International Journal for Parasitology 55 (2025) 569–579

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Zoonotic *Echinococcus granulosus sensu lato* genotypes G6 and G7: new insights from the global mitogenome analysis ☆

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ARTICLE INFO

Article history:
Received 23 October 2024
Received in revised form 3 March 2025
Accepted 11 April 2025
Available online 16 April 2025

Keywords:
Cystic echinococcosis
Echinococcus
Genotype G6
Genotype G7
Mitochondrial genome
Zoonoses

ABSTRACT

Cystic echinococcosis is a severe zoonotic disease caused by different species and genotypes belonging to the *Echinococcus granulosus sensu lato* (*s.l.*) complex. Among these, genotypes G6 and G7 are the second most common cause of human cystic echinococcosis. One of the very first steps towards understanding the epidemiology of G6 and G7 is to study their genetic and host diversity, population structure and phylogenetic relationships. For this, we sequenced near-complete mitochondrial genomes (12,850–12,856 bp) of 72 new G6 and G7 samples from eight countries and six host species, including humans. By adding 103 sequences from previous studies, the total dataset for further analyses comprised of 175 sequences from 20 countries and seven host species. This is the most comprehensive global mitogenome study of *Echinococcus granulosus s.l.* G6 and G7 to date. The results of this work revealed: (i) a new divergent haplogroup G6b from Mongolia; (ii) the subdivision of genotype G6 into two major haplogroups: G6a (the nominal haplogroup) and G6b (the Mongolian haplogroup); (iii) highly divergent haplotypes of G6 and G7; (iv) the first molecularly confirmed findings of genotype G7 in camel; (v) genotype G7 in sheep – a rare species for G7; (vi) the importance of using long DNA sequences in phylogenetic analysis.

1. Introduction

The tapeworm parasites belonging to the *Echinococcus granulosus sensu lato* (*s.l.*) species complex are the etiological agents of the life-threatening disease cystic echinococcosis (CE) (McManus et al., 2003; Casulli et al., 2023). These parasites have a worldwide geographical distribution, with the ability to infect wild and domestic animals, as well as humans (Deplazes et al., 2017; Romig et al.,

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank database under accession numbers PQ420728-PQ420799.

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https://doi.org/10.1016/j.ijpara.2025.04.014
0020-7519/

2017). CE is a major public health concern, particularly in areas where pastoralism is widely practised and free-roaming dogs are present. It has been estimated that approximately 207,000 new cases of CE occur globally each year (Yang et al., 2024), with 2,000 deaths/year and an estimated 184,000 disability-adjusted life-years (DALYs) each year being attributed to the disease (Torgerson et al., 2015). The economic damage caused by these parasites has been estimated US $3 billion annually due to the costs related to the treatment of human CE cases and loss of livestock (WHO, 2020; https://www.who.int/news-room/fact-sheets/detail/echinococcosis). Despite the considerable impact on public health, CE continues to be neglected and underdiagnosed in many endemic regions and controlling this disease has remained challenging. The World Health Organization (WHO) considers this disease among Neglected Tropical Diseases (NTDs) and seeks to have it under control by 2030 (WHO, 2020).

Echinococcus granulosus s.l. life cycle requires two different mammalian hosts: an intermediate host, primarily domesticated and wild ungulates, and a definitive host, primarily dogs, but also wild canids (Romig et al., 2017; Thompson, 2017). In the intermediate host, the parasite forms fluid-filled cysts, mainly in the liver and lungs, sometimes also in other organs or body parts such as the central nervous system, heart, kidney, spleen, muscles, etc. (McManus et al., 2003; Jenkins et al., 2005). In humans, cysts can cause significant health problems and, in severe cases, can even lead to death (Eckert et al., 2001). In the animal definitive host, the infection is asymptomatic. The worm attaches itself to the small intestine of the host, where it matures and reproduces; the parasite's eggs are released into the environment with the excrement of the host (Thompson, 2013). Humans can become accidental dead-end intermediate hosts for this parasite through the consumption of water or food that is contaminated with parasite eggs or direct contact with infected dogs (Torgerson et al., 2020).

Echinococcus granulosus s.l. is known to be genetically diverse and based on mitochondrial DNA (mtDNA) sequence data, this species complex has been divided into unique genotypes G1, G3–G8, G10, and *Echinococcus felidis* (Bowles et al., 1992; Bowles and McManus, 1994; Scott et al., 1997; Lavikainen et al., 2003; Hüttner et al., 2008; Kinkar et al., 2017). In addition, a unique and highly divergent genotype (Gomo) reported by Wassermann et al. (2016) further showcases the high genetic diversity of this species complex. Genotypes of *E. granulosus s.l.* may differ from each other in terms of host ranges, geographical distribution, pathogenicity and infectivity (Alvarez Rojas et al., 2014; Romig et al., 2015, 2017; Debiaggi et al., 2023). On the grounds of these differences, several of these genotypes have now been recognised as distinct species*: E. granulosus sensu stricto* (*s.s.*) (G1 and G3; Kinkar et al., 2017), *Echinococcus equinus* (G4), *Echinococcus ortleppi* (G5) (Thompson and McManus, 2002; Lymbery, 2017). The phylogeny and species status of genotypes G6-G8 and G10 continue to be debated today, with arguments being made for one (Nakao et al., 2006), two (Saarma et al., 2009; Laurimäe et al., 2018a) or three separate species (Lymbery et al., 2015). G6-G8/G10 may be currently referred to as genotypes of the *Echinococcus canadensis* cluster (Vuitton et al., 2020). However, until the taxonomic status of G6/G7 is resolved, we have opted to refer to G6/G7 as genotypes of the *E. granulosus s.l.* species complex.

The life cycle of genotypes G6 and G7 involves primarily livestock. The main intermediate hosts of genotype G6 are camels and goats, and it is mainly found in camel-rearing regions in northern Africa, the Middle East and Asia, and goat-rearing regions in South America (Cucher et al., 2016; Deplazes et al., 2017). The primary intermediate host of genotype G7 is the domestic pig, and is therefore most common in pig-rearing regions of Europe, Asia and Central America (Borhani et al., 2020; Casulli et al., 2022; Hua et al., 2022). The definitive host of both genotypes is usually the dog (Romig et al., 2017). While the life cycle of genotypes G6/G7 is predominantly tied to domestic animals, wild animals could act as a reservoir for this disease (Romig and Wassermann, 2024). These genotypes have been, among intermediate hosts, most often found in the wild boar (*Sus scrofa*) (e.g., Onac et al., 2013; Umhang et al., 2014; Laurimäe et al., 2019; Sgroi et al., 2019), and of definitive hosts in the grey wolf (*Canis lupus*) (e.g., Guerra et al., 2013; Ito et al., 2013; Kilinc et al., 2023). Genotypes G6/G7 are zoonotic and known to infect humans, posing a significant risk to human health. The most common cause of human infections in the world is *Echinococcus granulosus s.s.* (G1 and G3), with the second most common being genotypes G6/G7 (Alvarez Rojas et al., 2014). In Europe, G6/G7 infections have been estimated to be more numerous than previously thought, with 21.6% of European cases being caused by these genotypes (Casulli et al., 2022).

The genetic diversity and phylogeography of these parasites have been primarily studied using rather short sequences such as mitochondrial gene fragments and complete gene sequences. These have provided valuable insights into the genetic diversity, population structure and phylogeography of *E. granulosus s.l.* However, short sequences set certain limitations – while helpful for the identification of some genotypes (G4, G5, G8, G10), they are not always reliable in distinguishing closely related genotypes such as G1 and G3 (Kinkar et al., 2017, 2018a) or G6 and G7 (Laurimäe et al., 2018b, 2019). Yet, accurate genotype identification of these highly zoonotic taxa is crucial for helping to design control measures for CE, as they allow for accurate assessment of the epidemiological situation such as the host range, geographical spread and infectivity of the genotypes involved. Moreover, short sequences have limited analytical power. For example, phylogenetic networks based on short sequences often produce 'star-like' patterns, with most samples clustering into a few central haplotypes, making it difficult to analyse their actual genetic diversity and phylogenetic subdivision. The analysis of near-complete (>8,000 bp) or complete mitochondrial genome sequences (>13,000 bp) has helped to overcome these problems (Laurimäe et al., 2016, 2018b, 2023; Kinkar et al., 2017, 2018c; Ohiolei et al., 2020; Saarma et al., 2023; Guo et al., 2024). Although for genotypes G6 and G7 the mitogenomic data remain scarce (Laurimäe et al., 2018b; Ohiolei et al., 2020), these studies have already provided a significantly improved phylogenetic resolution, leading to a better understanding of the genetic diversity, phylogeny and phylogeographic patterns, which remained elusive with short sequences. For example, in the analysis of complete mitochondrial genomes, Laurimäe et al. (2018b) found that genotype G7 is divided into two distinct haplogroups, G7a and G7b. However, when using only the commonly applied *cox1* gene fragment (366 bp), some of the members of haplogroup G7b were erroneously identified as part of genotype G6. Even complete *cox1* (1,608 bp) sequences could not distinguish haplogroups G7a and G7b. The low diagnostic power of *cox1*/*nad1* genes created a need for a new set of genotype defining markers for accurate genotyping. A combination of *nad2* (714 bp) and *nad5* (680 bp) has been proposed as a reliable tool for the identification of genotypes G6 and G7 (Laurimäe et al., 2019), whereas *nad5* (680 bp) alone is sufficient for distinguishing G1 and G3 (Kinkar et al., 2018a).

With the use of longer mitochondrial sequences, it has become clear that the genetic diversity of genotypes G6 and G7 is higher than previously shown (Laurimäe et al., 2018b). Nevertheless, geographically broader sampling is necessary to further assess the genetic diversity, phylogeny and phylogeography of G6/G7. Of special interest is Mongolia, where a highly divergent haplotype Gmon was found previously by Laurimäe et al. (2018b). However, due to the limited sampling, it was not possible to say whether this haplotype belongs to a separate haplogroup or is an outlier. The aim of this study was to analyse, based on near-complete mitogenomes,

the genetic diversity and phylogenetic relations of G6/G7 samples at a wide geographical scale and from different host species, including humans.

2. Materials and methods

2.1. Parasite material

In this study, a total of 72 new *E. granulosus s.l.* G6/G7 isolates were sequenced. These samples originated from eight countries worldwide: Argentina (n = 32), Mongolia (n = 19), Poland (n = 3), Iran (n = 5), Türkiye (n = 2), France (n = 9), Moldova (n = 1) and Pakistan (n = 1) (Fig. 1; Supplementary Table S1). Samples were from six different intermediate hosts: pig (n = 32), goat (n = 5), cattle (n = 3), camel (n = 18), human (n = 13), sheep (n = 1). For the data analysis, an additional 103 sequences of G6/G7 from the GenBank database were included (Supplementary Table S1). Of these, 94 sequences were from Laurimäe et al. (2018b) with samples from Argentina (n = 19), Mexico (n = 10), Sudan (n = 14), Kenya (n = 3), Mauritania (n = 2), Iran (n = 4), Mongolia (n = 1), Spain (n = 1), France (n = 27), Italy (n = 2), Serbia (n = 1), Romania (n = 2), Ukraine (n = 2), Poland (n = 5), and Lithuania (n = 1). The rest of the samples were from Ohiolei et al. (2019) with samples from China (n = 2), Ohiolei et al. (2020) from Nigeria (n = 5) and Nakao et al. (2006) from Poland (n = 1) and Kazakhstan (n = 1).

2.2. DNA extraction, PCR amplification and sequencing of the mitochondrial genome

DNA was extracted from protoscoleces or cyst membranes using High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany), following the manufacturer's protocols. PCR amplification was carried out using the set of 13 primer pairs described in Laurimäe et al. (2018b) to amplify the entire mitochondrial genome. The PCR was carried out in a 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, 1 U Advantage 2 Polymerase mix (BD Biosciences) and <1 µg of template DNA. Touchdown PCR was carried out as described in Laurimäe et al. (2018b): 95 °C for 1 min, followed by 10 cycles of 95 °C for 20 s, 55 °C for 45 s (with annealing temperature reduced in each cycle by –0.5 °C) 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 finished with a final elongation step at 68 °C for 3 min. Of the PCR products, 10 µL were examined on a 1% agarose gel electrophoresis. The remaining 10 µL of the PCR products were purified using 1 U FastAP thermosensitive alkaline phosphatase and 1 U exonuclease I (both from Thermo Scientific, Waltham, USA). The mixture was incubated at 37 °C for 30 min, followed by 80 °C for 15 min to inactivate the enzymes. Sequencing was performed at the Core Facility of Genomics (Institute of Genomics,

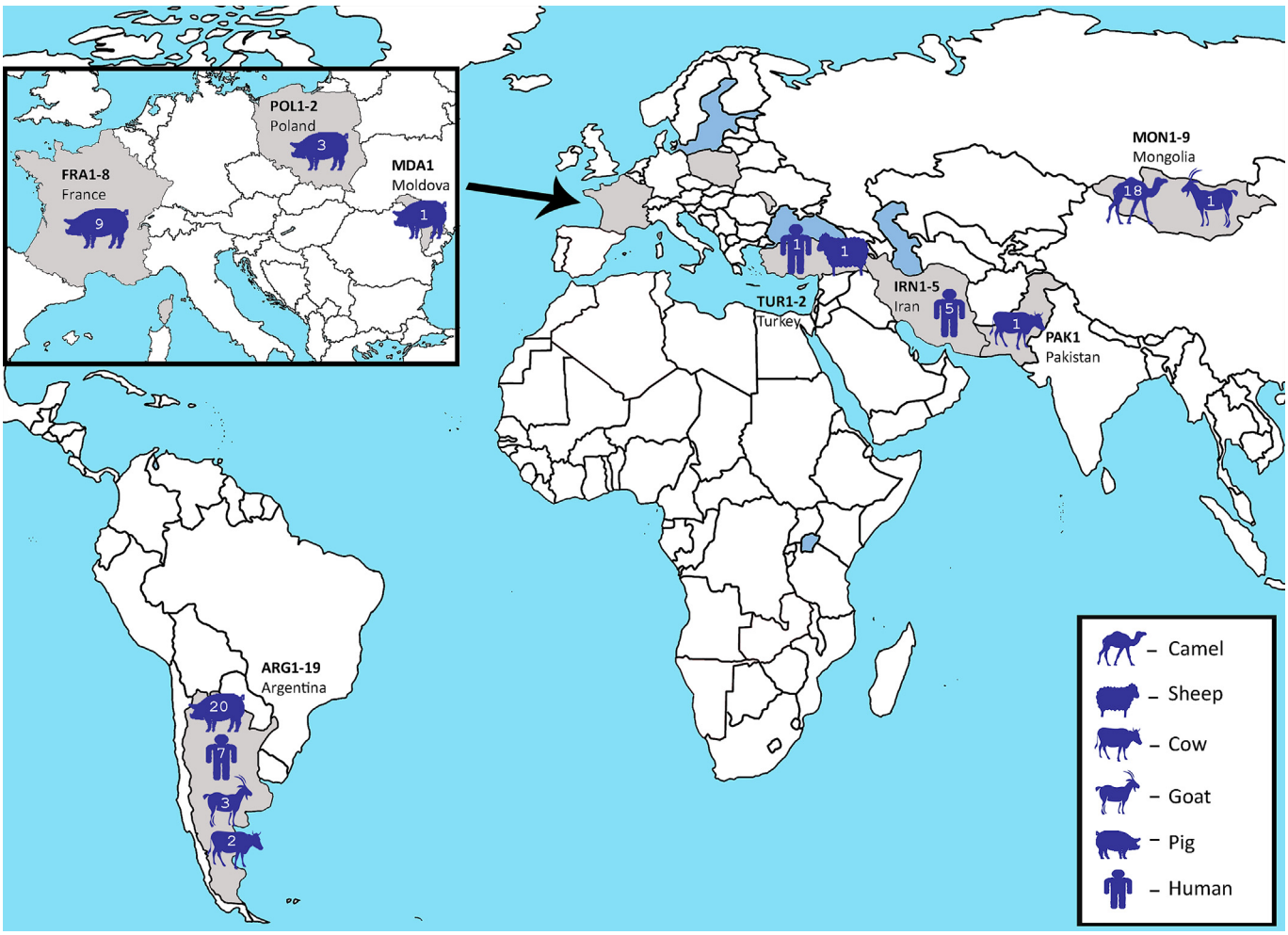

Fig. 1. Geographic locations, intermediate hosts, number of samples and haplotype names for the isolates of *Echinococcus granulosus sensu lato* (genotypes G6 and G7) sequenced in this study. Haplotype names are according to the country ISO 3166 standard codes.

University of Tartu, Estonia) using Sanger sequencing of both forward and reverse strands. The same set of primers as for the initial PCR and an additional primer S11f (GGTGCTAATTTTAGGTTTGTATAGACA) were used for the sequencing.

2.3. Sequence assembly and quality control

Consensus sequences were assembled in Codon Code Aligner v.11.0.1 and each polymorphic position was verified manually by viewing the chromatogram data. Sequences were aligned in Bioedit v.7.7.1 (Hall, 1999), using Clustal W (Thompson et al., 1994). For initial species and/or genotype identification, the samples were aligned with sequences of different genotypes from GenBank.

2.4. Phylogenetic network and Bayesian phylogeny

Median-joining networks were calculated using program Network v.10.2.0.0 (Bandelt et al., 1999; https://www.fluxus-engineering.com, Fluxus Technology Ltd., 2004), with both indels and point mutations considered. The best-fit nucleotide substitution model for the dataset was determined using PartitionFinder v.2.1.1 (Lanfear et al., 2012, 2017). The Bayesian phylogeny for the dataset was found using the program BEAST v1.10.4 (Drummond et al., 2012). The posterior distribution of parameters was assessed using Markov Chain Monte Carlo (MCMC) sampling with a chain length of 50,000,000 states, and 10% was discarded as burn-in. Parameters were logged every 1000 states, and the resultant log files were assessed for parameter behaviour in Tracer v1.7.1. Phylogenetic trees were summarised and annotated in TreeAnnotator v.1.8.4, and the program FigTree v.1.4.4 was used to display the tree (https://tree.bio.ed.ac.uk/software/figtree).

2.5. Population indices

The programme DnaSP v.6.12.03 (Librado and Rozas, 2009; Rozas et al., 2017) was used to calculate population diversity indices (number of haplotypes, haplotype diversity and nucleotide diversity) for the near-complete sequences. Neutrality indices Tajima's D and Fu's Fs, and the pairwise fixation index were estimated using the Arlequin software package (Tajima, 1989; Fu, 1997; Excoffier et al., 2007). The Tajima's D and Fu's Fs test whether the observed pattern of polymorphisms is consistent with a neutral model of evolution, allowing estimation of rapid population expansions and bottlenecks (Tajima, 1989; Fu, 1997). Tajima's D preforms better under the assumption that there is unknown recombination rate in the dataset, whereas Fu's Fs performs best for non-recombining regions of the genome (Ramírez-Soriano et al., 2008). Mitochondrial genomes are widely accepted as non-recombinant in most animals, but rare cases of recombination have been reported (e.g., Rokas et al., 2003). Therefore, both indices were used in this work. The pairwise fixation index, on the other hand, is a measure of population differentiation due to genetic structure, allowing for deeper insight into the differentiation of genotypes and haplogroups.

The indices were calculated separately for genotypes G6 and G7, haplogroups G7a, G7b and G6b (Mongolia), and for countries represented by more than five samples. Fst values were similarly calculated between genotypes (G6/G7), haplogroups (G7a/G7b/G6b) and countries with more than five samples.

3. Results

The final length of the near-complete mitochondrial genomes sequenced in this study was between 12,850 and 12,856 bp. Of the 72 samples, 39 corresponded to the genotype G7, and 33 to G6. Together with an expanded dataset, that also included previously published complete mitochondrial genome sequences of genotypes G6 and G7 (Nakao et al., 2006; Laurimäe et al., 2018b; Ohiolei et al., 2019, 2020), the total number of sequences used in further analyses was 175. In this expanded dataset, 68 samples belonged to G6 and 107 to G7.

3.1. A new haplogroup, G6b, found in Mongolia

The genotype G6 was formed of 45 haplotypes, which were divided into two separate haplogroups, G6a and G6b (Fig. 2). G6a included 36 haplotypes, 13 of which were from this work, and G6b with 9 haplotypes, of which eight were from this work. In haplogroup G6a, geographically close samples generally clustered together: e.g., Argentinian samples (ARG4, ARG9, ARG15 and others) formed a monophyletic cluster. However, there are also examples of geographically distant samples clustering together, such as Iran (IRN7), which is separated by a single mutation from a sample from Nigeria (NGA1). It is worth mentioning that two haplotypes of Iranian origin (IRN1 and IRN3) and one from Kazakhstan (KAZ1) are highly diverged from the main G6a.

One of the main results of this study was the discovery of a distinct haplogroup G6b, comprising of samples only from Mongolia. On the phylogenetic network (Fig. 2), G6b was clearly separate from both the nominal G6 (referred to as G6a in this study) and the G7. Closest haplotypes of G6a and G6b are separated by 25 mutations (NGA1 and MON6), whereas the closest of G6b and G7 are separated by 31 mutations (MON6 and MON4). The genetic distance between the samples of G6b varied significantly and the most distant were separated by 35 mutations (MON1/MON3 from MON2).

Genotype G7 was represented by 55 haplotypes, which were divided into two distinct haplogroups, G7a and G7b (Fig. 2). G7a was represented by 48 haplotypes, 15 of which were from this study and three that were a combination of both this study and others. G7b was represented by seven haplotypes, three of which were from this study and one in a combination. The distance between the two haplogroups was a minimum of 20 mutations (ITA1 and MON4). Geographically close samples were generally also genetically close, but not always. For example, the Argentinian samples were divided into two distinct monophyletic clusters (ARG10 and three others; ARG5 and six others). Similarly, the French samples were divided between G7a (FRA3 and others) and G7b (FRA7 and others). Note that several haplotypes of G7a (e.g., TUR1, TUR2, IRN4, MON4, MEX9, POL7) are relatively highly diverged.

3.2. Bayesian phylogenetic analysis provides further support for the clustering of G6/G7

The best-fit nucleotide substitution model calculated with the PartionFinder was TRN + I + G. The Bayesian phylogenetic analysis, integrating this model, divided the 175 samples into two major clades, corresponding to genotypes G6 and G7 (Fig. 3). These were further divided into four large clades, corresponding to haplogroups G6a, G6b, G7a, G7b. All these clades were highly supported (posterior probability PP = 1.00), except for G7a, where the highly divergent haplotypes from Türkiye (TUR1, TUR2), Mongolia (MON4) and Iran (IRN4) received lower support. In genotype G6, both haplogroups G6a and G6b were well supported, as well as the subclades from Argentina (ARG4 and others), Kenya (KEN1-KEN3) and Iran/Pakistan (IRN6, IRN7, PAK1). The distinct Iranian cluster (IRN1 and IRN3) and a haplotype from Kazakhstan (KAZ1) positioned as sister clades to the rest of G6a.

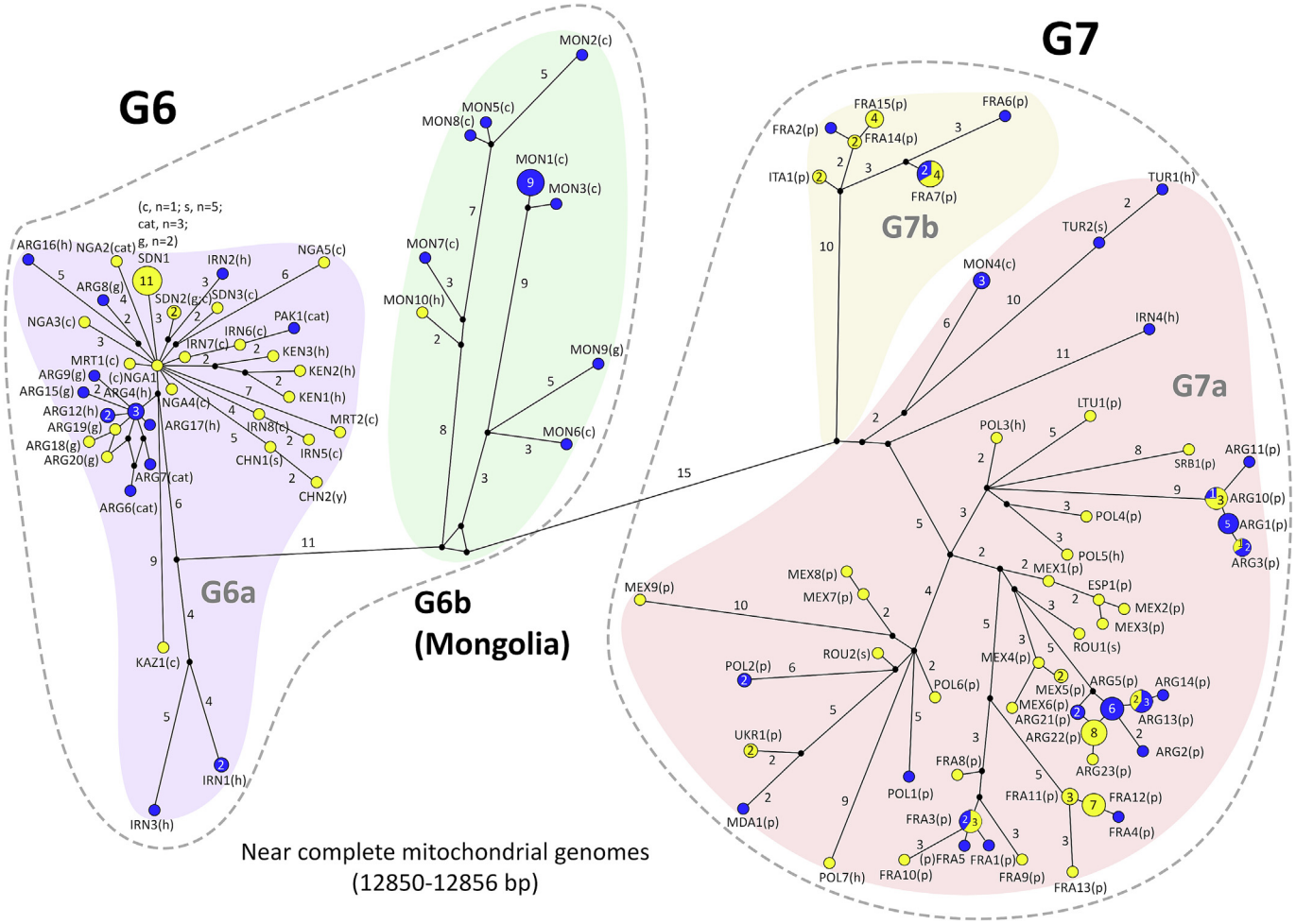

Fig. 2. Median-joining network based on near-complete mtDNA genome sequences of *Echinococcus granulosus sensu lato* isolates, belonging to genotypes G6 and G7 (n = 175; 12,850–12,856 bp). Dark blue in circles represents samples sequenced in this work and yellow are sequences from GenBank. Intermediate host species: h – human, g – goat, c – camel, cat – cattle, p – pig, s – sheep, y – yak. Numbers in the circles represent the number of samples in the haplotype, and the numbers next to the line represent the number of mutational steps. Haplogroups: G6a is depicted in purple, the Mongolian haplogroup G6b in green, G7a in red and G7b in orange. For interpretation of colours, the reader is referred to the online version of this article.

3.3. Differences in genetic diversity among haplogroups

The haplotype diversity Hd for genotype G6 was 0.956, and the nucleotide diversity π was 0.00138 (Table 1). Haplogroup G6a was also characterised by a relatively high haplotype diversity (Hd = 0.952). In contrast, haplogroup G6b had a significantly lower Hd (0.735). For genotype G7, the haplotype and nucleotide diversities were slightly higher compared with G6 (Hd = 0.975; π = 0.00155). Haplogroup G7a had also high haplotype diversity (Hd = 0.972), whereas for haplogroup G7b it was somewhat smaller (Hd = 0.808). Among countries, genotype G6 had the highest haplotype diversity in Nigeria (Hd = 1.00) and the lowest in Sudan (Hd = 0.385). For genotype G7, the highest haplotype diversity was found for samples from Mexico (Hd = 0.978) and the lowest for Argentina (Hd = 0.887).

3.4. Rapid radiation of haplogroups G6a and G7a

Genotype G6 had statistically significant ($P \leq 0.05$) Tajima's D and Fu's Fs values of −1.445 and −11.162, respectively, suggesting rapid radiation (Table 1). While haplogroup G6a had both statistically highly significant ($P \leq 0.0001$) Tajima's D and Fu's Fs values (−2.386 and −20.820, respectively), haplogroup G6b, on the other hand, had no statistically significant neutrality indices. This suggests that the radiation has not been a rapid process. Genotype G7 and haplogroup G7a had statistically significant Tajima's D values (−1.495 and −1.693, respectively). For haplogroup G7b, the neutrality indices were statistically not significant. Among the countries, only genotype G6 from Argentina had statistically significant neutrality indices (Tajima's D = −1.788; Fu's Fs = −6.223).

3.5. Relatively high genetic differentiation of genotypes and haplogroups

The pairwise fixation index between genotypes G6 and G7 (Fst = 0.565) was statistically highly significant ($P \leq 0.00001$) (Supplementary Table S2). The Fst values between the majority of haplogroups was also statistically significant ($P \leq 0.01$), ranging from 0.515 for G7a and G7b, up to 0.833 for G6a and G7b. The Fst values were also high between G6 from Mongolia and other countries, ranging from 0.586 to 0.742, suggesting relatively ancient separation of G6 from the others (Supplementary Table S4).

3.6. Comparison of shorter and longer sequences revealed differences in genotype identification and phylogenetic clustering

To compare the phylogenetic power of datasets with different sequence lengths, five different phylogenetic networks were con-

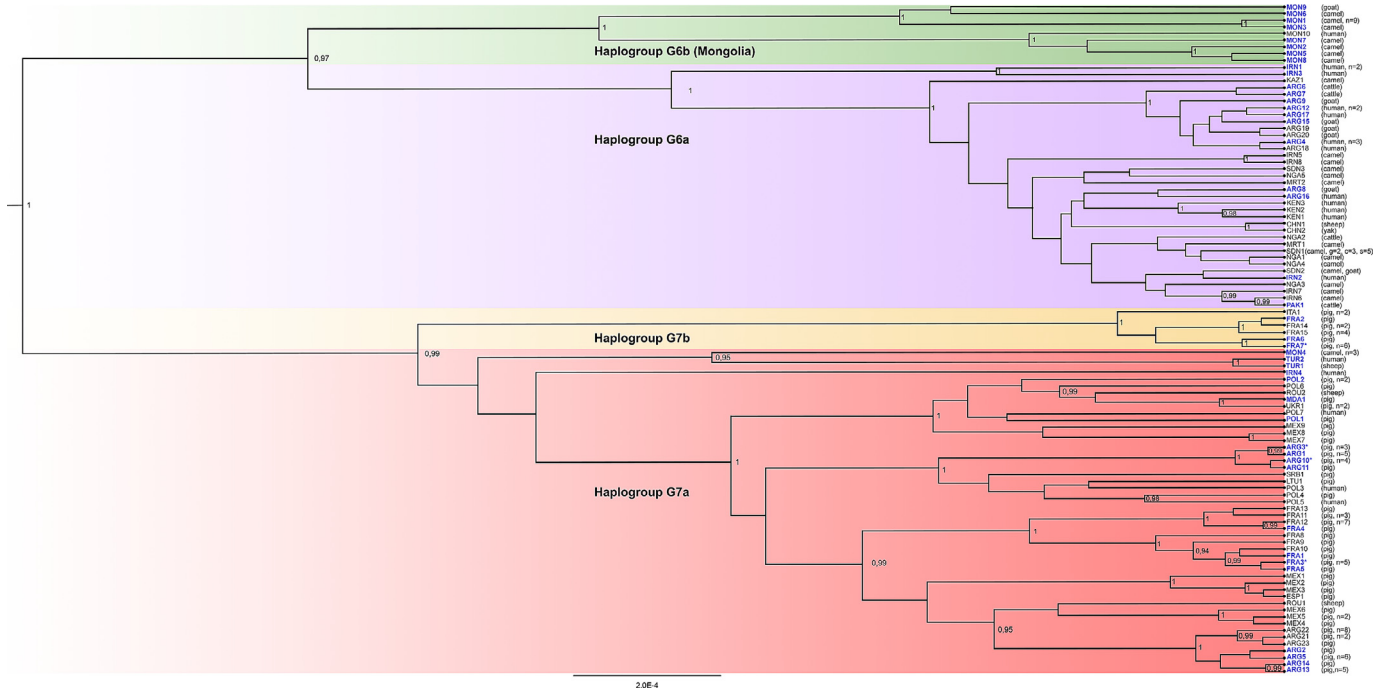

Fig. 3. Bayesian phylogenetic tree inferred from near-complete mitogenome sequences (n = 175; 12,850–12,856 bp) of *Echinococcus granulosus sensu lato* isolates, belonging to genotypes G6 and G7. Haplogroups: G6a is depicted in purple, the Mongolian haplogroup G6b in green, G7a in red and G7b in orange. The samples sequenced in this study are marked in blue. Posterior probability values over 0.90 are depicted at the nodes. For interpretation of colours, the reader is referred to the online version of this article.

Table 1
Diversity and neutrality indices for *Echinococcus granulosus sensu lato* genotypes G6 and G7, and their haplogroups, based on near-complete mitochondrial genomes (12,850–12,856 bp) and 175 samples.

	Diversity				Neutrality	
	n	Hn	Hd ± SD	π ± SD	Tajima's D	Fu's Fs
Genotype						
G6	68	45	0.956 ± 0.015	0.00138 ± 0.00012	**−1.445**[a]	**−11.162**[a]
G7	107	54	0.975 ± 0.005	0.00155 ± 0.00005	**−1.495**[a]	−7.940
G6 haplogroups						
G6b	17	9	0.735 ± 0.117	0.00106 ± 0.00018	0.491	2.945
G6a	51	36	0.952 ± 0.023	0.00060 ± 0.00007	**−2.386**[c]	**−20.820**[c]
G7 haplogroups						
G7a	91	48	0.972 ± 0.007	0.00126 ± 0.00004	**−1.693**[a]	−8.334
G7b	16	6	0.808 ± 0.069	0.00027 ± 0.00002	1.020	1.867
G7 countries						
Argentina	36	11	0.887 ± 0.024	0.00082 ± 0.00009	1.958	4.308
France (Corsica)	36	15	0.911 ± 0.024	0.00137 ± 0.00007	1.791	3.975
Mexico	10	9	0.978 ± 0.054	0.00086 ± 0.00015	−0.229	−1.383
Poland	9	8	0.972 ± 0.064	0.00113 ± 0.00013	−0.982	−0.266
G6 countries						
Argentina	15	11	0.933 ± 0.054	0.00027 ± 0.00007	**−1.788**[a]	**−6.223**[b]
Iran	8	7	0.964 ± 0.077	0.00099 ± 0.00015	0.176	0.026
Nigeria	5	5	1.000 ± 0.126	0.00047 ± 0.00013	**−1.219**[a]	−1.061
Sudan	14	3	0.385 ± 0.149	0.00016 ± 0.00006	−0.707	2.650

n, number of samples; Hn, number of haplotypes; Hd, haplotype diversity; π, nucleotide diversity.
[a] Significant *P*-value ($P \leq 0.05$).
[b] Significant *P*-value ($P \leq 0.01$).
[c] Highly significant *P*-value ($P \leq 0.0001$).

structed: (a) near-complete mitogenomic sequences (12,850–12,856 bp; Fig. 2); (b) complete *cox1* gene (1,608 bp; Fig. 4); (c) *cox1* gene fragment (366 bp; Fig. 5); (d) complete *nad1* gene (894 bp; Supplementary Fig. S1), and (e) *nad1* gene fragment (471 bp; Supplementary Fig. S2).

Based on the near-complete mitogenomic network, haplogroups G6a, G6b, G7a, and G7b are represented as distinct clusters (Fig. 2). By contrast, using the *cox1* gene sequences, there was no clear distinction between genotypes G6 and G7, nor their haplogroups (Fig. 4). In the case of G6b, seven of the 16 samples were grouped together with the other samples in the central haplotype of the G6. In the network based on the *cox1* fragment (366 bp; Fig. 5), the divergence was very low: majority of G6 samples clustered in a single haplotype and the same was evident for G7 samples. Only a few additional haplotypes were found. Note that all G7b samples clustered into the central G6 haplotype, as found also by Laurimäe et al. (2018a,b).

The phylogenetic network based on the *nad1* gene (894 bp) yielded distinguishable genotypes, with three mutations separating G6 and G7 (Supplementary Fig. S1). Moreover, there was

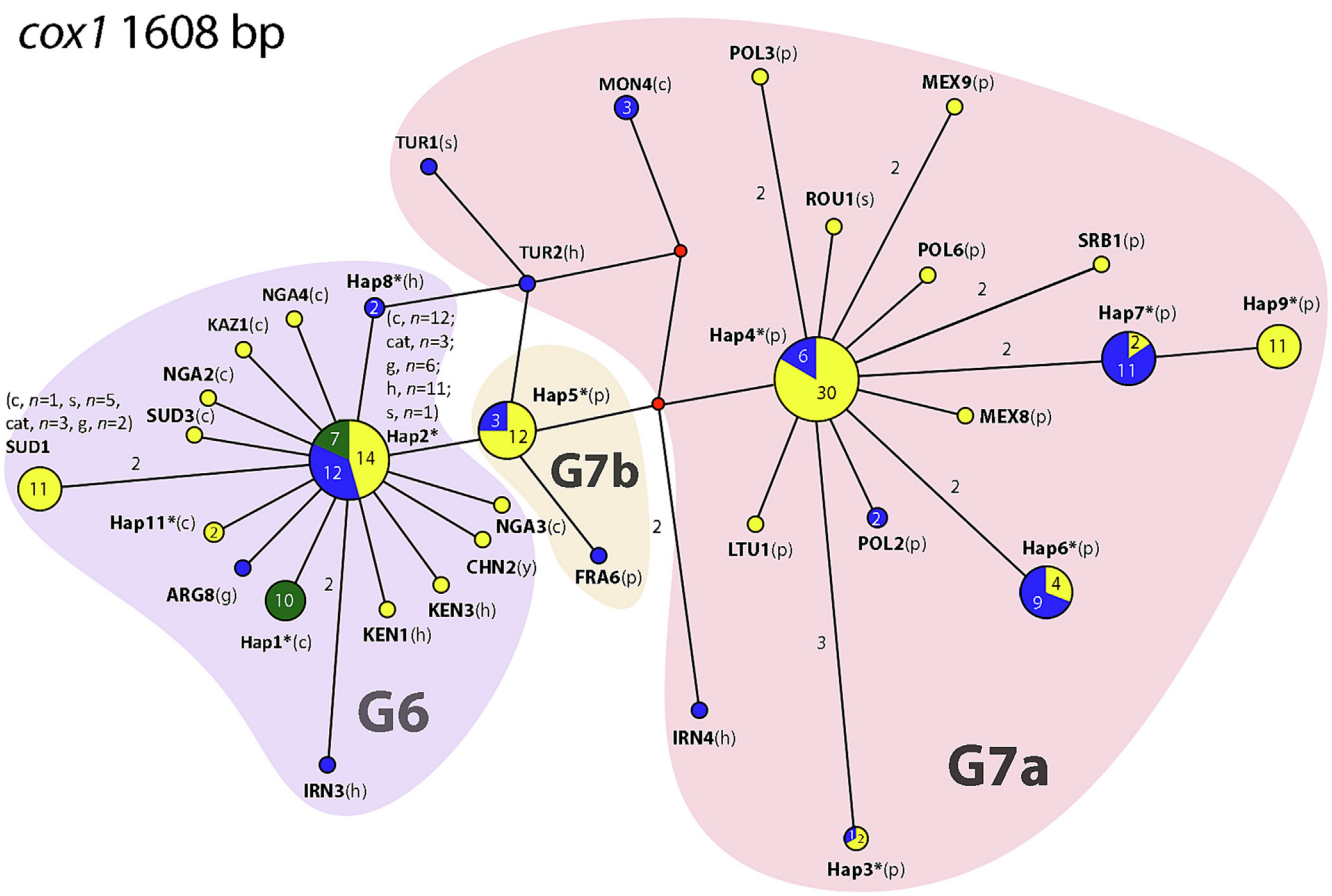

Fig. 4. Median-joining network of *Echinococcus granulosus sensu lato* isolates (n = 175) of genotypes G6 and G7, based on full-length cytochrome *c* oxidase subunit 1 sequences (*cox1*, 1608 bp). Dark blue in circles represents samples sequenced in this work, yellow in circles represents sequences from GenBank, and green circles represent samples of haplogroup G6b. Letters in parentheses next to the haplotype names represent the intermediate hosts: c – camel, cat – cattle, h – human, g – goat, s – sheep, p – pig, y – yak. Numbers inside the circles depict the number of samples that belong to the haplotype, and the numbers near the lines represent the number of mutational steps. Haplotypes marked with an asterisk are formed of haplotypes that were separated in the near-complete mitogenome network (Fig. 2). For interpretation of colours, the reader is referred to the online version of this article.

separation between G6a and G6b, albeit defined only by a single nucleotide difference. On the phylogenetic network based on the *nad1* fragment (471 bp; Supplementary Fig. S2), genotypes G6 and G7 were distinguishable, but not G6a and G6b.

4. Discussion

One of the most noteworthy of the findings in this work is the discovery of a distinct haplogroup from Mongolia (G6b). The first mitogenome sequence of this haplogroup was published by Laurimäe et al. (2018b). However, as in their work only a single sample was analysed, it was difficult to determine if this was a single highly diverged haplotype or potentially a member of a bigger haplogroup. The analysis of this work revealed the existence of a highly divergent haplogroup, comprising samples from goats (MON9), camels (MON1-3/5-8) and humans (MON10). Although on the phylogenetic network the Mongolian haplogroup G6b positioned between G6 and G7, it was genetically closer to G6a (25 mutational steps) than to G7 (31 steps) (Fig. 2). The Bayesian phylogenetic analysis also confirmed that the Mongolian haplogroup was part of the genotype G6 (named therefore G6b). It is also interesting to note that not all samples from Mongolia belonged to haplogroup G6b. Three samples from camels, with identical sequences (haplotype MON4), clustered into haplogroup G7a. This is clear evidence that G6 and G7 are present in Mongolia and camels can be intermediate hosts for both genotypes. The genetic diversity and evolutionary history of *E. granulosus s.l.* appears to have an intriguing background. Many haplotypes from Mongolia are separated from each other by a relatively large genetic distance. For example, the number of mutational steps between MON1 and MON2 in haplogroup G6b is 35. This is evidence of a long evolutionary history of haplogroup G6b and it remains to be studied, whether G6b is present outside Mongolia or is, for some reason, specific to this country. Note that the new haplogroup G6b was identified using near-complete mitogenome sequences, whereas the commonly used markers of *cox1* (1,608 bp and 366 bp; Figs. 4 and 5; and *nad1*, 894 bp and 471 bp; Supplementary Figs. S1 and S2) could not reliably distinguish G6b from the nominal G6 (here G6a).

Haplotypes from Iran (IRN4), Türkiye (TUR1/TUR2) and Mongolia (MON4) were identified within the genotype G7. However, this genotype is not common in these regions due to its preferred intermediate host, the domestic pig, rarely being reared in these countries (Sadjjadi, 2006; Lymbery et al., 2015; Gilbert et al., 2018). Nonetheless, several cases of genotype G7 have been reported previously in Türkiye, both in humans and sheep (Šnábel et al., 2009; Eryildiz and Sakru, 2012; Mehmood et al., 2020). G7 has been found also in a dog in Cyprus, that is geographically close to Türkiye (Santoro et al., 2024). Similarly, there have been reports of genotype G7 being found in goats in Iran (Fadakar et al., 2015), and putatively in camels in Mongolia, though the authors argued that additional analysis is required for confirmation (Bold et al., 2019). As these are not the regular hosts for genotype G7,

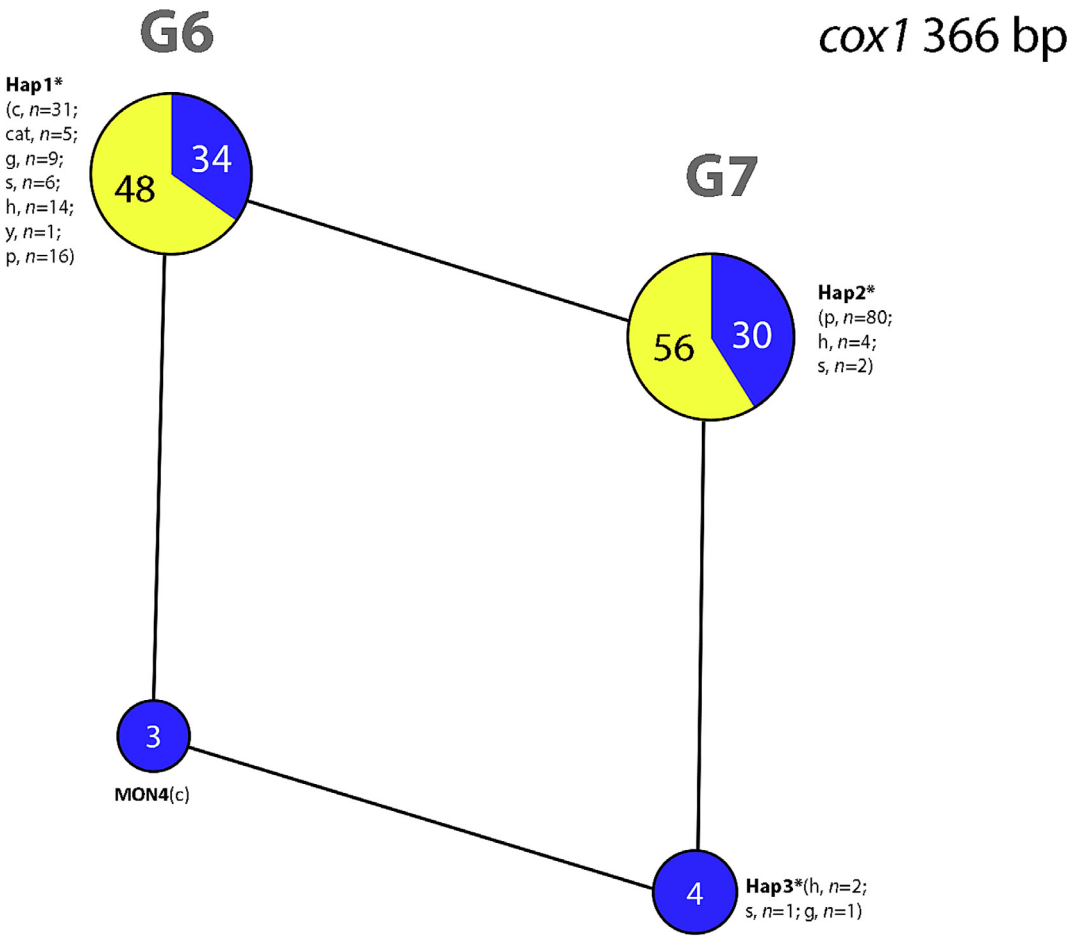

Fig. 5. Median-joining network of *Echinococcus granulosus sensu lato* isolates (n = 175) of genotypes G6 and G7, based on partial cytochrome *c* oxidase subunit 1 gene sequences (*cox1*, 366 bp). Dark blue in circles represents samples sequenced in this work and yellow in circles represents sequences acquired from GenBank. Letters in parentheses next to the haplotype names represent the intermediate hosts: c – camel, cat – cattle, h – human, g – goat, s – sheep, p – pig, y – yak. Numbers inside the circles depict the number of samples that belong to the haplotype, and the numbers beside the lines represent the number of mutational steps. Haplotypes marked with an asterisk are haplotypes that were separate in the near-complete mitogenome network. Note that Hap1* includes all the samples of haplogroup G7b based on the near-complete mitochondrial genome sequences (Fig. 2). For interpretation of colours, the reader is referred to the online version of this article.

the infection usually results in the formation of infertile cysts. In Iran, adult G7 worms have also been reported in dogs (Mirbadie et al., 2019), implying that a suitable intermediate host(s) should exist for the parasite's full lifecycle to take place in this region. The wild boar (*Sus scrofa*) is a likely candidate for the intermediate host and is relatively abundant in Türkiye, Cyprus, Iran and Mongolia (Keuling and Leus, 2019). Evidence of boars with fertile G7 cysts has been found in Europe (Onac et al., 2013; Umhang et al., 2014; Sgroi et al., 2019; Kilinc et al., 2024). In addition to (free-roaming) dogs, grey wolves (*Canis lupus*) could fulfil the role of the primary definitive hosts in this scenario, as they have been found to be hosts to adult genotype G7 worms (Guerra et al., 2013; Ito et al., 2013; Kilinc et al., 2023). As such, the entire lifecycle could occur in the wild. Potential intermediate hosts could include also goats, sheep or camels, as fertile cysts of G7 have been reported for Greece, Namibia and Sudan (Varcasia et al., 2007; Addy et al., 2017; Aschenborn et al., 2022).

It is important to note that the geographical distribution of *E. granulosus s.l.* has strongly been influenced by human-related factors, such as animal trading and travel, leading to a degree of uncertainty for geographically tied data. Yet, it has also been demonstrated that with sufficient genetic and epidemiological evidence, we can trace back the origins of the parasite. An example of such a case is a human CE diagnosed in Finland that was genetically linked back to Algeria, the country of origin of the patient, currently living in Finland (Kinkar et al., 2018b). This showcases a need for collecting for each sample as much data as possible, to accurately assess the spread of these parasites.

In haplogroup G6a, samples from the same region often cluster together (e.g., from Kenya: KEN1-3), whereas there are also samples that are genetically similar but geographically distant, for example some of the samples from Sudan (SDN2) and Iran (IRN2). The same is evident for haplogroup G7a. While the genetic similarity is due to the shared evolutionary history in the same region, the genetic affinity of geographically distant samples can be explained by animal transportation. This is in accord with former theories proposing that intensive animal trade could explain some of the phylogenetic structure of *E. granulosus s.l.* (Andresiuk et al., 2013; Laurimäe et al., 2016; Kinkar et al., 2017, 2018b).

This work has found multiple highly divergent haplotypes. For example, haplotypes of G7a from Türkiye (TUR1 and TUR2), Iran (IRN4) and Poland (POL7). These divergent haplotypes can potentially be part of larger sub-haplogroups and in future more samples are needed to test this. Similarly to the G7a, highly divergent haplotypes exist also in haplogroup G6a (from Iran and Kazakhstan), and as mentioned above, in haplogroup G6b.

Charting the mitochondrial haplotypes, based on long sequences, and their relations is important as it provides essential

data for the geographical distribution of haplotypes, haplogroups, genotypes and evolutionary relations between them, as well as helping to better understand their host ranges and infections in humans. However, it is unclear to what extent the mitogenome data can reflect differences in biological traits of parasites belonging to different genotypes or haplogroups. Initial evidence of different pathogenicity in humans has already been found between *E. granulosus s.s.* and genotype G6 (Debiaggi et al., 2023), highlighting the need for further epidemiological and genomic studies to better understand these differences. We acknowledge that such in-depth analysis of the mitochondrial genome has yet to find more value in practical use. However, this work and several previous studies have demonstrated the practical use of long sequences in accurate genotype identification and tracing back the origin of infection (see above). In addition, amassing mitogenomic data across the parasite distribution range builds a solid foundation for the future research of a more practical nature.

In conclusion, this work has provided important new findings for genotypes G6 and G7. We discovered a new haplogroup G6b that is, according to current data, found only in Mongolia. We also refined knowledge of the hosts, geographical spread, genetic diversity and evolutionary relations of isolates belonging to genotypes G6 and G7. Moreover, we highly recommend using complete or near-complete mitogenome sequences, as these are significantly more informative in phylogenetic and geographical analyses, and in some instances also crucial in correct genotype identification.

CRediT authorship contribution statement

Anti Biedermann: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Teivi Laurimäe:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation. **Liina Anijalg:** Writing – review & editing, Validation, Supervision, Software, Methodology, Investigation. **Laura Kamenetzky:** Writing – review & editing, Resources, Investigation. **Silvia V. Soriano:** Writing – review & editing, Resources, Investigation. **Nora Pierangeli:** Writing – review & editing, Resources, Investigation. **Lorena E. Lazzarini:** Writing – review & editing, Resources, Investigation. **Gérald Umhang:** Writing – review & editing, Resources, Investigation. **Bolor Bold:** Writing – review & editing, Resources, Investigation. **Chimedtseren Bayasgalan:** Writing – review & editing, Resources, Investigation. **Jacek Karamon:** Writing – review & editing, Resources, Investigation. **Małgorzata Samorek-Pieórg:** Writing – review & editing, Resources, Investigation. **Sami Simsek:** Writing – review & editing, Resources, Investigation. **Figen Celik:** Writing – review & editing, Resources, Investigation. **Majid F. Harandi:** Writing – review & editing, Resources, Investigation. **Saeid Nasibi:** Writing – review & editing, Resources, Investigation. **Naunain Mehmood:** Writing – review & editing, Resources, Investigation. **Oleg Chihai:** Writing – review & editing, Resources, Investigation. **Adriano Casulli:** Writing – review & editing, Investigation, Funding acquisition. **Urmas Saarma:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

2.6. Data availability

All sequences of this study have been deposited into the GenBank database: accession numbers PQ420728-PQ420799. The full list of data for samples and additional statistical values can be found in the supplementary files. Sequence alignment file is available in the Mendeley Data repository (Mendeley Data, https://doi.org/10.17632/4cjpz79kf4.1).

Acknowledgements

We would like to thank M.A. Cucher and M.C. Rosenzvit for helping with the Argentinean sampling. This work was supported by funding from the Estonian Ministry of Education and Research (grants PRG1209 and TK215), and by funding from the European Union's Horizon 2020 Research and Innovation programme under grant agreement No. 773830: One Health European Joint Programme (MEME project; https://onehealthejp.eu/jrp-meme/); PNUD PGTF/INT/21/K09, UBACYT 20020190100261BA, and The National Scientific and Technical Research Council, Argentina (CONICET) (to L.K.).

Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.ijpara.2025.04.014.

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