Global phylogeography and genetic diversity of the zoonotic tapeworm Echinococcus granulosus sensu stricto genotype G1

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ABSTRACT

Echinococcus granulosus sensu stricto (s.s.) is the major cause of human cystic echinococcosis worldwide and is listed among the most severe parasitic diseases of humans. To date, numerous studies have inves- tigated the genetic diversity and population structure of E. granulosus s.s. in various geographic regions. However, there has been no global study. Recently, using mitochondrial DNA, it was shown that E. gran- ulosus s.s. G1 and G3 are distinct genotypes, but a larger dataset is required to confirm the distinction of these genotypes. The objectives of this study were to: (i) investigate the distinction of genotypes G1 and G3 using a large global dataset; and (ii) analyse the genetic diversity and phylogeography of genotype G1 on a global scale using near-complete mitogenome sequences. For this study, 222 globally distributed E. granulosus s.s. samples were used, of which 212 belonged to genotype G1 and 10 to G3. Using a total sequence length of 11,682 bp, we inferred phylogenetic networks for three datasets: E. granulosus s.s. (n = 222), G1 (n = 212) and human G1 samples (n = 41). In addition, the Bayesian phylogenetic and phy- logeographic analyses were performed. The latter yielded several strongly supported diffusion routes of genotype G1 originating from Turkey, Tunisia and Argentina. We conclude that: (i) using a considerably larger dataset than employed previously, E. granulosus s.s. G1 and G3 are indeed distinct mitochondrial genotypes; (ii) the genetic diversity of E. granulosus s.s. G1 is high globally, with lower values in South America; and (iii) the complex phylogeographic patterns emerging from the phylogenetic and geographic analyses suggest that the current distribution of genotype G1 has been shaped by intensive animal trade.

Introduction

The species complex Echinococcus granulosus sensu lato (s.l.) is the causative agent of cystic echinococcosis (CE), which is one of the most important zoonoses worldwide and a significant global public health concern (e.g., Dakkak, 2010; Wahlers et al., 2012; Marcinkute et al., 2015; Zhang et al., 2015; Cucher et al., 2016; Budke et al., 2017). CE is listed amongst the most severe parasitic diseases in humans, ranking second in the list of food-borne para- sites globally (FAO/WHO, 2014) and representing one of the 17 neglected tropical diseases prioritised by the World Health Organization (WHO, 2015). The life-cycle of the parasite involves two mammalian hosts – a definitive host, which harbours the adult worm in the small intestine, and an intermediate host, in which the larval stage develops in the form of cysts, predominantly in liver and lungs, causing CE (Eckert et al., 2001). While definitive hosts are most commonly dogs and wild carnivores, a wide range of domestic and wild mammals, but also humans, act as interme- diate or accidental hosts (Romig et al., 2017). Segments containing eggs or free eggs are passed into the environment with carnivore faeces, and intermediate or accidental hosts acquire infection by ingesting eggs (Moro and Schantz, 2009). The eggs are resilient and can survive up to a year in a suitable environment (Eckert et al., 2001).

Echinococcus granulosus s.l. exhibits considerable variation in terms of morphology, host range, infectivity to humans, pathogenicity and other aspects (e.g., Eckert et al., 2001; Thompson, 2008; Gholami et al., 2011; Romig et al., 2015).Molecular studies have identified and characterised a number of geno- types/species within the E. granulosus s.l. complex (Bowles et al., 1992, 1994; Thompson and McManus, 2002; Lavikainen et al., 2003; Thompson, 2008; Saarma et al., 2009; Knapp et al., 2011), which are relatively closely related to other species within the genus Echinococcus (Knapp et al., 2015). The accurate identification and differentiation of genotypes has important epidemiological implications and informs about the zoonotic potential of particular genotypes. Earlier, the complex was considered to consist of geno- types G1–G8, G10 and Echinococcus felidis (see Bowles et al., 1992, 1994; Lavikainen et al., 2003; Hüttner et al., 2008), however G2 is no longer considered a valid genotype (Vural et al., 2008; Kinkar et al., 2017). Currently, the genotypes regarded as distinct species are E. granulosus sensu stricto (s.s.; genotypes G1 and G3; Kinkar et al., 2017), Echinococcus equinus (G4), Echinococcus ortleppi (G5) (Thompson and McManus, 2002), whereas the species status of genotypes G6–G10 remains contentious (Moks et al., 2008; Thompson, 2008; Saarma et al., 2009; Knapp et al., 2011, 2015; Lymbery et al., 2015; Nakao et al., 2015). A study by Yanagida et al. (2017) using two nuclear loci, suggested the sharing of nuclear alleles between genotypic groups G6/G7 and G8/G10, whereas recent data representing six nuclear loci suggest that G6/G7 and G8/G10 are two distinct species (Laurimäe et al., 2018). Echinococcus granulosus s.s. (genotypes G1 and G3) is wide- spread globally, with highly endemic foci in South America, the Mediterranean basin and Asia, and particularly affects rural livestock-raising areas (Jenkins et al., 2005; Jabbar et al., 2011; Hajialilo et al., 2012; Cardona and Carmena, 2013; Rostami et al., 2015; Cucher et al., 2016; Deplazes et al., 2017; Ito and Budke, 2017). Some of the main factors contributing to the persistence of CE include the frequent illegal and home slaughtering of animals for food, feeding raw offal to dogs, low public awareness of the dis- ease, large populations of stray dogs and poor hygiene conditions (Eckert et al., 2001; Torgerson and Budke, 2003; Varcasia et al., 2011; Possenti et al., 2016). According to a recent estimate by Alvarez Rojas et al. (2014), E. granulosus s.s. is also the most fre- quently implicated causative agent of CE of humans (88% of cases) worldwide, and thus deserves particular attention.

To date, numerous studies have explored the genetic diversity and population structure of E. granulosus s.s. in various geographic regions (e.g., Nakao et al., 2010; Casulli et al., 2012; Rostami Nejad et al., 2012; Yanagida et al., 2012; Andresiuk et al., 2013; Yan et al., 2013; Boufana et al., 2014, 2015; Romig et al., 2015; Kinkar et al., 2016, 2018; Laurimäe et al., 2016;

Hassan et al., 2017). However, there has been no global study. In addition, the analytical power has been low in most studies as the analyses have been based lar- gely on short sequences of mitochondrial DNA (mtDNA), most often on a single gene, e.g., the cytochrome C oxidase subunit 1 gene (cox1; 1609 bp; Yanagida et al., 2012; Alvarez Rojas et al., 2016, 2017) or partial sequence of the cox1 or nad1 (e.g., Casulli et al., 2012; Andresiuk et al., 2013). Few studies used considerably longer mtDNA sequences (8270 bp; Kinkar et al., 2016; Laurimäe et al., 2016) and demonstrated significantly better phylogenetic resolution. Due to the variable sequence lengths used thus far (a few hundred bp up to 8270 bp), the results from different studies and geographic regions are not directly comparable. Therefore, an analysis of near-complete mitogenome sequences on a large geo- graphical scale is required to gain better insight into the global patterns of diversity and phylogeography. Furthermore, the sequences of relatively short mtDNA regions most commonly used to date cannot unequivocally differentiate genotypes G1 and G3 due to limited phylogenetic signal (e.g., Casulli et al., 2012; Andresiuk et al., 2013; Romig et al., 2015). Thus, although short mtDNA sequences have been widely used in phylogeographic studies and to develop methods for identifying Echinococcus spp. and genotypes (e.g., Bowles et al., 1992; Bowles and McManus, 1993; Boubaker et al., 2013; Laurimaa et al., 2015; Boubaker et al., 2016), one has to be cautious when interpreting the results based on short mtDNA sequences.
By contrast, using near-complete mitogenome sequences (11,443 bp), Kinkar et al. (2017) provided evidence that G1 and G3 are distinct mitochondrial genotypes. As a relatively small number of samples was used in Kinkar et al. (2017), a larger sam- ple size would be preferable to confirm the distinction of the two genotypes (G1 and G3). Therefore, in the present study, we (i) investigated the distinction of the E. granulosus s.s. genotypes G1 and G3 using a large global dataset (n = 222), and (ii) analysed the genetic diversity and phylogeography of genotype G1 on a world-wide scale using near-complete mitochondrial genome sequences.

Materials and methods

1.1. Parasite material

We sequenced 221 E. granulosus s.s. samples and included an additional sequence from GenBank (AB786664; genotype G1 from China; Nakao et al., 2013). Of the 221 samples, 114 were newly sequenced, whereas the rest were from Kinkar et al. (2016, 2017) and Laurimäe et al. (2016). We have listed the corresponding hap- lotype names for genotype G1 in Supplementary Tables S1 and S2. The origin and host species of the G1 samples are shown in Figs. 1 and 2, Table 1 and Supplementary Table S3. Ten G3 samples repre- sented haplotypes FRA1, FRA2, IRA1, IRA3, SPA2, SPA3, SPA4, TUR1, TUR2 and IND1 (cf. Kinkar et al., 2017). However, additional mtDNA loci were sequenced for all of the previously published samples in this study. The samples were obtained during routine parasite inspections or from hospital cases and were ethanol-preserved at —20 °C until further use.

1.2. DNA extraction, PCR amplification, sequencing and assembly

Total genomic DNA was extracted from protoscoleces, cyst membranes or adult worms of E. granulosus using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Ger- many), following the manufacturer's protocols. For PCR amplifica- tion we used 12 primer pairs described in Kinkar et al. (2017). Sequencing was performed using the same primers as for the ini- tial PCR amplification. Cycle parameters for PCR and sequencing were as described in Kinkar et al. (2016). Sequences were assem- bled using the program CodonCode v6.0.2 and manually curated in BioEdit v7.2.5 (Hall, 1999). All G1 sequences were deposited in the GenBank database under accession nos. MG672124– MG672293; the alignment is

available at Mendeley Data (DOI: https://doi.org//10.17632/tytzhyk93w.1).

1.3. Phylogenetic analyses

Phylogenetic networks were calculated for three mtDNA sequence datasets: (1) all samples of E. granulosus s.s. (n = 222), (2) sequences representing genotype G1 only (n = 212) and (3) sequences representing genotype G1 from humans (n = 41) using Network v4.6.1.5 (Bandelt et al., 1999), http://www.fluxusengi- neering.com, Fluxus Technology Ltd., 2004. Networks were con- structed considering both indels and point mutations. The Bayesian phylogenetic analysis for the whole dataset (n = 222 samples) was performed in the program BEAST 1.8.4 (Drummond et al., 2012) using BEAUti v1.8.4 to generate the initial xml file for BEAST. The general time-reversible nucleotide- substitution model with a proportion of invariable sites and gamma distributed rate variation (GTR+I+G; Tavaré, 1986; Gu et al., 1995) was determined as the best-fit model of sequence evo- lution using the program PartitionFinder 2.1.1 (Guindon et al., 2010; Lanfear et al., 2012, 2016). Exponential growth coalescent prior (Griffiths and Tavaré, 1994) was chosen for the tree, and a strict molecular clock was assumed owing to the intraspecific nat- ure of the data (Drummond and Bouckaert, 2015). The posterior distribution of parameters was estimated by Markov Chain Monte Carlo (MCMC) sampling. MCMC chains were run for 10 million states, sampled every 1000 states with 10% burn-in. Log files were analysed using the program Tracer v1.6 (Rambaut et al., 2014; http://tree.bio.ed.ac.uk/software/tracer). The tree was produced using TreeAnnotator v1.8.4 and displayed in FigTree v.1.4.3 (Ram- baut, 2014; http://tree.bio.ed.ac.uk/software/figtree).

1.4. Population indices

The population diversity indices, such as the number of haplo- types, haplotype diversity and nucleotide diversity, were calculated using the program DnaSP v5.10.01 (Librado and Rozas, 2009). Haplotype diversity is the probability that two randomly sampled haplotypes are different (Nei, 1987), and nucleotide diver- sity is the average number of nucleotide differences per site between two randomly chosen DNA sequences (Nei and Li, 1979). Neutrality indices Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997), and the pairwise fixation index (Fst) were calculated using the Arlequin 3.5.2.2 software package (Excoffier et al., 2005). Tajima's D and Fu's Fs are widely used statistics for demo- graphic analyses. Specifically, those test whether the observed pat-tern of polymorphism in a set of DNA sequences is consistent with a neutral model of evolution (Tajima, 1989; Fu, 1997). The pairwise fixation index (Fst) measures population differentiation and pro- vides insight into the genetic structure of populations. The indices were calculated for four different datasets representing genotype G1: (a) all sequences (n = 212); (b) the three most numerous host species in this study (cattle, sheep and human), (c) five regions (South America, Africa, Asia/Australia, Europe and the Middle East), and (d) eight countries for which the sample size exceeded 10: Algeria, Argentina, Brazil, Iran, Italy (comprising continental Italy and Sardinia), Spain, Tunisia and Turkey. In addition, the pairwise fixation index was calculated between genotypes G1 and G3.

1.5. Bayesian phylogeographic analysis

The phylogeographic diffusion patterns of genotype G1 were analysed using a Bayesian discrete phylogeographic approach (Lemey et al., 2009). This approach estimates ancestral locations from the set of sampled locations and annotates the discrete loca- tion states to tree nodes (Lemey et al., 2009; Faria et al., 2011). The standard Markov model is extended using a Bayesian Stochastic Search Variable Selection (BSSVS) procedure, which offers a Baye- sian Factor (BF) test to identify the most parsimonious description of the phylogeographic diffusion

process (Lemey et al., 2009). Specifically, the intial xml file generated in BEAUti in the Bayesian phylogenetic analysis (see Section 2.3) was edited according to the 'Discrete phylogeographic analysis' tutorial available on the Beast website (http://beast.bio.ed.ac.uk/tutorials – accessed in June 2017). The analysis was performed in BEAST 1.8.4 (Drummond et al., 2012) using the BEAGLE library (Ayres et al., 2012). MCMC chains were run for 50 million states, sampled every 5000 states with 10% burn-in. The effective sampling size (ESS) of estimates was assessed using Tracer v1.6 (Rambaut et al., 2014; http://tree. bio.ed.ac.uk/software/tracer), and the tree was produced using TreeAnnotator v1.8.4 and displayed in FigTree v.1.4.3 (Rambaut, 2014; http://tree.bio.ed.ac.uk/software/figtree). The program SpreaD3 v0.9.6 (Bielejec et al., 2016) was used to visualise the out- put from the Bayesian phylogeographic analysis and to calculate the Bayes Factor supports. Three independent runs were conducted and geographic links that yielded an average value of BF >10 were displayed.

2. Results

Near-complete mitogenome sequences representing E. granulosus s.s. samples (n = 221) were aligned (length of alignment 11,682 bp). Most sequences were 11,675 bp in length, but some varied from 11,674 bp to 11,678 bp. An additional sequence from Gen- Bank (see Section 2.1) was included, totalling 222 sequences in the analysis.

2.1. The phylogenetic network of E. granulosus s.s.

The 222 sequences divided into two haplogroups, separated by 37 mutations (Fig. 3). The larger haplogroup included 212 sequences, of which 145 represented the G1 sequence originally described by Bowles et al. (1992) (366 bp of the cox1 gene). The other haplogroup included 10 samples, of which five represented the G3 sequence sensu Bowles et al. (1992). Thus, the two hap- logroups corresponded to the E. granulosus s.s. mitochondrial genotypes G1 and G3, and were named accordingly. The 212 G1 samples were divided into 171 different haplotypes (Fig. 3). To the best of our knowledge, all human G1 samples used in the anal- ysis were autochthonic cases of CE, except for a Finnish sample, which originated from an Algerian patient who was living in Finland. Therefore the origin of the infection was most likely Algeria.

2.2. Bayesian phylogenetic analysis

The Bayesian phylogenetic analysis divided E. granulosus s.s. samples into two well-supported clades, corresponding to geno- types G1 and G3 (posterior probability value = 1.00; Fig. 4; Supple- mentary Fig. S1). The intraspecific phylogeny of G1 yielded clades with varying support values, of which several clades were well resolved (posterior probability values = 1.00).

2.3. The phylogenetic network for genotype G1

The phylogenetic network for genotype G1 was highly diver- gent (Fig. 5). Among the 171 haplotypes, 147 were represented by a single sample, 18 haplotypes included two samples, five hap- lotypes (IRA1, BRA1, TUR1, TUR3, TUN5) included three samples and one haplotype (ARB1) included 14 samples. The average num- ber of mutational steps between different G1 haplotypes was 16 and the maximum 32 (e.g., between TUR12 and ALB2).
Multiple haplogroups (monophyletic groups) could be distin- guished. Seven such haplogroups (named A–G, respectively) corresponded to the well-supported clusters in the Bayesian phylogenetic tree (posterior probability values = 1.00; see Figs. 4 and 5; see also Section 3.2).

Out of the nine haplogroups which are not labelled with letters (Fig. 5), seven were well-supported on the phylogenetic tree (posterior probability values = 1.00; Fig. 4).
In some of the monophyletic clusters in the network, haplo- types clustered together according to geographic origin (Fig. 5). For example, three monophyletic groups represented haplotypes only from Tunisia (TUN25, TUN11 and TUN1; TUN26 and TUN6; TUN13, TUN3 and TUN18). Another haplogroup (D) was of Middle-Eastern origin, comprising samples from Turkey (TUR8, TUR21, TUR18, TUR19) and Iran (IRA11). In addition, one group was of African origin and included samples from Tunisia (TUN5, TUN7) and Algeria (ALG9), and another group was of South American origin, including haplotypes from Brazil and Argentina (BRA4, ARG2, BRA6). Haplogroup B included a central haplotype ARB1, which comprised samples from Argentina and Brazil. The haplogroup also included 12 haplotypes from Argentina, four haplo- types from Brazil (BRA7 – BRA10), two haplotypes from Chile (CHI2 and CHI3) and one from Mexico (MEX1). In other monophyletic groups, samples from Eurasia clustered together, some of which comprised haplotypes that were geographically distant from each other, such as an Indian-Iranian group (IND1 and IRA16) and a Turkish-Spanish-Iranian group F (TUR12, TUR24,TUR27, TUR4, TUR9, IRA12 and SPA1). Haplogroup G from Eurasia represented haplotypes from Turkey (n = 12), Iran (n = 8), Albania (ALB1, ALB2), Moldova (MOL2) and Romania (ROM1), and haplogroup C represented haplotypes from Iran (IRA19, IRA6 and IRA5), Moldova (MOL3), Mongolia (MON1) and Romania (ROM2).
The geographically most distant haplotypes that clustered together into haplogroups originated from different continents, including two haplotypes from Australia (AUS1 and AUS2) and a haplotype originating from Algeria (ALG4) (Fig. 5). However, haplo- type AUS3 from Australia clustered together with 12 haplotypes from Africa and three haplotypes from Europe (SPA7, SPA4 and FIN1; group A). In addition, five haplotypes from Africa (ALG2, TUN15, MOR1, TUN27, ALG8) clustered with haplotype ARG8 from Argentina, and haplotypes ITA7, ITA6, ITA8, and TUN2 from Italy and Tunisia also clustered together.
No host-specific pattern was identified, as the majority of monophyletic clusters included samples from different host species. The most numerous host species in this study, cattle and sheep, were genetically closely related and some haplotypes (TUR17, TUN14 and ARB1) included samples from both hosts. The haplotypes representing 41 samples from humans did not cluster together and were in different haplogroups, together with samples from other hosts. Haplotype TUN5 from Tunisia represented three samples, one from sheep and two from humans, and haplotype TUN15, also from Tunisia, represented two samples, one from sheep the other from a human.

2.4. The phylogenetic network of human G1 samples

The 41 genotype G1 samples from humans represented 37 dis- tinct haplotypes (Fig. 6). Haplotypes from Tunisia and Algeria were frequently closely related (e.g., TUN22 and ALG12), but some were genetically very distant from one another (e.g. ALG7 and TUN27; separated by 30 mutations). Haplotype ALG1 from Algeria was most closely related to haplotype FIN1; FIN1 was from an Algerian CE patient who was living in Finland. Haplotype MON1, representing two samples from Mongolia, was within a monophyletic cluster with haplotype ROM2 from Romania and haplotype IRA3 from Iran with haplotype TUN21 from Tunisia.

2.5. Diversity and neutrality indices

The overall haplotype diversity (Hd) index for genotype G1 was very high (Hd = 0.994), while the nucleotide diversity (p) was 0.00133 (Table 2). The most numerous host species in this study – cattle, sheep and human – were represented by high haplotype diversity indices (0.987–

0.995), whereas nucleotide diversities ranged from 0.00128 to 0.00138. The haplotype diversity indices for genotype G1 from the five geographical regions were also high, ranging from 0.923 to 0.994, whereas the nucleotide diversities varied from 0.00083 to 0.00136, with samples from South America having the lowest values. Of the countries represented in the present analysis, Argentina had the lowest values of haplotype and nucleotide diversities (Hd = 0.832 and p = 0.00057), whilst the corresponding values for other countries were higher (ranging from 0.956 to 1.000 and p ranging from 0.115 to 0.00143).
Neutrality indices Tajima's D and Fu's Fs were negative and statistically highly significant for the whole G1 dataset (D = 2.77, Fs = 23.80; Table 2). Neutrality indices were similar among host species and in the majority of the regions (Africa, South America, Europe and the Middle East). However, neutrality indices were insignificant for Asia and Australia. Among the countries included, both neutrality indices were negative and statistically significant for Algeria, Argentina, Tunisia and Turkey, while only Tajima's D was significant for Iran. The neutrality indices calculated for Brazil, Italy and Spain were all negative, but statistically insignificant. Negative values of the neutrality indices Tajima's D and Fu's Fs sug- gest population expansion (Tajima, 1989; Fu, 1997).

2.6. Population differentiation

To assess population differentiation, we calculated the fixation indices (Fst) between different populations. Fst values can range from 0 to 1, where 0 means complete sharing of genetic material (panmixia) and 1 means that the two populations do not share any genetic diversity (relating to a lack of gene flow) (Holsinger and Weir, 2009). The Fst value between genotypes G1 and G3 was very high (0.711; $P < 0.00001$). By contrast, low Fst values were observed between cattle, sheep and human samples of G1 (Fst < 0.05; Table 3) and between most of the regions of G1 in this study (Africa, Asia and Australia, Europe and the Middle East), ranging from 0.022 to 0.068 (Table 4). However, higher Fst values (ranging from 0.184 to 0.213) were detected between South America and the other regions. Among countries, the highest Fst values were seen between Argentina and the Eurasian (Iran, Italy, Spain and Turkey) and African countries (Algeria and Tunisia), ranging from 0.269 to 0.359, while the value was slightly lower between Argentina and Brazil (0.124; Table 5). The Fst values between the remaining countries were mostly less than 0.100. Statistically insignificant values were observed between Europe and Asia- Australia (Table 4) and between Algeria and Tunisia (Table 5).

2.7. Bayesian phylogeographic analysis

The Bayesian discrete phylogeographic analysis yielded 18 well supported spatial diffusion routes for genotype G1, of which 11 had a Bayes Factor value of 10 to 100, whereas the BF value was very high (>100) for seven routes (Fig. 7). Values >3 are considered well supported (Lemey et al., 2009). A total of seven routes originated from Turkey, two of which had very high support (BF>100; between Turkey and Iran, and Turkey and Greece); six originated from Tunisia, three of which had BF values >100 (between Tunisia and Italy, Tunisia – Algeria and Tunisia – Argentina). Argentina was the ancestral location to Brazil (BF >100), Mexico and Chile, while Iran was ancestral to India. Algeria was identified as the origin of the sample from a human from Finland.

3. Discussion

The results of this study based on 222 near-complete E. granulosus s.s. mitogenome sequences from a worldwide distribution confirmed that genotypes G1 and G3 are indeed distinct genotypes, as reported recently by Kinkar et al. (2017) with a significantly smaller sample size

(n = 23). The analysis of the much larger data- set used in the present study also positioned genotypes G1 and G3 into distinct haplogroups, separated by 37 mutations (Fig. 3). This distinction was also well supported by the Bayesian phylogenetic analysis (Fig. 4) and by the high Fst value (0.711; $P < 0.00001$) between genotypes G1 and G3. As genotypes G1 and G3 represent distinct mitochondrial lineages and G1 is more widespread with a larger spectrum of hosts, it is possible that there are epidemiological differences between these genotypes. Although this proposal has not yet been explored, the use of up-to-date molecular meth- ods to identify and distinguish these genotypes will be the prerequisite to test this hypothesis. However, sequencing a large portion of the mitochondrial genome is often not feasible in most laboratories. Establishing a set of diagnostic nucleotides to confidently assign samples to genotypes G1 and G3 is an ongoing project.

The results of the present study demonstrated an extremely high global haplotype diversity within genotype G1 (Fig. 5): the 212 samples analysed represented a total of 171 haplotypes (over- all haplotype diversity 0.994; Table 2). High genetic diversity within E. granulosus s.s. has also been reported in various parts of the world based on shorter sequence lengths (e.g., Casulli et al., 2012; Sharma et al., 2013; Alvarez Rojas et al., 2016; Debeljak et al., 2016; Hassan et al., 2017) and >8000 bp of mtDNA (Kinkar et al., 2016; Laurimäe et al., 2016). However, this is the first study to demonstrate the diversity of G1 based on near-complete mito- genome data sets on a vast geographic scale. Haplotype diversities within genotype G1 were high for different host species, regions and countries (with values being mostly between 0.970 and 1.000; Table 2), whereas Fst values were low (mostly <0.1; Tables 3–5), pointing to a high genetic diversity and low genetic differentiation between G1 subpopulations globally, possibly due to rapid radiation. However, the South American samples showed slightly lower values of haplotype diversities (particularly Argentina; Hd = 0.832; Table 2) and higher values of Fst (ranging from 0.184 to 0.213 between South America and the other regions; Table 4), indicating lower genetic diversity and moderate genetic differentiation of samples from South America compared with those from Africa and Eurasia. This finding is also supported by the phylogenetic net- work wherein the South American samples (and one sample from Mexico) formed a haplogroup (B) with a dominant central haplo- type (Fig. 5), suggesting a bottleneck event in the past, while significant negative values of neutrality indices (D = 2.182, Fs = 12.1 90; Table 2) indicated a population expansion in South America. A possible explanation for this observation is the relatively recent arrival to and sudden expansion of domestic animals (cattle and sheep) in South America during the 15th and 16th Centuries (Rodero et al., 1992) compared with the domestication history in Africa and Eurasia, extending thousands of years BC (Zeder, 2008; Lv et al., 2015). However, as Argentina contributed more to the lower Hd value for South America, another possible reason for this could be that a relatively large number of the Argentinian samples (24 of 31) originated from the same geographical area (the Buenos Aires province in Argentina). However, the samples from Turkey used in this study also originated from one area in the east (Erzu- rum and Elazig provinces), but yielded very high haplotype diver- sity (Hd = 0.991; Table 2). Therefore, the results could reflect a more recent arrival and sudden expansion of E. granulosus s.s. genotype G1 in South America. Low genetic diversity within E. granulosus s.s. in South America, specifically in Peru, has been demonstrated by Yanagida et al. (2012) and Nakao et al. (2010) (Hd = 0.545 and 0.137, respectively), whereas higher haplotype diversity values were reported later in Chile (Hd = 0.875; Alvarez Rojas et al., 2017). However, due to the different sequence lengths used, the Hd values are not directly comparable. In addition, analysis of complete mtDNA data for genotype G7 has shown that hap- lotype diversity is limited in Argentina compared with other regions (Laurimäe et al., unpublished data), suggesting that the overall genetic diversity of different genotypes in South America is low. To elucidate the genetic diversity and population structure of the parasite in South America, further investigations are needed. In addition to the American haplogroup B, there were multiple other groups where samples clustered together according to

their geographical origin; for example, some of the African samples (Fig. 5). However, the opposite was also observed, and numerous well supported clusters on the phylogenetic tree comprised sam- ples from various geographic locations (e.g., in haplogroup A, in which African, Australian and European samples clustered together). These observed phylogeographical patterns (together with the low Fst values in Eurasia and Africa) are likely consequences of livestock diffusion and trade that have facilitated the dispersal of the parasite over vast geographic areas, as hypothesised previously (e.g., Kinkar et al., 2016; Laurimäe et al., 2016). Demographic analysis also supported this hypothesis: significant negative values of neutrality indices Tajima's D (2.771) and Fu's Fs (23.802) suggest rapid demographic expansion, particularly evident among subpopulations with larger sample sizes (the whole dataset, hosts, African and the Middle Eastern region, Turkey; Table 2). Similar results reflecting populations under expansion have been reported in previous studies in various geographic regions (e.g., Nakao et al., 2010; Casulli et al., 2012; Yanagida et al., 2012; Kinkar et al., 2016; Laurimäe et al., 2016; Hassan et al., 2017).

In this study, samples from humans did not cluster together and were frequently positioned with samples from various livestock species (e.g., sheep and goat in group C; sheep and cattle in groups A and F; see Figs. 4 and 5). The Fst values point to a slightly higher genetic similarity between sheep and human samples (Fst = 0.025) compared with cattle and human samples (Fst = 0.046). Interestingly, the majority of the E. granulosus s.s. cysts obtained from cattle are reported as sterile, whereas a high fertility rate is characteristic of sheep and human infections (e.g. McManus and Thompson, 2003; Andresiuk et al., 2013; Elmajdoub and Rahman, 2015; Kamelli et al., 2016). The higher genetic similarity between samples of human and sheep origin could indicate a more efficient transmission of G1 to humans via the sheep-dog cycle than via the cattle-dog cycle. However, it should be noted that samples from sheep were in excess compared with those from other hosts, but this is unavoidable, since the sheep is the main intermediate host of G1.

As a large portion (29 of 41) of the G1 samples from humans studied here originated from Africa, it is not surprising that most of these clustered together in the phylogenetic network (see Fig. 6). The sample from a CE patient in Finland who originated from Algeria, clustered together with another human sample from Algeria and the link between Algeria and Finland was also sup- ported by phylogeographic analysis (Fig. 7), suggesting that the individual was most likely infected in Algeria. The genetic diversity among samples from humans was very high (Hd = 0.995), almost equal to values calculated for cattle and sheep (Hd = 0.992 and 0.987, respectively; Table 2).

We performed a Bayesian phylogeographic analysis which, as an output, draws hypothetical ancestral locations on to a map. While these links could reflect the complex livestock trade circuits in relatively recent history, interestingly, some of these circuits seemed to follow the diffusion routes of livestock early in history. However, it should be emphasised that linking the well supported diffusion routes to a timescale remains speculative. The analysis revealed a number of well supported routes that seemed to follow the spread of livestock animals from the centre of domestication during Neolithic times (Zeder, 2008; Lv et al., 2015; Fig. 7). One ancestral location of genotype G1 was Turkey, from which several migration routes originated. The Fertile Crescent of the Middle East is considered one of the earliest centres of livestock domestication (mainly cattle, sheep, pigs and goats) from where the animals were later distributed east- and westwards during Neolithic times (Bruford et al., 2003; Zeder, 2008; Chessa et al., 2009; Lv et al., 2015; Rannamäe et al., 2016). The phylogeographic results of this study could reflect the early spread of livestock from this region together with E. granulosus s.s. genotype G1. The possible ancestral location of E. granulosus s.s. in the Middle East has been suggested before (e. g. Nakao et al., 2010; Casulli et al., 2012; Yanagida et al., 2012; Kinkar et al., 2016; Hassan et al., 2017), but has not been demonstrated using the discrete Bayesian phylogeographic approach. In addition, the migration routes from Tunisia to Mor-

occo and Algeria point to a westward movement of genotype G1 in North Africa which is also in accordance with the supposed direction of early dispersal of domesticated animals (cattle, sheep and goats) in this area (Gifford-Gonzalez and Hanotte, 2011).

Another location from which several diffusion routes originated was Tunisia: among others, three routes showed a possible migration of genotype G1 from Tunisia to Argentina, Australia and Tur- key which could be linked to human/livestock migration in later history. During the 15th and 16th Centuries, sheep and other live- stock were introduced to the Americas by Spanish and British colonizers. However, some animals that arrived to the Americas could have had an African origin as some of the livestock species (mostly pigs and goats) were taken aboard on the Canary Islands, which were colonised by people from North Africa (Rodero et al., 1992; Rando et al., 1999; also discussed in Alvarez Rojas et al., 2017), possibly explaining the significant diffusion route between Tunisia and Argentina. The ancestral position of Argentina could indicate its possible origin for the other American samples (Brazil, Chile and Mexico), although it should be noted that only a single sample was from Mexico. As this result is counter-intuitive in relation to the direction of livestock introduction to South America (Rodero et al., 1992), more samples are required from this region to address this issue. The connection between Tunisia and Australia could also be linked to relatively recent history: it is thought that the sources of Australian sheep could be Spain and/or North Africa, as Merinos raised in North Africa arrived in Australia in the beginning of the 19th Century, as discussed by Jenkins (2005).

The samples in the present study cover most of the global distribution range of E. granulosus s.s. G1 (Fig. 1) and highly endemic regions, which are of particular epidemiological importance, such as South America, the Mediterranean region and the Middle East, are represented with a larger sample size. However, it is important to take into account that samples from some geographical regions where G1 has been found to be highly prevalent were lacking or under-represented (e.g., Peru, Ethiopia, Kenya, Libya and central Asia) and samples from Argentina, Turkey and Tunisia were in excess compared with other regions (e.g., only three samples from Australia and one from Mexico). This aspect is important to con- sider in the context of the Bayesian phylogeographic analysis, which is highly dependent on sampling and, therefore, should be interpreted with some caution. The 'ancestral' locations in the analysis are drawn from the set of sampled locations. Taking this aspect into consideration, it is likely that some of the migrations proposed did not occur directly between the two locations, but were in reality much more complex, involving geographical loca- tions that were not represented in this study. While we are able to provide the first insight into the large-scale phylogeographic patterns of G1, these should be further tested using larger datasets. Taken together, based on the extensive research of E. granulosus

s.s. G1 thus far, it is evident that the current cosmopolitan distribution of this parasite has been highly influenced by humans. This trend could continue, further increasing the number of infections globally, if serious intervention methods are not implemented. As most control programs have largely been regional (Craig and Larrieu, 2006), attention should shift to implementing global intervention and control programs for G1 due to its worldwide distribution. The surveillance of CE in imported/exported livestock species should be encouraged. The diagnosis of CE in livestock currently relies largely on necropsy findings, as sero- or immuno-diagnosis is not sufficiently specific or sensitive (Craig et al., 2015). Mass ultrasound scanning for CE in small ruminants has also been shown as a relatively cost-effective and practical means of parasite detection (Sage et al., 1998; Dore et al., 2014; Craig et al., 2015), while the application of the Eg95 vaccine has been shown to be a promising preventative measure (e.g., Lightowlers et al., 1999; Larrieu et al., 2015).

In conclusion, this is the first study to explore the global pat- terns of genetic diversity and phylogeography of E. granulosus s.s.G1 using near-complete mitogenome sequences. We show that: (i) using a considerably larger G1 dataset than employed previously, E. granulosus s.s. genotypes G1 and G3 are clearly distinct mitochondrial genotypes; (ii) the genetic diversity

within genotype G1 is very high worldwide, with slightly lower values in South America; and (iii) the observed complex phylogeographic patterns emerging from the phylogenetic and geographic analyses suggest that the current distribution of E. granulosus s.s. genotype G1 has been shaped by the intensive animal trade.

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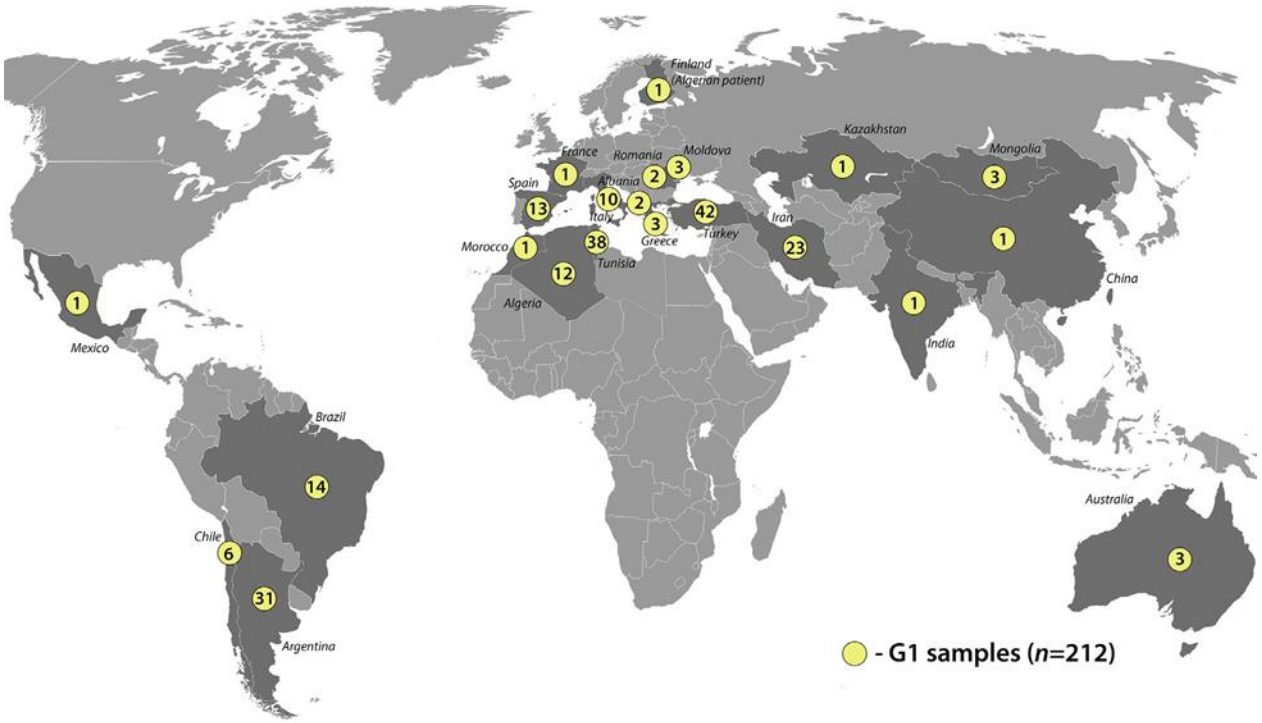

Fig. 1. Geographic locations of *Echinococcus granulosus* sensu stricto genotype G1 samples (*n* = 212) analysed in this study

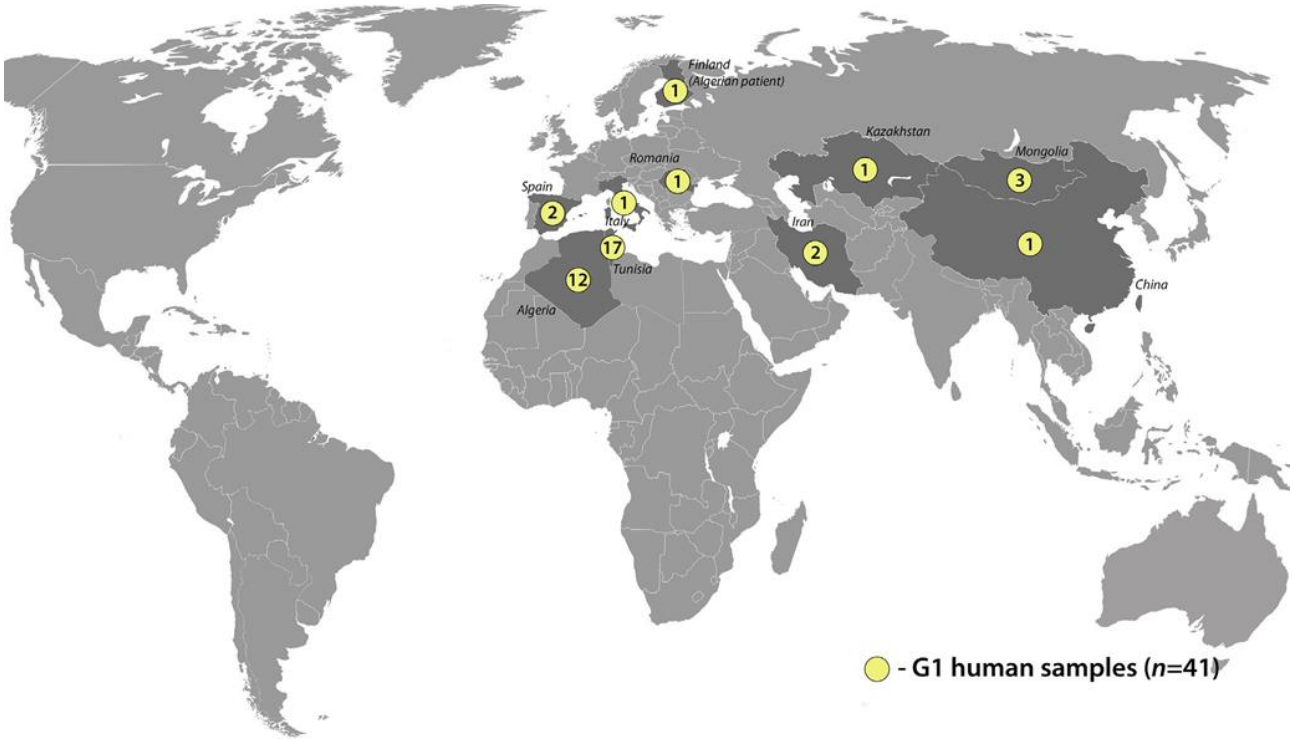

Fig. 2. Geographic locations of Echinococcus granulosus sensu stricto genotype G1 samples from humans (n = 41) used in this study

Table 1

Host data for 212 *Echinococcus granulosus* sensu stricto G1 isolates analysed in this study.

	Origin	Sheep	Cattle	Human	Goat	Swine	Wild boar	Dingo	Buffalo	Total
1.	Turkey	28	14							42
2.	Tunisia	17	4	17						38
3.	Iran	16	3	2	2					23
4.	Argentina	16	14			1				31
5.	Brazil		14							14
6.	Spain	6		2	3	1	1			13
7.	Algeria			12						12
8.	Italy	6	2	1	1					10
9.	Chile		6							6
10.	Australia							3		3
11.	Greece	3								3
12.	Mongolia		3							3
13.	Moldova	2	1							3
14.	Romania	1	1							2
15.	Albania	2								2
16.	Finland (Alg)		1							1
17.	France		1							1
18.	Kazakhstan		1							1
19.	China			1[a]						1
20.	India								1	1
21.	Mexico					1				1
22.	Morocco			1						1
Total		96	61	41	6	3	1	3	1	212

[a] Sequence was obtained from GenBank (AB786664; Nakao et al., 2013).

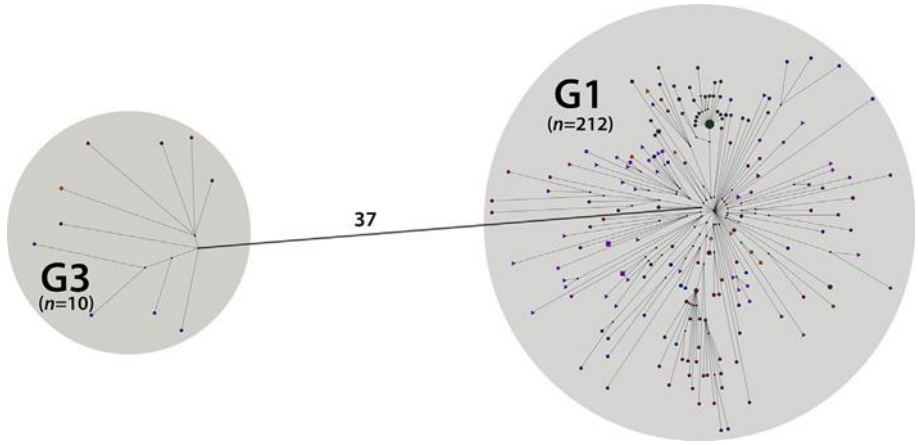

Fig. 3. Phylogenetic network of Echinococcus granulosus sensu stricto samples based on 11,682 bp of mtDNA. Small black circles are median vectors (i.e. hypothetical haplotypes: haplotypes not sampled or extinct). The larger haplogroup (n = 212) corresponds to the mitochondrial genotype G1 and the smaller haplogroup (n = 10) to G3. The small circles and triangles in the haplogroups represent haplotypes. The number on the line connecting the haplogroups indicates the mutational steps between genotypes G1 and G3

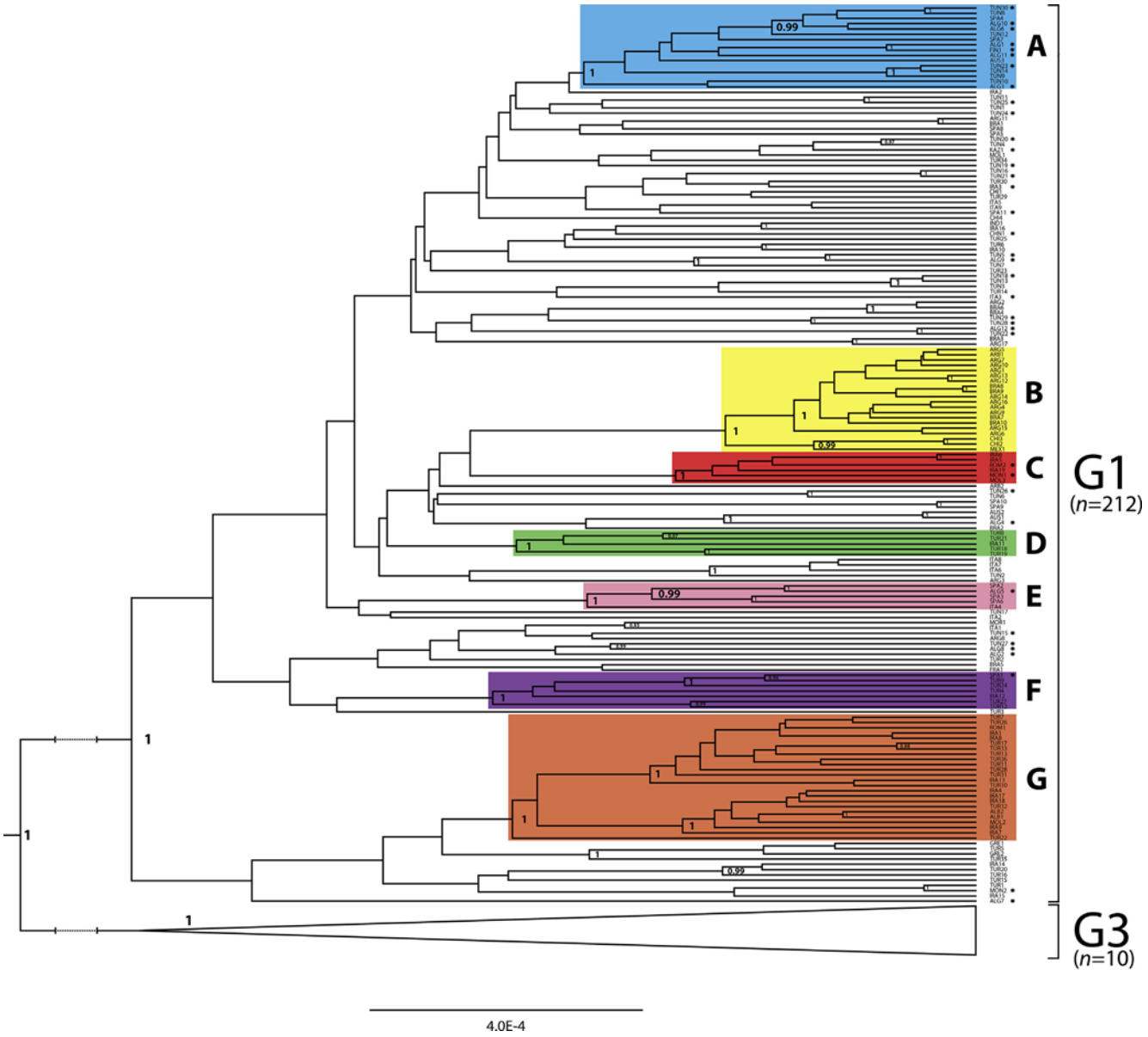

Fig. 4. Bayesian phylogenetic tree inferred from 222 Echinococcus granulosus sensu stricto samples. The larger clade (n = 212) corresponds to the mitochondrial genotype G1 and the smaller (n = 10) to G3. Posterior probability values >0.95 are indicated at the nodes. The asterisks indicate haplotypes obtained from humans. Seven clades labelled A– G, respectively, illustrate clades that received a posterior probability value >0.95 and in which the sample size was equal or higher than 5. Note that the lengths of two branches are reduced (dashed line); for the figure with actual branch lengths, see Supplementary Fig. S1.

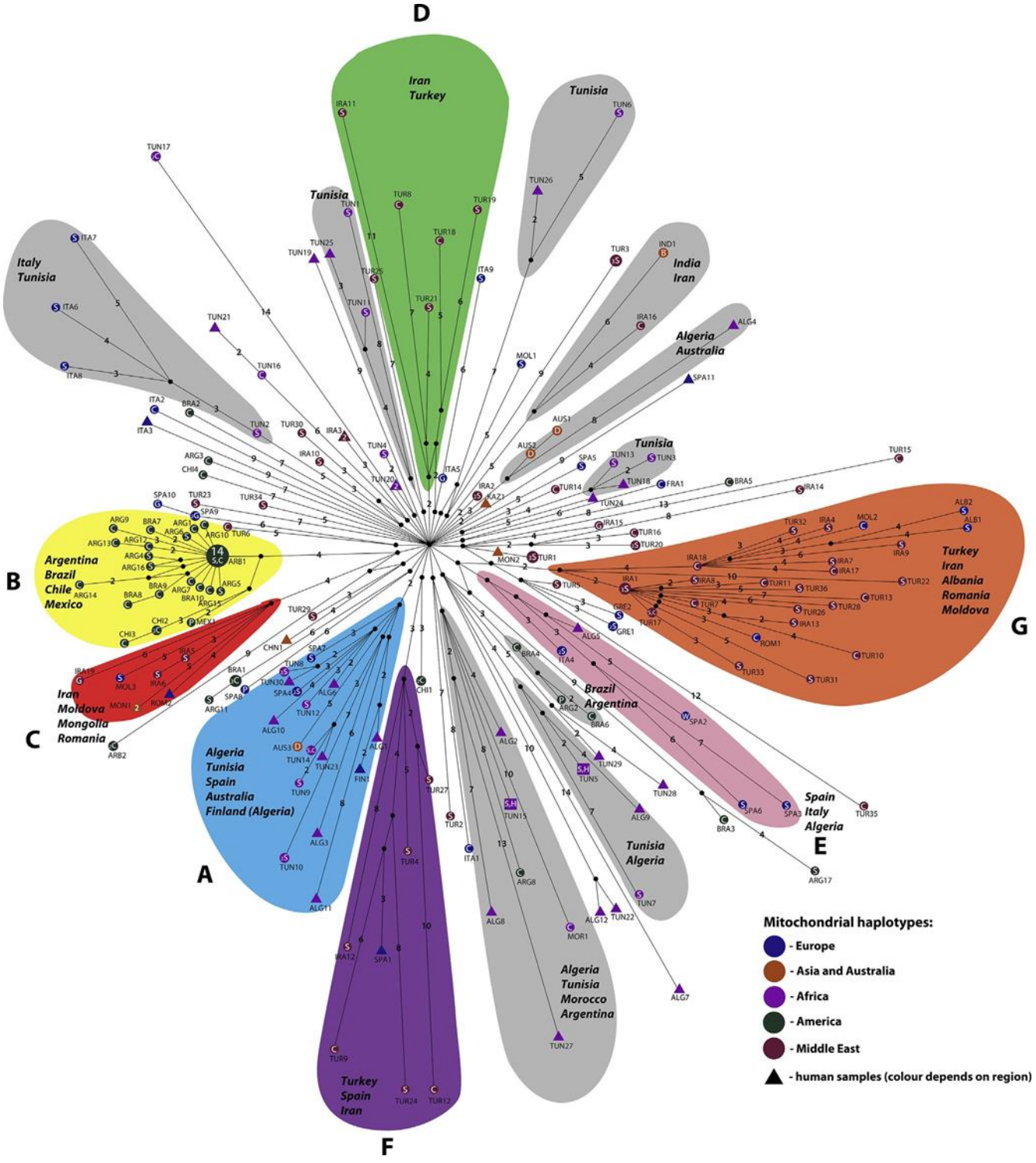

Fig. 5. Phylogenetic network of Echinococcus granulosus sensu stricto G1 samples based on 11,682 bp of mtDNA. Circles represent haplotypes obtained from livestock and wild animals, triangles represent haplotypes of human origin. Haplotype colours (in electronic version) represent different geographical regions: purple – Africa, green – America, orange – Asia and Australia, blue – Europe, dark red – the Middle East. Haplotype names represent their geographical origin (ALB – Albania, ALG – Algeria, ARG – Argentina, AUS – Australia, BRA – Brazil, CHI – Chile, CHN – China, FIN – Finland (patient from Algeria), FRA – France, GRE – Greece, IND – India, IRA – Iran, ITA – Italy, KAZ – Kazakhstan, MEX – Mexico, MOL – Moldova, MON – Mongolia, MOR – Morocco, ROM – Romania, SPA – Spain, TUN – Tunisia, TUR – Turkey), followed by sample ID number. Host species are indicated with letters inside the haplotypes (C – cattle, S – sheep, H – human, P – pig, G – goat, D – dingo, W – wild boar, B – buffalo). The small number inside

haplotypes indicates the frequency of the haplotype. Numbers on the lines represent the number of mutations (single mutations are not marked with a number). Labels A–G correspond to the well-supported clusters in Fig. 4 and Supplementary Fig. S1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

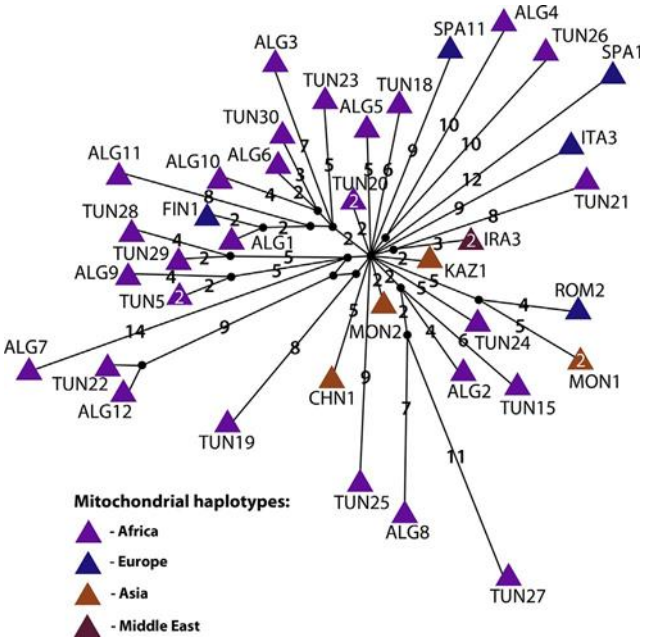

Fig. 6. Phylogenetic network of Echinococcus granulosus sensu stricto G1 human samples based on 11,682 bp of mtDNA. Triangles represent haplotypes. Haplotype colours (in the electronic version) represent different geographical regions: purple – Africa, orange – Asia, – Europe and dark red– the Middle East. Haplotype names represent different geographical origins (ALG – Algeria, CHN – China, FIN – Finland (Algerian patient), IRA – Iran, ITA – Italy, KAZ – Kazakhstan, MON – Mongolia, ROM – Romania, SPA – Spain, TUN – Tunisia), followed by sample ID number. The numbers inside some triangles indicate the frequencies of the haplotypes. Numbers on the lines represent the number of mutations (single mutations are not marked with a number). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Diversity and neutrality indices for *Echinococcus granulosus* sensu stricto G1 samples based on 11 682 bp mtDNA sequences.

	Diversity				Neutrality
	n	Hn Fs	Hd ± S.D.	p ± S.D.	*D*
Total	212 −23.80242[b]	171	0.994 ± 0.002	0.00133 ± 0.00004	−2.77109[a]
Host					
Cattle	61 −24.20117[a]	52	0.992 ± 0.005	0.00138 ± 0.00007	−2.56626[a]
Sheep	96 −24.12005[a]	74	0.987 ± 0.006	0.00128 ± 0.00005	−2.65309[a]
Human	41 −18.96890[a]	37	0.995 ± 0.007	0.00130 ± 0.00008	−2.61502[a]
Region					
Africa	51 −20.46636[a]	43	0.993 ± 0.006	0.00136 ± 0.00007	−2.50107[a]
Asia & Australia	9 −0.73526	8	0.972 ± 0.064	0.00099 ± 0.00014	−1.16779
Europe	35 −12.30737[b]	31	0.993 ± 0.009	0.00136 ± 0.00008	−2.40214[a]
South America	51 −12.19018[b]	33	0.923 ± 0.032	0.00083 ± 0.00010	−2.18228[a]
Middle East	65 −24.21632[a]	55	0.994 ± 0.004	0.00132 ± 0.00007	−2.60935[a]
Country					
Algeria	12 −3.17349[c]	12	1.000 ± 0.034	0.00143 ± 0.00014	−1.98613[b]
Argentina	31 −5.29367[c]	19	0.832 ± 0.070	0.00057 ± 0.00014	−2.38545[a]
Brazil	14 −1.67741	12	0.956 ± 0.045	0.00115 ± 0.00012	−1.31585
Iran	23 −4.14849	19	0.980 ± 0.020	0.00120 ± 0.00011	−2.03201[b]
Italy	10 −0.77495	9	0.978 ± 0.054	0.00126 ± 0.00014	−1.32335
Tunisia	38 −8.60682[c]	30	0.987 ± 0.009	0.00132 ± 0.00008	−2.25318[b]
Turkey	42 −15.01834[a]	36	0.991 ± 0.008	0.00137 ± 0.00009	−2.48392[b]
Spain	13 −0.92526	11	0.974 ± 0.039	0.00124 ± 0.00012	−1.61222

n, number of isolates examined, Hn, number of haplotypes, Hd, haplotype diversity, p, nucleotide diversity, *D*, Tajima's D, Fs, Fu's Fs.
[a] Highly significant *P* value (*P* 0.001).
[b] Highly significant *P* value (*P* < 0.01).
[c] Significant *P* value (*P* < 0.05).

Table 3
Pairwise fixation index (Fst) values between *Echinococcus granulosus* sensu stricto genotype G1 hosts based on 11,682 bp of mtDNA.

	Cattle	Sheep	Human
Cattle	–		
Sheep	0.01171[a]	–	
Human	0.04620[a]	0.02477[a]	–

[a] Significant *P* value ($P < 0.05$).

Table 4
Pairwise fixation index (Fst) values between *Echinococcus granulosus* sensu stricto genotype G1 regions based on 11,682 bp of mtDNA.

	Africa	Asia & Aus	Europe	South America	Middle East
Africa	–				
Asia & Australia	0.02603[a]	–			
Europe	0.02844[a]	0.02243	–		
South America	0.18353[a]	0.21320[a]	0.18837[a]	–	
Middle East	0.06808[a]	0.04671[a]	0.02998[a]	0.20516[a]	–

[a] Significant *P* value ($P < 0.05$).

Table 5
Pairwise fixation index (Fst) values between *Echinococcus granulosus* sensu stricto genotype G1 countries based on 11,682 bp of mtDNA.

	Algeria	Argentina	Brazil	Iran	Italy	Tunisia	Turkey	Spain
Algeria	–							
Argentina	0.32670[a]	–						
Brazil	0.08251[a]	0.12434[a]	–					
Iran	0.08940[a]	0.33548[a]	0.12860[a]	–				
Italy	0.04580[a]	0.35853[a]	0.10146[a]	0.10366[a]	–			
Tunisia	0.00410	0.26940[a]	0.07992[a]	0.08233[a]	0.05166[a]	–		
Turkey	0.06763[a]	0.27984[a]	0.09946[a]	0.01280[a]	0.07387[a]	0.06480[a]	–	
Spain	0.02989[a]	0.34402[a]	0.10144[a]	0.08996[a]	0.06351[a]	0.04593[a]	0.06133[a]	–

[a] Significant *P* value ($P < 0.05$).

Fig. 7. Well-supported diffusion routes inferred from the Bayesian phylogeographic analysis based on 212 Echinococcus granulosus sensu stricto genotype G1 samples (11,682 bp of mtDNA). Narrow black lines represent significant links (BF >10), whereas red outlines (thick black lines) represent highly significant links (BF >100).