Parasitology Research (2019) 118:2193–2201
https://doi.org/10.1007/s00436-019-06346-2

GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

First detection of zoonotic tapeworm *Echinococcus granulosus* sensu lato genotype G7 in continental Italy

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Received: 14 February 2019 / Accepted: 30 April 2019 / Published online: 27 May 2019

Abstract

The larval stage of the species complex *Echinococcus granulosus* sensu lato (*s.l.*) is the cause of a widespread zoonotic disease known as cystic echinococcosis (CE). The disease is highly prevalent in southern Italy and represents a serious public health issue. The main aim of this study was to characterize *E. granulosus s.l.* genotypes from wild boar on a continental area of Italy (Campania region), using recently developed mtDNA markers of *nad2* and *nad5* for reliable identification of different genotypes. Here, *nad5* (680 bp) allowed for a clear identification of G1 and G3, whereas a combination of *nad2* (714 bp) and *nad5* (1394 bp in total) did the same for genotype G7 and its haplogroups G7a and G7b. The results of this study revealed for the first time the presence of genotype G7 in continental Italy. While haplogroup G7b was previously shown to be restricted to the islands of Corsica and Sardinia, here we demonstrate that haplogroup G7b is also present on the mainland of Italy. This work has implications in designing future strategies to reduce CE in Italy.

Keywords Cystic echinococcosis · *Echinococcus granulosus* sensu lato · Genotype G1 · Genotype G3 · Genotype G7 · Wild boar

Introduction

The larval stage of the tapeworms belonging to the species complex *Echinococcus granulosus* sensu lato (*s.l.*) are the etiological agents of a globally widespread zoonotic disease known as cystic echinococcosis (CE). The worldwide economic burden of CE is substantial, and it is estimated to reach approximately 3 billion US dollars each year due to losses to the livestock industry and treatment of human cases (Budke et al. 2006; WHO 2018). CE poses problems in various regions of the world, including different parts of Europe (Varcasia et al. 2007; Marcinkute et al. 2015; Oksanen and Lavikainen 2015) the Middle East, East and Central Asia (especially the Tibetan Plateau), southern regions of South America, and the Mediterranean area (Deplazes et al. 2017). The life cycle of this cestode requires two mammalian hosts: canids (mostly dogs and wolves) are the final hosts for the adult parasites, whereas numerous domestic and wild mammals act as the intermediate hosts for the larval stage of the parasite (Moks et al. 2006; Laurimaa et al. 2015; Thompson 2017; Scala et al. 2017; Poglayen et al. 2017). Humans are infected with the larval stage and are considered to be accidental dead-end hosts for *E. granulosus s.l.* (Alvarez Rojas et al. 2014; Thompson 2017). The larval stage in the intermediate host is a fluid-filled cyst, which is typically located in the liver or in the lungs, and causes various serious health issues for the infected intermediate host, as well as for humans (Eckert et al. 2001).

It is well established that *E. granulosus s.l.* exhibits high genetic diversity and has a complicated taxonomy and phylogeny that are not yet fully resolved (e.g., Moks et al. 2008; Saarma et al. 2009; Knapp et al. 2011, 2015; Lymbery 2017; Kinkar et al. 2017; Maldonado et al. 2017; Laurimäe et al. 2018a). On the basis of two mitochondrial DNA (mtDNA)

Section Editor: Bruno Gottstein

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gene fragments (*cox1*, 366 bp; *nad1*, 471 bp), ten different genotypes (G1–G10) were initially described (Bowles et al. 1992, 1994; Scott et al. 1997; Lavikainen et al. 2003). Genotypes G2 and G9 are, however, now classified as invalid: G2 is considered as a microvariant of G3 (Kinkar et al. 2017), and G9 as a microvariant of G7 (Kedra et al. 1999; Thompson 2008). Over the years, there has been accumulating evidence of morphological, life cycle, host range, and developmental rate differences between the genotypes (e.g., Thompson 2017; Romig et al. 2017). These differences together with genetic evidence have now provided grounds to regard a number of genotypes as separate species: G1 and G3 as *E. granulosus* sensu stricto (*s.s.*; Kinkar et al. 2017), G4 as *Echinococcus equinus*, and G5 as *Echinococcus ortleppi* (Thompson and McManus 2002; Lymbery 2017). The species status of genotypes G6–G10 is still problematic (e.g., Thompson 2008; Saarma et al. 2009; Knapp et al. 2011; Nakao et al. 2013; Lymbery 2017). Recently, based on the analysis of six nuclear genes, it was shown that genotypes G6/G7 form one species (species name under dispute), whereas G8/G10 can be regarded as a separate species, named *Echinococcus canadensis* (Laurimäe et al. 2018a).

CE is considered endemic in Italy, whereas different prevalence rates are reported from northern, central, and southern parts of the country (including the islands) (Piseddu et al. 2017). In northern Italy, CE is thought to be spread rather sporadically, while a number of regions in southern Italy are considered highly endemic areas for CE and the disease is highly prevalent also on the island of Sardinia (Varcasia et al. 2004; Scala et al. 2006a; Varcasia et al. 2006; Dore et al. 2014). Cystic echinococcosis represents a substantial public health issue also in the Campania region (Veneziano et al. 2004; Garippa 2006; Capuano et al. 2006; Cringoli et al. 2007; Rinaldi et al. 2008a, b; Sgroi et al. unpublished). Similarly to other parasitoses prevalent in Italy (e.g., *Taenia hydatigena*; Scala et al. 2015), the main reasons for the high prevalence and continued perpetuation of the life cycle of the parasite in these areas are the practice of home-slaughering without proper disposal of offals and dead animals, as well as shortcomings in the parasitological control of definitive hosts like shepard and stray dogs, but also wolves (Rinaldi et al. 2008a; Varcasia et al. 2011; Paoletti et al. 2019). To date, the genotypes and species identified in the mainland of Italy are as follows: *E. granulosus s.s.*—G1 and G3 (e.g., Capuano et al. 2006; Rinaldi et al. 2008a; Casulli et al. 2008, 2012; Gori et al. 2015; Paoletti et al. 2019), *E. equinus*—G4 (Scala et al. 2006b; Varcasia et al. 2008), and *E. ortleppi*—G5 (Casulli et al. 2008). However, to date, the *E. granulosus s.l.* genotype G7 has been recorded in Italy only on the island of Sardinia (Varcasia et al. 2006).

In recent years, wild boar (*Sus scrofa*) populations in Italy have been expanding closer to urban areas and farmlands (Paoletti et al. 2019), and therefore, their parasites merit close attention since the interaction between wild boars, domestic livestock species, and definitive hosts could be the cause for concern for the spread of different parasites (Di Nicola et al. 2015; Paoletti et al. 2019). Previous studies have identified *E. granulosus s.s.* (G1, G3) and *E. granulosus s.l.* genotypes G6/G7 in wild boar from a variety of regions in Europe, including the southern regions of the continent: the island of Corsica, France (G6/G7; Umhang et al. 2014), Ukraine (G1, G7; Kedra et al. 2000), and Spain (G1; Martín-Hernando et al. 2008). Recent epidemiological study in the Marche region (central Italy) identified genotype G1 from wild boars, with an estimated prevalence of 1.0% (8 out of 765 animals; Paoletti et al. 2019), while from the Campania region, the prevalence of CE from wild boars was reported to be 4.4% (93 out of 2108 animals; Sgroi et al. unpublished).

In the past few years, several studies have emerged that have employed long mtDNA sequences or even near-complete/complete mitogenomes to identify population structures and phylogeographic patterns in the *E. granulosus s.l.* species complex, highlighting also the advantages of using longer sequences in such analyses (Kinkar et al. 2016, 2018a, c; Addy et al. 2017; Laurimäe et al. 2016, 2018b). However, if the purpose is solely genotype identification, shorter mtDNA sequences can be used. Although the widely used *cox1* and *nad1* gene sequences allow correct assignment in most cases, there are often isolates that are impossible to assign reliably with these markers, particularly G1 and G3 genotype samples, as well as G6 and G7 (reviewed in Romig et al. 2015). As a result, the authors have often opted to classify these ambiguous isolates as G1–G3 and G6/G7 (e.g., Andresiuk et al. 2013). Recently, based on a large sample size covering a wide geographical range, new mtDNA markers were developed that allow reliable allocation of samples into G1 and G3 (based on a fragment of *nad5* gene; Kinkar et al. 2018b), and into G6 and G7 (fragments of *nad2* and *nad5* genes; Laurimäe et al. unpublished). Moreover, Laurimäe et al. (2018b) demonstrated that genotype G7 is represented by two major haplogroups, G7a and G7b, and that G7b is specific to islands of Corsica (France) and Sardinia (Italy).

The main aim of this study was to characterize *E. granulosus s.l.* genotypes from cysts obtained during wild boar parasitological inspections in the Campania region of southern Italy, using mtDNA markers of *nad2* and *nad5* for correct genotype and haplogroup identification.

Material and methods

Samples

A total of 28 *E. granulosus s.l.* cysts were analyzed. The hydatid cysts were collected from wild boars during the meat inspection of the health monitoring project "Piano Emergenza

Cinghiali in Campania - PECC 2016-2019" in the southwest of Italy (Figs. 1 and 2). Only fertile cysts were used for further analysis.

DNA extraction and analysis of nad2 and nad5 sequences

Genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel GmbH & Co. KG, Düren, North Rhine-Westphalia, Germany) following the manufacturer's protocols. Primer pairs for PCR amplification for *nad2* and *nad5* mtDNA loci were as previously described in Kinkar et al. (2018b; *nad5*) and Laurimäe et al. (unpublished, *nad2*), with the resultant PCR products being 781 bp and 759 bp in length, respectively. PCR was carried out in a volume of 20 μl, using 1U x 5x FIREPol® Master Mix (Solis BioDyne, Tartu, Estonia), 0.25 μM of each primer, and 10 ng of purified genomic DNA. A touchdown protocol was used for PCR: initial denaturation at 95 °C for 15 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 72 °C for 1 min; followed by 25 cycles of 95 °C for 20 s, 50 °C for 45 s, and 72 °C for 1 min; and finishing with a final elongation step at 72 °C for 5 min. Sequencing was performed at the Institute of Genomics Core Facility using the same primers as for the initial PCR. BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) was used for sequencing, following the manufacturer's protocols. Cycling parameters were 96 °C 1 min, followed by 25 cycles of 96 °C 10 s, 50 °C 15 s, and 60 °C 4 min. Sequences were resolved on the ABI 3130xl sequencer (Applied Biosystems). Sequences were assessed for quality, and consensus sequences were assembled using the program Codon Code Aligner v.6.0.2, whereas BioEdit v.7.2.5 software was utilized for Clustal W multiple sequence alignment, and to manually check and correct the sequences for errors (Thompson et al. 1994; Hall 1999). After sequencing and trimming, the final length of the sequences was 714 bp (*nad2*) and 680 bp (*nad5*). All 23 new sequences were deposited in GenBank (accession numbers: MK682617–MK682658; Table 1).

Phylogenetic network

In order to place the sequence data obtained in this study into a wider phylogenetic context, we included 93 homologous sequences of *E. granulosus s.l.* genotypes G6 and G7 from GenBank (accession numbers: MH300929–MH300970; MH300972–MH301022; Laurimäe et al. 2018b), 212 genotype G1 sequences (AB786664, MG672124–MG672293; Nakao et al. 2013; Kinkar et al. 2018a), and 40 genotype G3 sequences (KJ559023, MG682511–MG682544; Wang et al. 2016; Kinkar et al. 2018c). Phylogenetic network analysis was performed separately for two datasets: (1) G7 samples and (2) G1 and G3 samples. Networks were constructed using Network v4.612 (Bandelt et al. 1999; http://www.fluxus-engineering.com, Fluxus Technology Ltd. 2004), with both indels and point mutations considered.

Results

For the genotype G7 phylogenetic network calculations, the sequences of *nad2* (714 bp) and *nad5* (680 bp) gene fragment sequences were concatenated, resulting in a total length of 1394 bp. For genotypes G1 and G3, only *nad5* (680 bp) was used for network calculations, as this gene fragment has been shown to be sufficient for correct identification of these two genotypes (Kinkar et al. 2018b). Of the 23 successfully

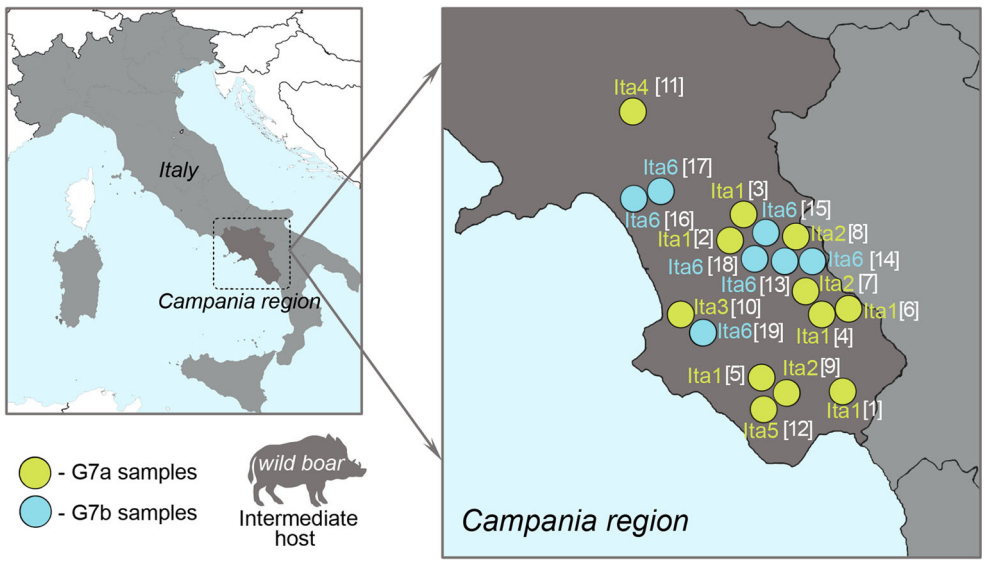

Fig. 1 Geographic locations of 19 samples obtained from the Campania region (Italy) that were identified as genotype G7. The intermediate host for all of the samples was the wild boar (*Sus scrofa*). Green dots represent haplogroup G7a samples, and blue dots depict haplogroup G7b samples. White numbers inside the brackets are sample numbers ('Sample no.' in Table 1)

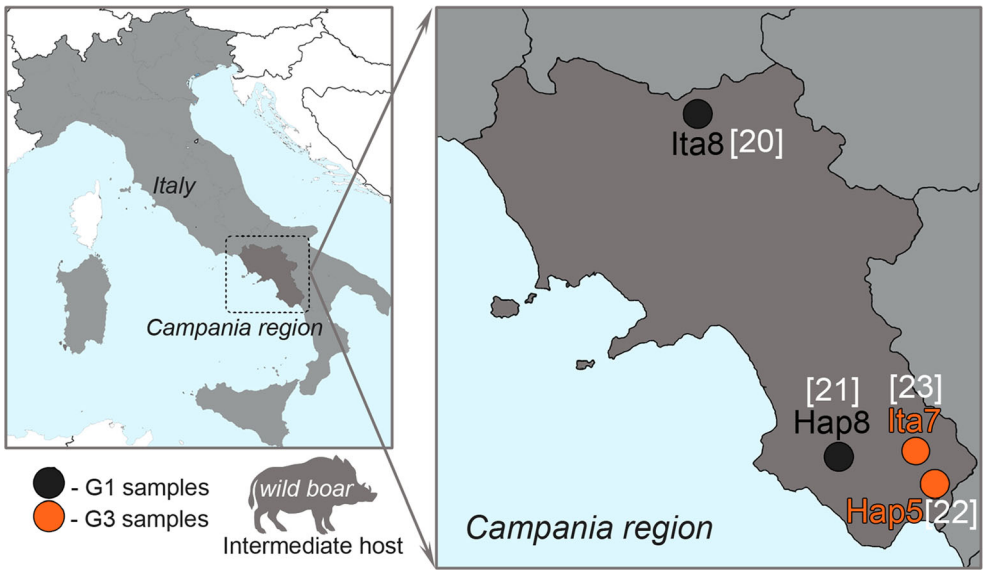

Fig. 2 Geographic locations of four samples obtained from the Campania region (Italy) that were identified as *E. granulosus s.s.* (G1 and G3). The intermediate host for all samples was the wild boar (*Sus scrofa*). Black dots represent genotype G1 samples, whereas orange depicts genotype G3 samples. White numbers inside the brackets are sample numbers ('Sample no.' in Table 1)

sequenced samples obtained from the Campania region, 19 clustered together with genotype G7 samples obtained from GenBank (Fig. 3), whereas sequences of two samples grouped together with published G1, and two with G3 samples (Fig. 4). The remaining five samples did not yield a PCR product.

Genotype G7

Genotype G7 samples from Italy were divided into six separate haplotypes (Fig. 3). Out of these, five haplotypes Ita1–Ita5 (12 samples) grouped together with G7a haplotypes

Table 1 Data for the 23 samples analyzed in the current study, collected from the Campania region, Italy. The intermediate host for all the samples was the wild boar (*Sus scrofa*)

Sample no	Haplotype	Location	Genotype	Haplogroup	Accession number *nad2*	Accession number *nad5*
1	Ita1	Caselle in Pittari	G7	G7a	MK682617	MK682636
2	Ita1	Postiglione	G7	G7a	MK682618	MK682637
3	Ita1	Petina	G7	G7a	MK682619	MK682638
4	Ita1	Sala Consilina	G7	G7a	MK682620	MK682639
5	Ita1	Montano Antilia	G7	G7a	MK682621	MK682640
6	Ita1	Sala Consilina	G7	G7a	MK682622	MK682641
7	Ita2	San Rufo	G7	G7a	MK682623	MK682642
8	Ita2	Petina	G7	G7a	MK682624	MK682643
9	Ita2	Montano Antilia	G7	G7a	MK682625	MK682644
10	Ita3	Cicerale	G7	G7a	MK682626	MK682645
11	Ita4	Serino	G7	G7a	MK68262	MK682646
12	Ita5	Futani	G7	G7a	MK682628	MK682647
13	Ita6	Petina	G7	G7b	MK682629	MK682648
14	Ita6	Polla	G7	G7b	MK682630	MK682649
15	Ita6	Sicignano Degli Alburni	G7	G7b	MK682631	MK682650
16	Ita6	Pontecagnano	G7	G7b	MK682632	MK682651
17	Ita6	Pontecagnano	G7	G7b	MK682633	MK682652
18	Ita6	Sicignano Degli Alburni	G7	G7b	MK682634	MK682653
19	Ita6	Cicerale	G7	G7b	MK682635	MK682654
20	Ita8	Morcone	G1	–	–	MK682655
21	Hap8	Vallo Della Lucania	G1	–	–	MK682656
22	Hap5	Casaletto Spartano	G3	–	–	MK682657
23	Ita7	Sanza	G3	–	–	MK682658

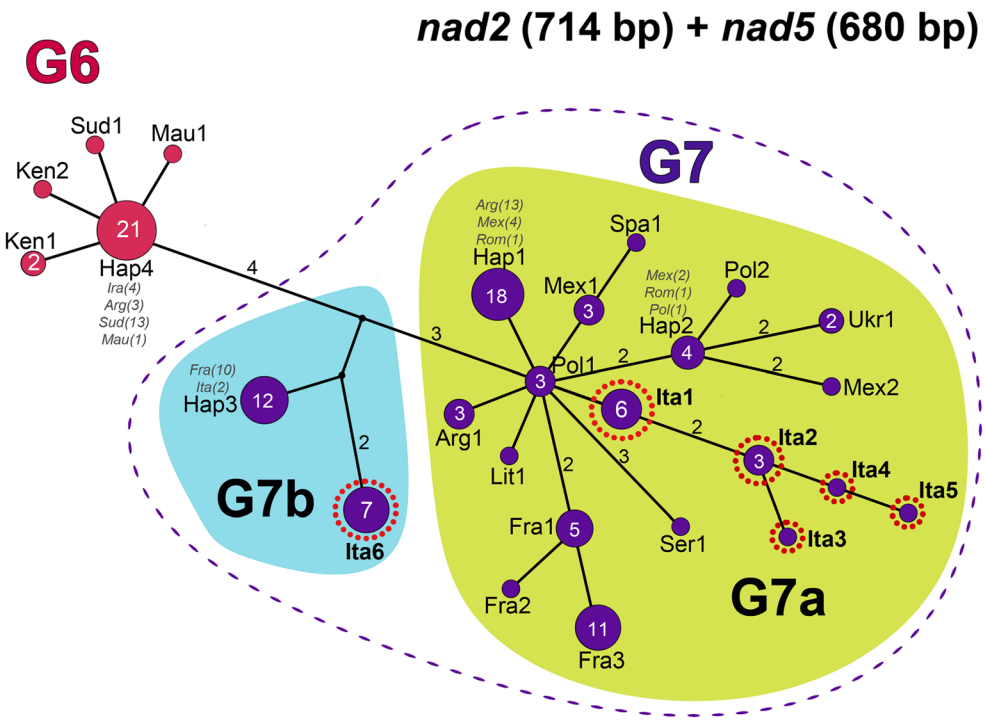

Fig. 3 Phylogenetic network of *nad2* (714 bp) and *nad5* (680 bp) gene sequences for the genotype G7 samples sequenced in this study ($n = 19$), with the additional 93 G6 ($n = 26$) and G7 ($n = 67$) sequences obtained from GenBank (Laurimäe et al. 2018b). Haplotypes sequenced in this study are marked with a red dotted circle. Red represents genotype G6 samples and purple genotype G7 samples. Haplogroup G7a is marked with a green background, and haplogroup G7b is marked with a blue background. Numbers inside the haplotypes represent the number of samples, and numbers above the lines depict the number of mutations. Haplotypes comprising samples from a single country are marked with three-letter abbreviations of respective country. Haplotypes comprising samples from multiple countries are named "Hap," whereas the origin of samples is written above the haplotype name in italics, and the number of samples per country is written in the brackets. Country abbreviations are as follows: Ita—Italy; Mex—Mexico; Pol—Poland; Ukr—Ukraine; Ser—Serbia; Fra—France; Lit—Lithuania; Arg—Argentina; Ken—Kenya; Sud—Sudan; Mau—Mauritania; Ira—Iran; Rom—Romania

obtained from GenBank. These GenBank samples were, based on complete mitogenome data, previously identified as belonging to haplogroup G7a (Laurimäe et al. 2018b). The remaining haplotype Ita6 (7 samples) was shown to be closely related to haplotype Hap3, which comprised samples that were previously shown to represent haplogroup G7b (Laurimäe et al. 2018b). The haplotype Hap3 samples from GenBank originated from Corsica and Sardinia.

At the local level, the phylogenetic network revealed that the samples collected from the southern Campania (Ita1—sample nos. 1 and 5; Ita2—sample no. 9; Ita5—sample no. 12) clustered into haplogroup G7a (Fig. 1). However, the samples obtained from the middle part of the Campania region were mixed, with eight samples belonging to haplogroup G7a (Ita1—sample nos. 2–4 and 6; Ita2—sample nos. 7 and 8; Ita3—sample no. 10; Ita4—sample no. 11) and seven samples clustering into G7b (Ita6—sample nos. 13–19).

Genotypes G1 and G3

Out of the two samples from the Campania region, which were identified as belonging to genotype G1, one clustered into the central G1 haplotype Hap8 (Fig. 4). The haplotype Hap8 comprised samples originating from 18 different countries worldwide (e.g., Romania, Moldova, Spain, Chile, Argentina, and Brazil). The second G1 sample from Italy formed a separate haplotype (Ita8), separated from haplotype Hap9 by a single point mutation. Hap9 representatives from GenBank were from Spain and Turkey.

Of the two G3 samples, one clustered into the central haplotype Hap5, whereas the other formed a single haplotype Ita7 (Fig. 4). Furthermore, representatives of Hap5 from GenBank were from nine countries: China, Turkey, Iran, India, Bulgaria, Albania, Spain, and Romania. The phylogenetic network revealed that Ita7 was separated from the central haplotype Hap5 only by one mutation.

Discussion

Recently, several studies have highlighted the advantages of using long mtDNA sequences for the genetic diversity and phylogenetic studies of *E. granulosus s.l.* (e.g., Addy et al. 2017; Kinkar et al. 2018a; Laurimäe et al. 2018b). However,

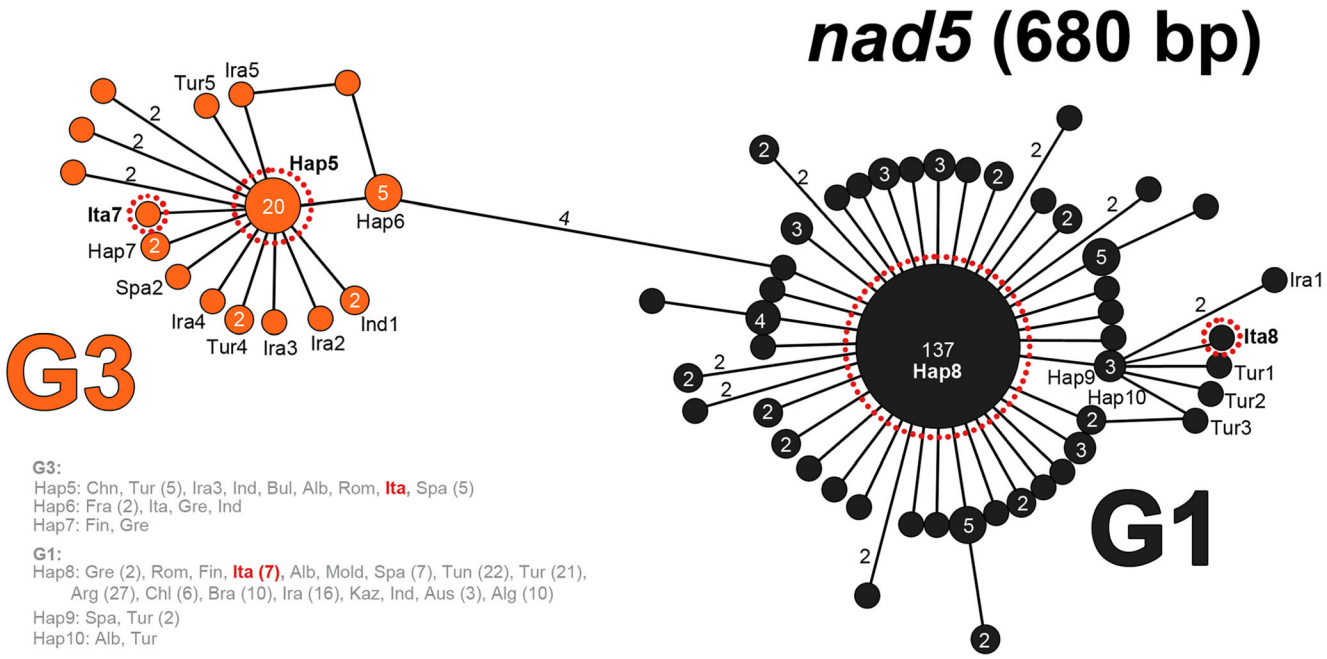

Fig. 4 Phylogenetic network of *nad5* (680 bp) gene sequences for *E. granulosus s.s.* genotype G1 ($n = 2$) and G3 ($n = 2$) samples from the Campania region (Italy), with the additional 252 sequences of genotype G1 ($n = 212$) and G3 ($n = 40$) obtained from GenBank. The figure configuration is essentially the same as in Kinkar et al. (2018b), but with the added four Italian samples sequenced in this study, which are surrounded by red dotted lines (note that both Hap5 and Hap8 include one newly sequenced Italian sample). Black represents genotype G1 samples, and orange genotype G3. Numbers inside the haplotypes represent the number of samples, and numbers above the lines depict the number of mutations. Only the haplotypes most closely related to the newly sequenced Italian samples were marked. Haplotypes comprising samples from a single country are marked with country three-letter abbreviations. Haplotypes comprising samples from multiple countries are named "Hap" and their sample origins are listed in the bottom left corner (numbers in brackets beside the country name represent the number of samples)

for purposes such as genotype/haplogroup identification, the use of complete or near-complete mtDNA sequences is not required. In the present study, we employed recently developed mtDNA markers to confidently distinguish between *E. granulosus s.s.* genotype G1 and G3 (*nad5*, 680 bp; Kinkar et al. 2018b) samples, and between *E. granulosus s.l.* genotype G6 and G7, as well as between haplogroup G7a and G7b (joint analysis of *nad2* and *nad5*, 714 bp and 680 bp, respectively; Laurimäe et al. unpublished).

E. granulosus s.l. genotype G7

The results of the present study identified that 19 samples collected from wild boars in the Campania region belonged to genotype G7 (Figs. 1 and 3). While a number of studies have previously identified several genotypes/species from continental Italy (e.g., Scala et al. 2006b; Rinaldi et al. 2008a; Casulli et al. 2008, 2012; Paoletti et al. 2019), this is the first study to identify *E. granulosus s.l.* genotype G7 from continental Italy, since previously genotype G7 has only been identified in Italy from the island of Sardinia (e.g., Varcasia et al. 2006; Laurimäe et al. 2018b). The reason why G7 has not been identified earlier in continental Italy could likely be the result of (i) insufficient number of studies on wild boars or domestic pigs and (ii) those studies that have attempted genotyping have typically employed the commonly used *cox1* and *nad1* mtDNA gene fragments that in some cases do not allow to distinguish between G6 and G7, resulting in a classification of samples as G6/7.

Our results revealed that the analyzed G7 samples clustered into two haplogroups—G7a and G7b (Fig. 3). It is important to note that when genotype assignment is made based on the original gene fragment of *cox1* (366 bp) according to Bowles et al. (1992), the G7b samples would be classified as genotype G6 (Laurimäe et al. 2018b). The existence of G7a and G7b haplogroups was previously firmly demonstrated using complete mitogenomes (Laurimäe et al. 2018b), where G7b was found only from the islands of Corsica and Sardinia. A possible explanation for the existence of G7b in these islands was long-term geographical isolation of the intermediate host (the pig), which has lived in relative isolation since its introduction to the islands (Addy et al. 2017; Laurimäe et al. 2018b). The results of this study, however, suggest that the haplogroup G7b might be more widespread than previously thought, since it is also present on continental Italy. Although there is still not enough data available to elaborate on the origin of haplogroup G7b, it is possible that G7b originated from Sardinia and was subsequently transported to the mainland through dogs or through livestock trade (pigs). However, confirming any hypothesis regarding the origin of G7b requires further sampling

from a significantly wider geographical area. In this regard, it also remains to be further studied whether G7b has a wider geographical range and whether there is any epidemiological significance related to these haplogroups. The genotype G7 is most likely maintained in this part of Italy due to semi-domestic cycle: the stray and hunting dogs, having access to raw offal (sheep, wild boar, and/or pig), have contaminated the environment with *E. granulosus s.l.* eggs through feces, and as a result, wild boars acquire the infection by foraging in such areas (Paoletti et al. 2019).

E. granulosus s.s. genotypes G1 and G3

The four *E. granulosus s.s.* samples sequenced in the present study were firmly assigned to genotypes G1 and G3 using 680 bp of the *nad5* gene (Figs. 2 and 4), which once again indicates the reliability of *nad5* as a genetic marker for distinguishing *E. granulosus s.s.* genotypes G1 and G3. All four G1 ($n = 2$) and G3 ($n = 2$) samples were closely related to other globally distributed haplotypes, indicating that the samples are likely not highly divergent in a worldwide context. Similarly to genotype G7 infections identified here from wild boar, the *E. granulosus s.s.* G1 and G3 infections are likely due to spillover from the domestic cycle.

Conclusion

In conclusion, the present study showed the following: (i) for the first time, the presence of genotype G7 on continental Italy; (ii) haplogroup G7b is not restricted to the islands of Corsica and Sardinia, but is present also on continental Italy; (iii) the previously newly designed mtDNA markers of *nad2* and *nad5* allowed reliable identification of G1 and G3 (*nad5*, 680 bp), as well as of G7 and its haplogroups G7a and G7b (*nad2*, 714 bp; and *nad5*, 680 bp). The G1, G3, and G7 infections we report in the present study in wild boar are possible spillovers from the domestic cycle and therefore likely reflect a semi-domestic life cycle of *E. granulosus s.l.* in Italy. In the future, it will be relevant to further characterize *E. granulosus s.l.* samples to the genotype and even haplogroup level from other regions in Italy as well. This would allow more accurate evaluation of the distribution of *E. granulosus s.l.* genotype G7 and haplogroup G7b in continental Italy.

Acknowledgements We would like to thank all who helped to collect hydatid cysts from wild boars in frame of the project "Piano Emergenza Cinghiali in Campania - PECC 2016-2019."

Financial support This study was supported by institutional research funding (IUT20-32) from the Estonian Ministry of Education and Research; by the Estonian Doctoral School of Ecology and Environmental Sciences; and by the Italian research grant from Regione Campania Piano Emergenza Cinghiali in Campania (PECC 2016-2019).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement The hydatid cysts in the current study were collected from wild boars during post mortem mandatory inspection visit by official veterinaries according to a specific agreement with Government of Campania Region, UOD Wild Boar Emergency Plan in Campania (2016–2019).

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