

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

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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 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 slaughterhouses 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 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 μ L, using 1 \times BD Advantage-2 PCR buffer (BD Biosciences, Franklin Lakes, NJ, USA), 0.2 mM dNTP (Fermentas, Vilnius, Lithuania), 0.25 μ M 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 μ L were examined on 1.2% agarose gel electrophoresis. The remaining 10 μ L 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; 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 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 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 ($H_d = 0.997$), whereas nucleotide diversity was rather low ($\pi = 0.00143$) (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 ($H_d = 0.999$, $\pi = 0.00152$ and $H_d = 0.991$, $\pi = 0.00131$, 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; $H_d = 0.920$; Table S1), whereas considerably lower for the 351 bp dataset ($H_d = 0.596$; Table S2). Low nucleotide diversities were observed for both of the reduced datasets: $\pi = 0.00196$ based on full *cox1* gene (1674 bp) and $\pi = 0.00219$ for the partial *cox1* gene (351 bp; Tables S1 and S2).

Neutrality indices such as Tajima's *D* and Fu's *F_s* 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 *F_{st}* values were observed among different localities (Table S3). The *F_{st}* value for 8274 bp dataset was statistically significant only between Spain and Turkey ($F_{ST} = 0.04130$, $P < 0.05$). Relatively low *F_{st}* values ($F_{ST} = 0.01180$, $P < 0.05$) 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 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 land-bridge 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 $F_{ST} = 0.041$, $P < 0.05$) 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 ($H_d = 0.997$, $\pi = 0.0014$ for overall population) suggest rapid demographic expansion, supported by significant negative values of neutrality indices Tajima's D (-2.69) and Fu's F_s (-24.32) (Avice, 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, $P < 0.05$) 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 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 much 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.

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

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Supplementary Information

Table S1. Diversity and neutrality indices for *E. granulosus* s. s. genotype G1 in Europe based on 1674 bp (*cox1*) 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	49	0.920 0.023	± 0.00196 0.00019	± -2.49144**	-26.27676**
<i>Origin</i>						
Turkey	69	37			-2.30562*	-26.23723**
Spain	10	7	0.939	± 0.00202	± -1.96119*	-9.51463**
Italy	7	3	0.019	0.00021	-1.35841	-9.21700**
South Eur	21	12	0.867 0.107	± 0.00143 0.00048	± -2.13632*	-26.54614**
<i>Host</i>						
Cattle	42	24	0.209	± 0.00025	± -2.11559**	-26.41819**
Sheep	43	26	0.786 0.096	± 0.00159 0.00043	± -2.28186**	-26.02824**
			0.937 0.024	± 0.00171 0.00020	±	
			0.905 0.039	± 0.00217 0.00031	±	

Abbreviations: number of isolates examined (n), number of haplotypes (Hn), haplotype diversity (Hd), nucleotide diversity (π), Tajima's D (D), Fu's Fs (Fs), and standard deviation (S.D.).

** Highly significant P value (P < 0.000001).

* Significant P value (P < 0.05).

Table S2. Diversity and neutrality indices for *E. granulosus* s. s. genotype G1 in Europe based on 351 bp (*coxI*) of mtDNA. The Southern European samples (South Eur) included all samples except Turkish and Finnish (Algerian).

	Diversity				Neutrality	
	n	Hn	Hd \pm S.D.	$\pi \pm$ S.D.	<i>D</i>	Fs
Total	91	11	0.596 0.043	\pm 0.00219 0.00025	\pm -1.43098*	-7.065
<i>Origin</i>						
Turkey	69	8			-0.83662	-3.396
Spain	10	4	0.621	\pm 0.00231	\pm -1.56222	-1.964
Italy	7	1	0.042	0.00028	-	-
South Eur	21	5	0.533 0.180	\pm 0.00171 0.00067	\pm -1.28742	-2.365
<i>Host</i>						
Cattle	42	8	0.486	\pm 0.00174	\pm -0.83916	-3.514
Sheep	43	6	0.124	0.00054	-1.16481	-2.720
			0.677	\pm 0.00271	\pm	
			0.049	0.00040		
			0.520	\pm 0.00176	\pm	
			0.070	0.00031		

Abbreviations: number of isolates examined (n), number of haplotypes (Hn), haplotype diversity (Hd), nucleotide diversity (π), Tajima's D (*D*), Fu's Fs (Fs), and standard deviation (S.D.).

** Highly significant P value ($P < 0.000001$).

* Significant P value ($P < 0.05$).

Table S3. Pairwise fixation index (Fst) values between *E. granulosus* s. s. genotype G1 subpopulations based on mtDNA datasets of different length. The Southern European samples (South Eur) included all samples except Turkish and Finnish (Algerian).

	1	2
8274 bp (mtDNA)		
1. Turkey		
2. Italy	0.02008	
3. Spain	0.04064*	0.00447
4. South Eur	0.01642	
1674 bp (full <i>coxI</i>)		
1. Turkey		
2. Italy	0.00443	
3. Spain	0.00868	-0.3714
4. South Eur	0.01272*	
351 bp (partial <i>coxI</i>)		
1. Turkey		
2. Italy	0.10934	
3. Spain	0.07072	-0.03960

4. South Eur	0.03297
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* Significant P value ($P < 0.05$).

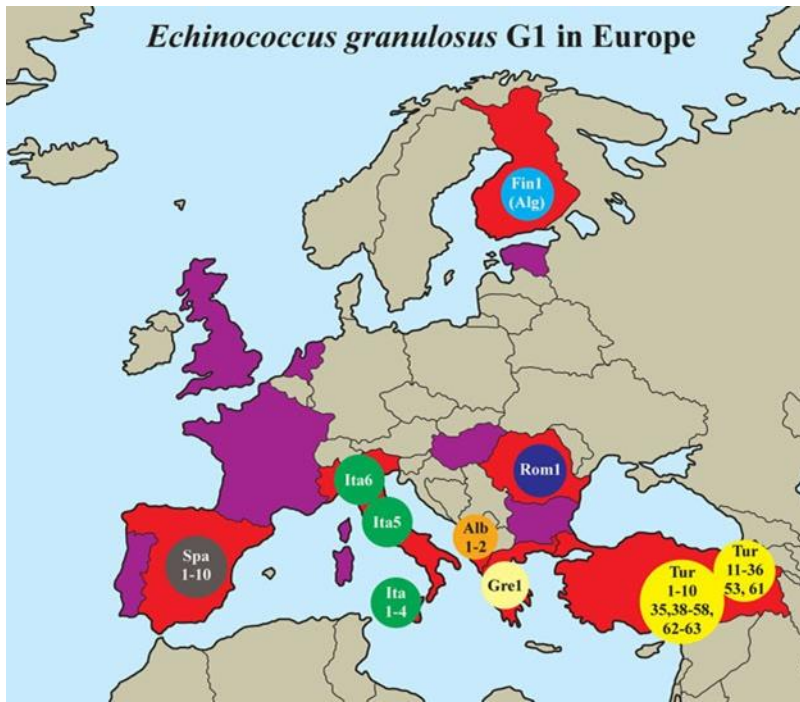


Fig. 1. Geographic locations of *Echinococcus granulosus* s. s. genotype G1 samples ($N=91$; red) from Europe analysed in this study. Additional distribution range of G1 in Europe is represented in pink.

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

Geographical origin	Cattle	Sheep	Human	Host			Total
				Wild boar	Pig	Goat	
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

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	GATGCTGTAACTTCAAGAAATG	6034...6056	1053 bp
E2r2	CTCAAAGCATTCAAACGC	7054...7071	
E3fn	GTTGATTCGTGTTAATTTTTGGAG	6874...6898	722 bp
E3rn	GAAAACATAGCAAACAACAACCC	7574...7596	
E4f2	GTGATCCTATTTTATTTCAAC	7461...7481	1516 bp
E4r	TGCTACCTTGCACAGTCAA	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	TTGTGGTGTTTTTATGATG	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	
	<i>n</i>	Hn	Hd ± s.d.	π ± s.d.	<i>D</i>	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 ±	-2.44968**	-24.56183**

0.00009

n, number of isolates examined; *H_n*, number of haplotypes; *H_d*, haplotype diversity; π , nucleotide diversity; *D* (*D*), Tajima's *D*; *F_s*, Fu's *F_s*; *s.d.*, standard deviation.

** Highly significant *P* value ($P < 0.000001$).

* Significant *P* value ($P < 0.05$).

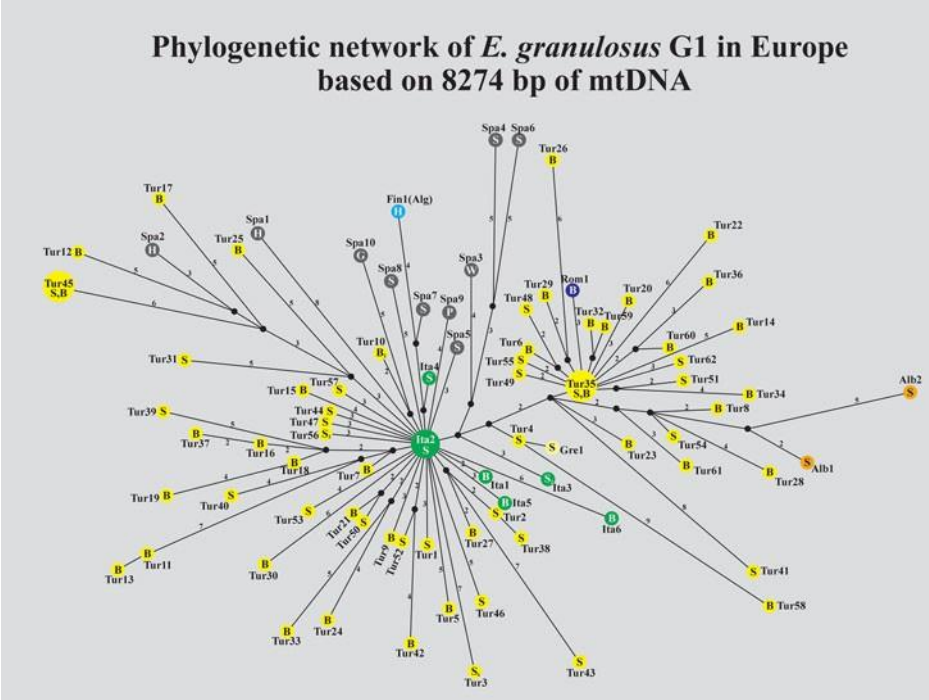


Fig. 2. Phylogenetic network of *Echinococcus granulosus* s. s. genotype G1 based on 8274 bp of mtDNA. Circles represent haplotypes. Haplotype names and colours represent different geographical origins: Tur (yellow) – Turkey, Rom (dark blue) – Romania, Fin-Alg (light blue) – Finland (a patient from Algeria), 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.

