the most frequent intermediate host for *E. granulosus* G1, recent data based on ancient DNA analysis have revealed that the proportion of rarer haplotypes have declined during the expansion of sheep from the Near Eastern domestication centre towards Europe (Rannamäe *et al*. 2016). As the lifecycle of *E. granulosus* genotype G1 is maintained mainly by domestic animals, their distribution is subject to anthropogenic effects, most likely extensive animal trade along the Mediterranean shore, resulting in high degree of genetic diversity across this region. Although wild animals can also distribute *E. granulosus* G1, animal transportation can help to spread the parasite with significantly higher pace. Moreover, the narrow landbridge connecting Turkey to the rest of Europe has posed, at least to some extent, a migration barrier for wild animals.

The importance of animal trade is further endorsed by lack of genetic segregation between different countries. Several Turkish samples were more closely related to Spanish, Romanian, Albanian and Greek samples than with geographically close other Turkish samples (Fig. 2). Furthermore, low *F*st values between different localities (e.g. Spain and Turkey $F_{ST} = 0{\cdot}041$, $P < 0{\cdot}05$) suggest relatively moderate genetic divergence between Mediterranean countries. Therefore, these observed phylogeographical patterns might also be shaped by livestock trade that has facilitated the parasite dispersal over vast areas. Demographic analysis also supported this hypothesis. High haplotype diversity coupled with relatively low nucleotide diversity values observed in this study (Hd = 0·997, $\pi = 0{\cdot}0014$ for overall population) suggest rapid demographic expansion, supported by significant negative values of neutrality indices Tajima's D (−2·69) and Fu's Fs (−24·32) (Avise, 2000). In addition to the efficient distribution of livestock (infected with G1) by humans, population bottlenecks can also cause the rapid demographic expansion. However, the relatively high divergence of haplotypes is better explained by livestock trade, since demographic bottleneck would rather result in a star-like network structure where majority of haplotypes are identical or very closely related and geographically linked.

The effect of large-scale animal trade on *E. granulosus* haplotype distribution has been discussed also by others (e.g. Casulli *et al*. 2012; Yanagida *et al*. 2012). Casulli *et al*. (2012) considered the effect of animal trade negligible compared with thousands of years of diffusion. The phylogeography of *E. granulosus* G1 based on high-resolution network in this study suggests that the observed pattern is likely due to both factors: trade and diffusion. However, their role on the genetic diversity and distribution of genotype G1 in Europe remains largely unresolved and requires further investigations using more elaborate sampling and coverage of the entire G1 distribution range in Europe.

The results of this study indicated the absence of host-specific phylogeography of G1 according to host species (Fig. 2), supported also by low *F*st value (0·0118, $P < 0{\cdot}05$) of G1 between cattle and sheep. As the samples in this study were mostly from livestock animals, the rapid expansion of G1 isolates observed in this study has most likely been facilitated by the intensive (shepherd) dog-livestock transmission cycle. These results support efficient transmission of G1 between different hosts via dogs (and to lesser extent by other definitive hosts) and suggest that different host species are not particularly susceptible to any specific mtDNA haplotype. Analysis of the nuclear genome is required to address this question in more detail.

On the phylogenetic network (Fig. 2), haplotype Ita2 originating from southern Italy and Turkish haplotype Tur35 from east of the country, both assumed central positions in the network, suggesting that they are ancestral to many other haplotypes (note, however, that samples from Turkey are in excess compared with other regions). The ancestral position of these haplotypes might reflect early arrival of *E. granulosus* with sheep and other livestock to Europe via eastern Turkey, which lies at the immediate vicinity of a domestication centre for the majority of livestock species, and via southern

http://dx.doi.org/10.1017/S0031182016001530

Italy. However, this scenario remains to be further tested with a larger set of samples.

The main value of this study lies largely on the high-resolution approach based on relatively long mtDNA sequences. Also, we were able to provide preliminary results on what valuable information could be lost when using must shorter sequences, which is useful for future research. However, it is important to note that in this study samples from Turkey were in excess compared with other regions, as well as cattle and sheep samples that were in excess compared with other hosts. Therefore, the results of this study are biased towards Turkey, which should be taken into account. On the other hand, the relatively large number of samples from Turkey represents a value in itself, since this area, as part of a domestication centre for the majority of livestock, is likely to represent large part of G1 genetic diversity in Europe and can therefore provide valuable insight into the phylogeography of G1. Also, as cattle and sheep are the most common hosts for genotype G1, it was inevitable that the samples that we analyzed originated mostly from these species.

The longer sequences used in this study revealed significantly higher resolution compared with the shorter sequences. The networks based on shorter sequences both revealed two dominant haplotypes, whereas on the network based on longer sequences, no dominant haplotypes were highlighted. The shortest dataset based on 351 bp was able to separate 6 Turkish, 2 Spanish haplotypes and positioned all 7 Italian samples into the central haplotype (Fig. 3). The network based on 1674 bp separated 35 Turkish, 6 Spanish and 2 Italian haplotypes. However, in the 8274 bp network, Turkish samples were divided into 63 haplotypes, Spanish samples were all fully resolved and divided into 10 haplotypes and Italian samples were divided into 6 haplotypes (Fig. 2).

Although the resolution of the phylogenetic network based on different lengths of mtDNA was significantly higher for the 8274 bp dataset, the haplotype diversity index for the 1674 bp dataset was only slightly lower compared with the 8274 bp (Hd = 0·920 and Hd = 0·997, respectively) (Tables 3 and S1). It is interesting to note that nucleotide diversity increased with shorter sequences (Tables 2, S1 and S2) indicating that the average diversity of the *cox1* gene is higher compared with the 8274 bp of mtDNA. For the 8274 bp dataset, haplotype diversities were equally high for Turkey (part of the domestication area) and for Southern Europe, indicating that the genetic diversity of G1 has remained high after the expansion from the domestication area. However, using shorter sequences, haplotype diversities were lower in Southern Europe compared with Turkey, suggesting that using a single mtDNA gene or its fragment may not be sufficient to reveal the level of genetic diversity of G1 in different localities.

There were also significant differences regarding the origin and prevalence of central ancestral haplotypes. All three networks based on different sequence lengths revealed two ancestral haplotypes. However, in networks based on shorter sequences, a significant number of samples were positioned into the central ancestral haplotypes: 23 and 9 samples based on full *cox1* gene, also 52 and 25 samples based on 351 bp, respectively (Fig. 3). Both networks based on shorter sequences suggest a wide geographical spectra of samples in the ancestral haplotypes, whereas the dominant haplotypes in both networks based on shorter sequences were fully resolved in the 8274 bp network (Fig. 2), demonstrating that Ita2 and Tur35 are the ancestral haplotypes, originating from a specific country. This represents a good example how complex haplotypes can be resolved to the highest degree, revealing the ancestral sequences at which all others coalesce. Furthermore, in both networks based on shorter sequences, the most dominant haplotype is identical to the haplotype EG1 (Casulli *et al*. 2012), which has been found to be highly prevalent worldwide (Nakao *et al*. 2010; Yanagida *et al*. 2012; Boufana *et al*. 2014, 2015). However, the 8274 bp dataset showed that this haplotype is actually genetically highly diverse and was fully resolved, revealing the single ancestral haplotype Ita2 (Fig. 2).

The networks also show that the longer sequences have significantly more power to reveal the genetic relations between different haplotypes as the longer sequences positioned a number of haplotypes differently compared with shorter ones. For example, haplotypes Spa4, Tur43, Spa7 and Fin1 assumed different phylogenetic relations to each other (Figs 2 and 3). Based on 8274 bp, haplotypes Spa7 and Fin1 originate from the central Italian haplotype Ita2, whereas the network based on the full *cox1* gene suggests that the same haplotypes originate from the Turkish central haplotype Tur35. Furthermore, based on 351 bp, they were positioned into both of the ancestral haplotypes – Fin1 into the central dominant haplotype that contains Italian samples and Spa7 into the other ancestral haplotype. Also, based on 1674 bp, haplotype Tur43 was most closely related to Spanish haplotype Spa4, whereas based on 8274 bp, the haplotype formed a monophyletic group of 4 Turkish samples most closely related to central Italian haplotype Ita2.

Our results demonstrate that using longer mtDNA sequences for phylogeographic analysis has indeed clear advantages over commonly used shorter sequences. The same has been demonstrated also for other species, e.g. for the brown bear (Keis *et al*. 2013): the analysis of complete mitochondrial genomes on brown bear clearly demonstrated the advantage of using data from complete mitogenomes,

which allowed identifying spatio-temporal population processes that had not previously been detected using shorter mtDNA sequences, not even by those of ca 2 kb (Korsten *et al*. 2009). Therefore, analysis of genetic diversity and evolutionary trajectories of *E. granulosus* and other parasites are likely to benefit significantly from large-scale mitochondrial and nuclear genome sequencing. In time, the next-generation sequencing methods will most likely replace many of the Sanger-sequencing approaches, including the mitogenome analysis.

Our findings have obvious public health importance as knowledge of *E. granulosus* s. s. genetic diversity and geographic distribution is fundamental to understand how such life-threatening pathogens evolve. The level of genetic diversity forms a basis for future adaptations of pathogens, constituting a force towards the emergence of new host-parasite associations and potentially also for development of drug resistance (Morgan *et al*. 2012). Better understanding of *E. granulosus* G1 phylogeography may thus contribute to the advancement of effective strategies to control the spread of hydatid disease.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0031182016001530.

FINANCIAL SUPPORT

This work was supported by institutional research funding (IUT20-32) from the Estonian Ministry of Education and Research (to U.S.); by grant ESF-8525 from the Estonian Research Council (to U.S.); the European Union through the European Regional Development Fund (Centre of Excellence FIBIR) (to U.S.); and the Estonian Doctoral School of Ecology and Environmental Sciences to (L.K. and T.L.) the European Community's Seventh Framework Programme under the grant agreement 602051 (Project HERACLES; http://www.Heracles-fp7.eu/) (to A.C.); the Dutch Food and Consumer Product Safety Authority (NVWA) (to J.vd G.). The funding sources had no involvement in the preparation, ideas, writing, interpretation, or the decision to submit this article.

REFERENCES

Altintas, N. (2003). Past to present: echinococcosis in Turkey. *Acta Tropica* **85**, 105–112.

Alvarez Rojas, C. A., Romig, T. and Lightowlers, M. W. (2014). *Echinococcus granulosus* sensu lato genotypes infecting humans – review of current knowledge. *International Journal for Parasitology* **44**, 9–18.

Andresiuk, M. V., Gordo, F. P., Saarma, M., Elissondo, M. C., Taraborelli, A., Casalongue, C., Denegri, G. and Saarma, U. (2013). *Echinococcus granulosus* genotype G1 dominated in cattle and sheep during 2003–2006 in Buenos Aires province, an endemic area for cystic echinococcosis in Argentina. *Acta Tropica* **127**, 136–142.

Avise, J. C. (2000). *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, Massachusetts.

Badaraco, J. L., Ayala, F. J., Bart, J. M., Gottstein, B. and Haag, K. L. (2008). Using mitochondrial and nuclear markers to evaluate the degree of genetic cohesion among *Echinococcus* populations. *Experimental Parasitology* **119**, 453–459.

Bandelt, H. J., Forster, P. and Rohl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**, 37–48.

Bart, J. M., Morariu, S., Knapp, J., Ilie, M. S., Pitulescu, M., Anghel, A., Cosoroaba, I. and Piarroux, R. (2006). Genetic typing of *Echinococcus granulosus* in Romania. *Parasitology Research* **98**, 130–137.

Beato, S., Parreira, R., Calado, M. and Crácio, M. A. A. (2010). Apparent dominance of the G1–G3 genetic cluster of *Echinococcus granulosus* strains in the central inland region of Portugal. *Parasitology International* **59**, 638–642.

Boufana, B., Lahmar, S., Rebaï, W., Safta, Z. B., Jebabli, L., Ammar, A., Kachti, M., Aouadi, S. and Craig, P. S. (2014). Genetic variability and haplotypes of *Echinococcus* isolates from Tunisia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **108**, 706–714.

Boufana, B., Lett, W. S., Lahmar, S., Buishi, I., Bodell, A. J., Varcasia, A., Casulli, A., Beeching, N. J., Campbell, F., Terlizzo, M., McManus, D. P. and Craig, P. S. (2015). *Echinococcus equinus* and *Echinococcus granulosus* sensu stricto from the United Kingdom: genetic diversity and haplotypic variation. *International Journal for Parasitology* **45**, 161–166.

Bowles, J., Blair, D. and McManus, D. P. (1992). Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Molecular and Biochemical Parasitology* **54**, 165–173.

Bowles, J., Blair, D. and McManus, D. (1994). Molecular genetic characterization of the cervid strain ('northern form') of *Echinococcus granulosus*. *Parasitology* **109**, 215–221.

Breyer, I., Georgieva, D., Kudrova, R. and Gottstein, B. (2004). *Echinococcus granulosus* strain typing in Bulgaria: the G1 genotype is predominant in intermediate and definitive wild hosts. *Parasitology Research* **93**, 127–130.

Busi, M., Šnábel, V., Varcasia, A., Garippa, G., Perrone, V., De Liberato, C. and D'Amelio, S. (2007). Genetic variation within and between G1 and G3 genotypes of *Echinococcus granulosus* in Italy revealed by multilocus DNA sequencing. *Veterinary Parasitology* **150**, 75–83.

Cardona, G. A. and Carmena, D. (2013). A review of the global prevalence, molecular epidemiology and economics of cystic echinococcosis in production animals. *Veterinary Parasitology* **192**, 10–32.

Carmena, D., Sánchez-Serrano, L. and Barbero-Martínez, I. (2008). *Echinococcus granulosus* infection in Spain. *Zoonoses and Public Health* **55**, 156–165.

Casulli, A., Manfredi, M. T., La Rosa, G., Di Cerbo, A. R., Genchi, C. and Pozio, E. (2008). *Echinococcus ortleppi* and *E. granulosus* G1, G2 and G3 genotypes in Italian bovines. *Veterinary Parasitology* **155**, 168–172.

Casulli, A., Interisano, M., Sreter, T., Chitimia, L., Kirkova, Z., La Rosa, G. and Pozio, E. (2012). Genetic variability of *Echinococcus granulosus* sensu stricto in Europe inferred by mitochondrial DNA sequences. *Infection Genetics and Evolution* **12**, 377–383.

Chaligiannis, I., Maillard, S., Boubaker, G., Spiliotis, M., Saratsis, A., Gottstein, B. and Sotiraki, S. (2015). *Echinococcus granulosus* infection dynamics in livestock of Greece. *Acta Tropica* **150**, 64–70.

Chessa, B., Pereira, F., Arnaud, F., Amorim, A., Goyache, F., Mainland, I., Kao, R., Pemberton, J., Beraldi, D., Stear, M., Alberti, A., Pittau, M., Iannuzzi, L., Banabazi, M., Kazwala, R., Zhang, Y., Arranz, J. J., Ali, B., Wang, Z., Uzun, M., Dione, M., Olsaker, I., Holm, L.-E., Saarma, U., Ahmad, S., Marzanov, N., Eythorsdottir, E., Holland, M., Ajmone-Marsan, P., Bruford, M., Kantanen, J., Spencer, T. and Palmarini, M. (2009). Revealing the history of sheep domestication using retrovirus integrations. *Science* **324**, 532–536.

Dakkak, A. (2010). Echinococcosis/hydatidosis: a severe threat in Mediterranean countries. *Veterinary Parasitology* **174**, 2–11.

Daniel-Mwambete, K., Ponce-Gordo, F. and Cuesta-Bandera, C. (2004). Genetic identification and host range of the Spanish strains of *Echinococcus granulosus*. *Acta Tropica* **91**, 87–93.

Daumerie, D., Savioli, L., Crompton, D. W. T. and Peters, P. (2010). *Working to Overcome the Global Impact of Neglected Tropical Diseases: First WHO Report on Neglected Tropical Diseases*. World Health Organization, France.

Deplazes, P., van Knapen, F., Schweiger, A. and Overgaauw, P. A. (2011). Role of pet dogs and cats in the transmission of helminthic zoonoses in Europe, with a focus on echinococcosis and toxocarosis. *Veterinary Parasitology* **182**, 41–53.

Dore, F., Varcasia, A., Pipia, A. P., Sanna, G., Pinna Parpaglia, M. L., Corda, A., Romig, T. and Scala, A. (2014). Ultrasound as a monitoring tool for cystic echinococcosis in sheep. *Veterinary Parasitology* **203**, 59–64.

Eckert, J., Gemmell, M. A., Meslin, F.-X. and Pawlowski, Z. S. (eds.) (2001). *WHO/OIE Manual on Echinococcosis in Humans and Animals: A Public Health Problem of Global Concern*. WHO/OIE, Paris.

Excoffier, L., Laval, G. and Schneider, S. (2005). Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47–50.

FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization]. (2014). Multicriteria-based ranking for risk management of food-borne parasites. Microbiological Risk Assessment Series No. 23. Rome. 302 pp.

Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**, 915–925.

Garippa, G. and Manfredi, M. T. (2009). Cystic echinococcosis in Europe and in Italy. *Veterinary Research Communications* **33**, 35–39.

Giannetto, S., Poglayen, G., Brianti, E., Sorgi, C., Gaglio, G., Canu, S. and Virga, A. N. (2004). An epidemiological updating on cystic echinococcosis in cattle and sheep in Sicily, Italy. *Parassitologia* **46**, 423–424.

González, L. M., Daniel-Mwambete, K., Montero, E., Rosenzvit, M. C., McManus, D. P., Gárate, T. and Cuesta-Bandera, C. (2002). Further molecular discrimination of Spanish strains of *Echinococcus granulosus*. *Experimental Parasitology* **102**, 46–56.

Haag, K. L., Araujo, A. M., Gottstein, B., Siles-Lucas, M., Thompson, R. and Zaha, A. (1999). Breeding systems in *Echinococcus granulosus* (Cestoda; Taeniidae): selfing or outcrossing? *Parasitology* **118**, 63–71.

Kedra, A. H., Swiderski, Z., Tkach, V., Dubinsky, P., Pawlowski, Z., Stefaniak, J. and Pawlowski, J. (1999). Genetic analysis of *Echinococcus granulosus* from humans and pigs in Poland, Slovakia and Ukraine. A multicenter study. *Acta Parasitologica* **44**, 248–254.

Keis, M., Remm, J., Ho, S. Y. W., Davison, J., Tammeleht, E., Tumanov, I. L., Saveljev, A. P., Männil, P., Kojola, I., Abramov, A. V., Margus, T. and Saarma, U. (2013). Complete mitochondrial genomes and a novel spatial genetic method reveal cryptic phylogeographic structure and migration patterns among brown bears in north-western Eurasia. *Journal of Biogeography* **40**, 915–927.

Knapp, J., Nakao, M., Yanagida, T., Okamoto, M., Saarma, U., Lavikainen, A. and Ito, A. (2011). Phylogenetic relationships within *Echinococcus* and *Taenia* tapeworms (Cestoda: Taeniidae): an inference from nuclear protein-coding genes. *Molecular Phylogenetics and Evolution* **61**, 628–638.

Knapp, J., Gottstein, B., Saarma, U. and Millon, L. (2015). Taxonomy, phylogeny and molecular epidemiology of *Echinococcus multilocularis*: from fundamental knowledge to health ecology. *Veterinary Parasitology* **213**, 85–91.

Konyaev, S. V., Yanagida, T., Ivanov, M. V., Ruppel, V. V., Sako, Y., Nakao, M. and Ito, A. (2012). The first report on cystic echinococcosis in a cat caused by *Echinococcus granulosus* sensu stricto (G1). *Journal of Helminthology* **86**, 391–394.

Korsten, M., Ho, S. Y. W., Davison, J., Pähn, B., Vulla, E., Roht, M., Tumanov, I. L., Kojola, I., Andersone-Lilley, Z., Ozolins, J., Pilot, M., Mertzanis, Y., Giannakopoulos, A., Vorobiev, A. A., Markov, N. I., Saveljev, A. P., Lyapunova, E. A., Abramov, A. V., Männil, P., Valdmann, H., Pazetnov, S. V., Pazetnov, V. S., Rõkov, A. and Saarma, U. (2009). Sudden expansion of a single brown bear lineage in northern continental Eurasia: a general model for mammals after the last ice age? *Molecular Ecology* **18**, 1963–1979.

Laurimaa, L., Davison, J., Süld, K., Plumer, L., Oja, R., Moks, E., Keis, M., Hindrikson, M., Kinkar, L., Laurimäe, T., Abner, J., Remm, J., Anijalg, P. and Saarma, U. (2015). First report of highly pathogenic *Echinococcus granulosus* genotype G1 in European Union urban environment. *Parasites & Vectors* **8**, 182.

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

Lavikainen, A., Lehtinen, A., Laaksonen, S., Agren, E., Oksanen, A. and Meri, S. (2006). Molecular characterization of *Echinococcus* isolates of cervid origin from Finland and Sweden. *Parasitology* **133**, 565–570.

Librado, P. and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**, 1451–1452.

Marcinkute, A., Šarkunas, M., Moks, E., Saarma, U., Jokelainen, P., Bagrade, G., Laivacuma, S., Strupas, K., Sokolovas, V. and Deplazes, P. (2015). *Echinococcus* infections in the Baltic region. *Veterinary Parasitology* **213**, 121–131.

Martin-Hernando, M. P., González, L. M., Ruiz-Fons, F., Garate, T. and Gortazar, C. (2008). Massive presence of *Echinococcus granulosus* (Cestoda, Taeniidae) cysts in a wild boar (*Sus scrofa*) from Spain. *Parasitology Research* **103**, 705–707.

Mitrea, I. L., Ionita, M., Costin, I. I., Predoi, G., Avram, E., Rinaldi, L., Maurelli, M. P., Cringoli, G. and Genchi, C. (2014). Occurrence and genetic characterization of *Echinococcus granulosus* in naturally infected adult sheep and cattle in Romania. *Veterinary Parasitology* **206**, 159–166.

Moks, E., Jõgisalu, I., Saarma, U., Talvik, H., Järvis, T. and Valdmann, H. (2006). Helminthologic survey of the wolf (*Canis lupus*) in Estonia, with an emphasis on *Echinococcus granulosus*. *Journal of Wildlife Diseases* **42**, 359–365.

Moks, E., Jõgisalu, I., Valdmann, H. and Saarma, U. (2008). First report of *Echinococcus granulosus* G8 in Eurasia and a reappraisal of the phylogenetic relationships of 'genotypes' G5-G10. *Parasitology* **135**, 647–654.

Morgan, E., Clare, E., Jefferies, R. and Stevens, J. (2012). Parasite epidemiology in a changing world: can molecular phylogeography help us tell the wood from the trees? *Parasitology* **139**, 1924–1938.

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

Nakao, M., Li, T., Han, X., Ma, X., Xiao, N., Qiu, J., Wang, H., Yanagida, T., Mamuti, W. and Wen, H. (2010). Genetic polymorphisms of *Echinococcus* tapeworms in China as determined by mitochondrial and nuclear DNA sequences. *International Journal for Parasitology* **40**, 379–385.

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

Nakao, M., Lavikainen, A. and Hoberg, E. (2015). Is *Echinococcus intermedius* a valid species? *Trends in Parasitology* **31**, 342–343.

Oksanen, A. and Lavikainen, A. (2015). *Echinococcus canadensis* transmission in the North. *Veterinary Parasitology* **213**, 182–186.

Onac, D., Győrke, A., Oltean, M., Gavrea, R. and Cozma, V. (2013). First detection of *Echinococcus granulosus* G1 and G7 in wild boars (*Sus scrofa*) and red deer (*Cervus elaphus*) in Romania using PCR and PCR-RFLP techniques. *Veterinary Parasitology* **193**, 289–291.

Rannamäe, E., Lõugas, L., Niemi, M., Kantanen, J., Maldre, L., Kadõrova, N. and Saarma, U. (2016). Maternal and paternal genetic diversity of ancient sheep in Estonia from the late bronze age to the post-medieval period, and comparison with other regions in Eurasia. *Animal Genetics* **47**, 208–218.

Romig, T., Dinkel, A. and Mackenstedt, U. (2006). The present situation of echinococcosis in Europe. *Parasitology International* **55**, S187–S191.

Romig, T., Ebi, D. and Wassermann, M. (2015). Taxonomy and molecular epidemiology of *Echinococcus granulosus* sensu lato. *Veterinary Parasitology* **213**, 76–84.

Saarma, U., Jõgisalu, I., Moks, E., Varcasia, A., Lavikainen, A., Oksanen, A., Simsek, S., Andresiuk, V., Denegri, G. and González, L. M. (2009). A novel phylogeny for the genus *Echinococcus*, based on nuclear data, challenges relationships based on mitochondrial evidence. *Parasitology* **136**, 317–328.

Scala, A., Garippa, G., Varcasia, A., Tranquillo, V. M. and Genchi, C. (2006). Cystic echinococcosis in slaughtered sheep in Sardinia (Italy). *Veterinary Parasitology* **135**, 33–38.

Schneider, R., Gollackner, B., Schindl, M., Tucek, G. and Auer, H. (2010). *Echinococcus canadensis* G7 (pig strain): an underestimated cause of cystic echinococcosis in Austria. *American Journal of Tropical Medicine and Hygiene* **82**, 871–874.

Sherifi, K., Rexhepi, A., Hamidi, A., Behluli, B., Zessin, K. H., Mathis, A. and Deplazes, P. (2011). Detection of patent infections of *Echinococcus granulosus* ("sheep-strain", G1) in naturally infected dogs in Kosovo. *Berliner und Munchener Tierarztliche Wochenschrift* **124**, 518–521.

Simsek, S. and Cevik, A. (2014). First detection and molecular characterization of *Echinococcus equinus* in a mule in Turkey. *Acta Parasitologica* **59**, 773–777.

Simsek, S., Roinioti, E. and Eroksuz, H. (2015). First report of *Echinococcus equinus* in a donkey in Turkey. *Korean Journal of Parasitology* **53**, 731–735.

Sobrino, R., González, L. M., Vicente, J., Fernández de Luco, D., Garate, T. and Gortázar, C. (2006). *Echinococcus granulosus* (Cestoda, Taeniidae) in the Iberian wolf. *Parasitology Research* **99**, 753–756.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**, 585–595.

Thompson, R. (2008). The taxonomy, phylogeny and transmission of *Echinococcus*. *Experimental Parasitology* **119**, 439–446.

Thompson, R. A. and McManus, D. P. (2002). Towards a taxonomic revision of the genus *Echinococcus*. *Trends in Parasitology* **18**, 452–457.

Utuk, A. E. and Simsek, S. (2013). Molecular characterization of the horse isolate of *Echinococcus granulosus* in Turkey. *Journal of Helminthology* **87**, 305–308.

Varcasia, A., Canu, S., Lightowlers, M. W., Scala, A. and Garippa, G. (2006). Molecular characterization of *Echinococcus granulosus* strains in Sardinia. *Parasitology Research* **98**, 273–277.

Varcasia, A., Canu, S., Kogkos, A., Pipia, A. P., Scala, A., Garippa, G. and Seimenis, A. (2007). Molecular characterization of *Echinococcus granulosus* in sheep and goats of Peloponnesus, Greece. *Parasitology Research* **101**, 1135–1139.

Varcasia, A., Garippa, G., Pipia, A. P., Scala, A., Brianti, E., Giannetto, S., Battelli, G., Poglayen, G. and Micagni, G. (2008). Cystic echinococcosis in equids in Italy. *Parasitology Research* **102**, 815–818.

Varcasia, A., Tanda, B., Giobbe, M., Solinas, C., Pipia, A. P., Malgor, R., Carmona, C., Garippa, G. and Scala, A. (2011). Cystic echinococcosis in Sardinia: farmers' knowledge and dog infection in sheep farms. *Veterinary Parasitology* **181**, 335–340.

Vural, G., Baca, A. U., Gauci, C. G., Bagci, O., Gicik, Y. and Lightowlers, M. W. (2008). Variability in the *Echinococcus granulosus* cytochrome C oxidase 1 mitochondrial gene sequence from livestock in Turkey and a re-appraisal of the G1-3 genotype cluster. *Veterinary Parasitology* **154**, 347–350.

Xhaxhiu, D., Kusi, I., Rapti, D., Kondi, E., Postoli, R., Rinaldi, L., Dimitrova, Z. M., Visser, M., Knaus, M. and Rehbein, S. (2011). Principal intestinal parasites of dogs in Tirana, Albania. *Parasitology Research* **108**, 341–353.

Yan, N., Nie, H.-M., Jiang, Z.-R., Yang, A.-G., Deng, S.-J., Guo, L., Yu, H., Yan, Y.-B., Tsering, D. and Kong, W.-S. (2013). Genetic variability of *Echinococcus granulosus* from the Tibetan plateau inferred by mitochondrial DNA sequences. *Veterinary Parasitology* **196**, 179–183.

Yanagida, T., Mohammadzadeh, T., Kamhawi, S., Nakao, M., Sadjjadi, S. M., Hijjawi, N., Abdel-Hafez, S. K., Sako, Y., Okamoto, M. and Ito, A. (2012). Genetic polymorphisms of *Echinococcus granulosus* sensu stricto in the Middle East. *Parasitology International* **61**, 599–603.

Yang, Y. R., Rosenzvit, M. C., Zhang, L. H., Zhang, J. Z. and McManus, D. P. (2005). Molecular study of *Echinococcus* in west-central China. *Parasitology* **131**, 547–555.

Zeder, M. A. (2008). Domestication and early agriculture in the Mediterranean Basin: origins, diffusion, and impact. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 11597–11604.