

Research Article

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Dispersal and molecular characterisation of the *Echinococcus granulosus* (Batsch, 1786) complex isolated from various intermediate hosts in the Calabria region, southern Italy

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Abstract: Cystic echinococcosis (CE) is a zoonotic disease caused by the tapeworms of the *Echinococcus granulosus* sensu lato complex, which have worldwide distribution. No data on the circulation of genotypes of the *E. granulosus* complex in intermediate hosts in endemic areas in Calabria are available. The aims of our study were to evaluate the dispersal of genotypes of the *E. granulosus* complex in Calabria and to characterise parasite isolates by Sanger sequencing and phylogenetic analysis. We collected 71 animal samples from pigs, wild boars, sheep, cattle and goats. The first PCR screening analysis targeted three partial genomic regions: the cytochrome c oxidase subunit 1 (*cox1*), calreticulin protein (*cal*) and NADH dehydrogenase subunit 1 (*nad1*); this identified 28 parasitic cysts. Bi-directional sequencing of *cox1* amplicons and phylogenetic analysis allowed us to characterise all isolates. Molecular analyses of 28 newly generated *cox1* sequences revealed that most wild boars (n = 16) and three pigs were parasitised by the larval stage of *Taenia hydatidena* Pallas, 1766, called cysticercus tenuicollis. Two isolates from wild boars were identified as *Echinococcus canadensis* Webster and Cameron, 1961 (G7), while five sheep and two goats were infected with *E. granulosus* G1 (sheep strain) and G1 microvariant (previously reported as G2 genotype or Tasmanian sheep strain), respectively. These molecular findings should prompt further and more extensive studies, to elucidate regional transmission patterns and to guide control programs.

Keywords: *Echinococcus canadensis* G7, G1 microvariant of *E. granulosus* s.s., Cystic echinococcosis, wild boars, goats, zoonoses, sanger sequencing, molecular characterisation, genotyping

Cystic echinococcosis (CE) is a cosmopolitan zoonosis caused by the larval stage of tapeworms of the *Echinococcus granulosus* (Batsch, 1786) sensu lato complex, and constitutes a serious health problem in a “one-health” perspective. Despite the heavy economic impact on public health and the livestock trade (Seimenis 2003), the real burden of this parasitic infectious disease, which is classified as neglected in the WHO’s European Region (Da Silva 2010, Deplazes et al. 2017), is poorly understood.

CE has a worldwide geographic distribution, being found in Europe, Africa, Australia and the Americas, and displays regional or local differences related to the spectrum of intermediate hosts and other factors (Eckert et al. 2001, Thompson and McManus 2002, EFSA 2018). In Italy, retrospective studies have revealed an increased risk of human CE, particularly in the Islands and the southern regions, where sheep breeding is widely practised, and the prevalence of CE is highest in sheep (Garippa 2006, Brundu et al. 2014).

The *E. granulosus* s.l. complex is genetically highly heterogeneous. Traditionally, the identification and characterisation of species and subspecies (variants or genotypes) was based on a combination of biological, epidemiological and morphological criteria (Thompson and McManus 2002). In recent years, however, progress in mitochondrial phylogenetic analyses has allowed the different genotypes to be grouped into five species: *E. granulosus* sensu stricto (*E. granulosus* s.s.: G1–G3), *Echinococcus equinus* (Williams and Sweatman, 1963) (G4), *Echinococcus ortleppi* (Lopez-Neyra and Soler Planas, 1943) (G5), *Echinococcus canadensis* (G6/G7/G8/G10) and *Echinococcus felidis* (Ortlepp, 1937), all of which cause unilocular echinococcosis (Nakao et al. 2007; Saarma et al. 2009).

The species included in *Echinococcus* genus show clear differences in terms of their intermediate hosts, geographical distribution, genetic variability and ability to infect humans (McManus 2013). However, the role of different genotypes in this complex and their impact on human and animal health still need further investigation (Hosseini-Sa-

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Table 1. List of primer of three PCR protocols used to discriminate between the *Echinococcus granulosus* complex, *E. granulosus* sensu stricto (G1/G3) and *Echinococcus canadensis* (G6/G7).

Primer name	Sequence (5'-3')	Gene marker	Amplicon size (bp)	Specificity	Reference
CO1-F	TTTTTGGGCATCCTGAGGTTTAT	<i>cox1</i>	466	<i>Echinococcus granulosus</i> complex	Bowles et al. 1992
CO1-R	TAAAGAAAGAACATAATGAAAATG				
Cal-F	CAATTTACGGTAAAGCAT	<i>cal</i>	1001	<i>E. granulosus</i> s.s. (G1/G3)	Boubaker et al. 2017
Cal-R	CCTCATCTCCACTCTCT				
NDI-F	CTGCAGAGGTTTGCC	<i>nad1</i>	339	<i>Echinococcus canadensis</i> (G6/G7)	Boubaker et al. 2017
NDI-R	CACAACAGCTAAAGCG				

fa et al. 2016, Laurimäe et al. 2018a,b). Several studies have reported that *E. granulosus* s.s. and *E. canadensis* (G6/G7) are the most prevalent species that infect humans (Alvarez Rojas et al. 2014, Cucher et al. 2016).

Echinococcus granulosus s.s. has a cosmopolitan distribution and includes sheep (G1) and buffalo (G3) strains (Hosseini-Safa et al. 2016, Kinkar et al. 2017). It is responsible for the great majority of cases of human CE worldwide (88%), and is often associated with transmission via sheep as the intermediate host. Recently, Kinkar et al. (2017) conducted a study on the mitogenome and nuclear DNA of this species complex. This study revealed that genotypes G1 and G3 could be treated as distinct genotypes only at the mitochondrial level.

Echinococcus canadensis (G6/G7) has been reported in 11% of cases worldwide, with the G6 genotype being responsible for 7% of infections, whereas G7 strain for 4% of human cases transmitted mainly by pigs (Alvarez Rojas et al. 2014). However, *E. granulosus* (G6/G7) has also been reported in various hosts: moose, deer and caribou (Schurer et al. 2013), goats (Soriano et al. 2010), camels (Namaware and Dahiya 2018), cattle (Zhang et al. 1998, Wachira et al. 1993) and wild boars (Kedra et al. 2000, Mwambete et al. 2004).

In many European regions, the number of wild boars has increased considerably in recent years, and these animals have been reported to play an important role as intermediate hosts in the epidemiology and transmission of several diseases to livestock and humans (Meng et al. 2009, Casalinuovo et al. 2017, Fredriksson-Ahomaa 2019, Sgroi et al. 2019). Indeed, various Italian studies have reported evidence of the *E. granulosus* genotype complex in this wild ungulate (Di Paolo et al. 2017, Paoletti et al. 2019, Sgroi et al. 2019). To date, however, few studies have reported the presence of genotypes of the *E. granulosus* complex in wild boar, and data on their molecular characterisation and dispersal in endemic areas in southern Italy are absent.

The aims of the present study were therefore to determine the dispersal of CE in various domestic and wild intermediate hosts, and to characterise, by means of molecular approaches, the genotypes of the *E. granulosus* complex circulating in endemic areas of the Calabria region, southern Italy.

MATERIALS AND METHODS

Sample collection

From November 2017 to September 2019, a total of 71 samples were collected from pigs, wild boars, cattle, sheep and goats with

suspected hydatidosis during routine meat inspection in Calabria. In particular, we focused on the carcasses of home-slaughtered pigs and wild boars killed during the hunting season, which were examined by specialised veterinarians. Parasitised organs of intermediate hosts were transported within 24h to the Istituto Zooprofilattico Sperimentale del Mezzogiorno, Section of Catanzaro, Italy (IZSM), Catanzaro and carefully examined for the presence of presumptive hydatid cysts by means of visual inspection, palpation and serial section of the organs. Each cyst was treated as an isolate, transferred to a Petri dish and dissected. The germinal layers were washed three times with phosphate-buffered saline (PBS) pH 7.0 and stored in ethanol 70% until DNA extraction.

DNA extraction and PCR amplification

The DNA of each isolate was extracted from ethanol-preserved cysts by means of a Qiamp DNA Mini kit (Qiagen, Hilden, Germany) from approximately 25 mg of germinal layer, according to the manufacturer's recommendations. DNA was eluted with 150 µl of sterile water and stored at -20°C until molecular analysis. The *Echinococcus granulosus* complex and its different genotypes were identified by means of polymerase chain reaction (PCR) and DNA sequencing of partial mitochondrial and nuclear genes. The isolates were initially screened by implementing three PCR protocols for the amplification of the cytochrome c oxidase subunit 1 (*cox1*) (Bowles et al. 1992), calreticulin protein (*cal*) and NADH dehydrogenase subunit 1 (*nad1*) (Boubaker et al. 2017) in order to distinguish the *E. granulosus* complex from the G1/G2/G3 and G6/7 genotypes (Table 1). The PCR products were purified by means of the PCR Illustra MicroSpin S-300 Column HR (Gelifesciences, Amersham, UK) in accordance with the manufacturer's instructions.

DNA sequencing and phylogenetic analysis

The purified PCR *cox1* amplicons were sequenced by means of the ABI PRISM 3500 genetic analyser sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in both directions, using the same primers as in the three PCR protocols (see above), by means of the BigDye Terminator v1.1 cycle sequencing kit. Newly generated sequences were aligned by means of Clustal W (Thompson et al. 1994) and manually edited in Chromas v. 2.6.4. Blast software (<http://www.ncbi.nlm.nih.gov>) was used to compare the *cox1* sequences obtained with those deposited in GenBank, in order to preliminarily identify the genotype of the hydatid cyst isolates. A phylogenetic tree was generated by means of the MEGA v.7.0 program (Kumar et al. 2016). *Taenia saginata* (Goeze, 1782) was used as the out-group. The Neighbor-joining method (Saitou and Nei 1987) was used, together with the Tajima-Nei method (Tajima and Nei 1984), to compute evolutionary distances, which are presented in the units

Table 2. Characteristics of animal samples collected.

Animal host	Origin of parasite isolates	Numbers of samples
Wild boar	liver	20
	spleen	1
	mesenterium	1
	diaphragm	3
Sheep	liver	9
	lungs	5
Pig	liver	19
Cattle	liver	7
	lungs	4
Goat	liver	1
	lungs	1
Total samples		71

as the number of base substitutions per site. The reliability of phylogenetic clustering was evaluated by means of 1,000 bootstrap replicates. The *cox1* sequences were deposited in the GenBank database (Benson et al. 2014) under the accession numbers MN582430-MN582457.

RESULTS

Hydatid cyst collection

During routine meat inspection, 71 hydatid-like cysts were collected and their germinal layers fixed in 70% ethanol. The characteristics of the animal samples are summarised in Table 2. Parasite isolates were derived from several organs: liver, lung, spleen, mesenterium and diaphragm from pigs (n = 19), wild boars (n = 25), sheep (n = 14), cattle (n = 11) and goats (n = 2). Most of the wild boar samples that had proved positive on veterinary visual examination had hydatid-like cysts in the liver (20/25, 80%), diaphragm (3/25, 12%), mesenterium (1/25, 4%) and spleen (1/25, 4%); these organs often showed massive infection, with several “daughter” cysts. The examined organs are described in Table 2.

Amplification of *cox1*, *cal* and *nad1* partial genes

On initial molecular investigation, 28 cysts showed amplification on at least one of the PCR protocols (Table 3). All isolates were positive for *cox1* partial region, which identified all genotypes included in the *Echinococcus granulosus* complex. Of these, isolates from five sheep and two goats proved to be infected with *E. granulosus* s.s. (G1/G2/G3); two samples from wild boars, which were positive on the *nad1* PCR protocol, were identified as *Echinococcus canadensis* (G6/G7) isolates (Table 3). This first molecular approach did not allow us to distinguish between G1 and G3 or between G6 and G7 genotypes.

Molecular analyses

The initial molecular screening was carried out by means of PCR testing of three partial genomic regions (*cox1*, *nad1* and *cal*), followed by DNA Sanger sequencing and phylogenetic analysis. This allowed us to character-

ise and genotype all isolates. The 28 newly generated *cox1* sequences were analysed by means of BLAST similarity matches and multiple sequence alignment with reference sequences of genotypes of *E. granulosus* (downloaded from the GenBank database). Fig. 1 shows the phylogenetic tree of our isolates and the reference sequences. Molecular analyses revealed that most wild boar isolates and three pig isolates, which proved positive for the *E. granulosus* complex on PCR, were parasitised by another cestode; this larval stages of *Taenia hydatigena*, called cysticercus tenuicollis (Table 3). Out of a total of 18 wild boar sequences analysed, two isolates (ECHI 2 and ECHI 10) were *E. granulosus* (G7); five sheep (ECHI 32, ECHI 35, ECHI 36, ECHI 37, ECHI 54) and two goat samples (ECHI 72 and ECHI 73) were infected with *E. granulosus* G1 (sheep strain) and microvariant G1, respectively (Table 3). Overall, the phylogenetic analysis grouped seven of the 28 isolates (five sheep and two goats) in the clade with G1–G3 reference sequences, with bootstrap support of 95%. The two *E. granulosus* (G7) wild boar isolates, ECHI 2 and ECHI 10, formed a cluster, with bootstrap support of 69% (Fig. 1).

DISCUSSION

In agreement with previously reported European data (Loi et al. 2019), the findings of this study showed the prevalent occurrence of *Echinococcus granulosus* s.s. G1 in sheep. Our results also shed light on the epidemiology of CE, in that they revealed the presence of the *E. granulosus* s.s. G1 microvariant (previously reported as G2 genotype or Tasmanian sheep strain) in goats, and of *Echinococcus canadensis* (G7) in wild boar in southern Italy.

The accurate diagnosis of infections due to the *E. granulosus* complex in domestic and wild intermediate hosts is central to investigating the real burden of CE and underpins appropriate control programs. In Italy, CE is endemic or hyperendemic in several regions, and is responsible for significant economic losses in the public health and livestock sectors (Veneziano et al. 2004, Capuano et al. 2006, Scala et al. 2006, Cringoli et al. 2007, Rinaldi et al. 2008).

Recently, Piseddu et al. (2017) reported 12,619 cases of CE in hospitalised patients, emphasising the need for further national studies to improve *ad hoc* information on this under diagnosed and “neglected” disease. Indeed, reports from the EFSA and the ECDC highlight the fact that there is no system of surveillance of human CE in Italy. As a result, no official data are transmitted to European authorities (EFSA 2018).

It is noteworthy that the few official data available on wild intermediate hosts, particularly wild boar, substantially under-represent the dispersal of the different genotypes of the *E. granulosus* complex in this important reservoir. Several studies have reported the circulation of *E. granulosus* G1 and G3 in wild boars in various Italian regions (Varcasia et al. 2008, Di Nicola et al. 2015, Di Paolo et al. 2017). Moreover, concurrently with our sampling, Sgroi et al. (2019) isolated the *E. canadensis* (G7) strain in a wild boar population in the Campania region.

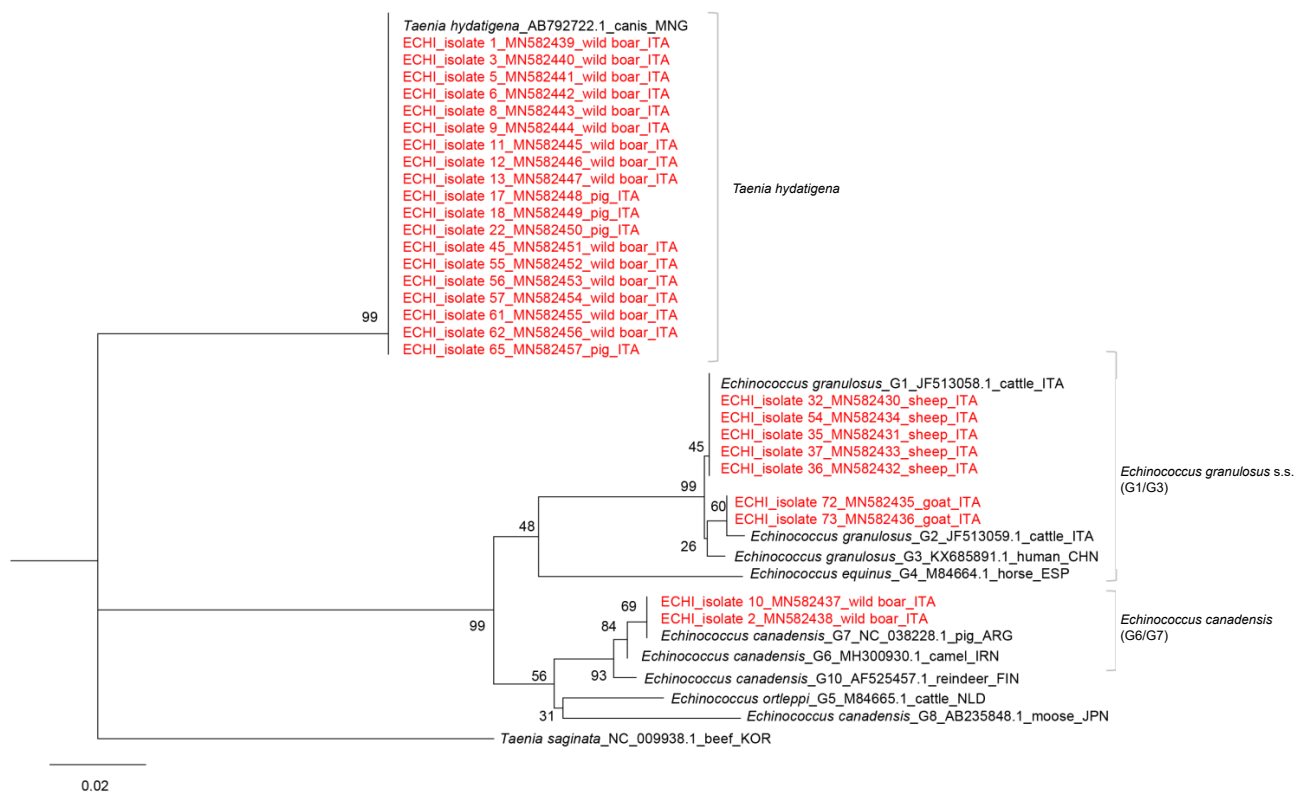


Fig. 1. The phylogenetic tree was estimated using reference sequences of the *Echinococcus granulosus* complex and *Taenia hydatigena* (black) downloaded from GenBank database and 28 parasite sequences isolated in this study (red). The tree was generated using Neighbor joining (NJ) method (Saitou and Nei 1984) based on an multi-alignment of partial sequences (466 bp) from cytochrome c oxidase I gene (*cox1*). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Tajima-Nei method (Tajima and Nei 1984) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. *Taenia saginata* (NC_009938.1) was used as an outgroup. Phylogenetic analyses were conducted in MEGA7 (Kumar et al. 2013). The nucleotide sequences are shown with: isolate name, Accession No., host and country code (ARG – Argentina, CHN – China, ESP – Spain, FIN – Finland, IRN – Iran, ITA – Italy, JPN – Japan, MNG – Mongolia, NLD – Netherlands, KOR – South Korea).

In our study, we characterised 28 isolates from parasitic cysts by means of molecular analyses; of these, two wild boar isolates were *E. canadensis* (G7). Of the total number of parasitised organs examined, hydatid-like cysts in wild boars were found in 80% of liver samples (20/25), 12% of diaphragm samples (3/25), and 4% of both mesenterium and spleen samples (1/25); these organs frequently presented massive infection, with several ‘daughter’ cysts being observed. Massive infection have also been reported in other studies, and seem to be associated with age and recurrent infections (Scala et al. 2006, Otero-Abad and Torgerson 2013).

In the last few years, several European countries, including Italy, have seen a ‘wild boar emergency’. Indeed, the overcrowding of these animals has led to a serious public health problem, as they are a crucial reservoir of various zoonoses (Casalinuovo et al. 2017). Moreover, it is important to point out that wild animals are possible indicators of the environmental spread of CE. The dispersion of infected animal carcasses (with parasitic cysts, tubercular lesions, etc.) during the hunting season could increase the load

of infectious parasites in the environment and promote a spill-over from wild to domestic animals (Otero-Abad and Torgerson 2013).

In this scenario, the *E. granulosus* complex may have adapted to wildlife, circulating in the environment and exposing to CE infection other wild or domestic animals that have so far not been considered susceptible. The surveillance of CE is more difficult in wild animals than in domestic one. Therefore, the presence of *E. canadensis* (G7) in wild boar could play a crucial role in spreading CE to intermediate hosts, including humans.

Our study also identified the presence of the *E. granulosus* s.s. G1 microvariant (previously reported as G2 genotype or Tasmanian sheep strain) in goats. In Italy, the G1 microvariant has frequently been isolated from humans, sheep, cattle and also goats, which testifies to the fact that the ‘sheep strain’ can adapt well to other reservoirs over time. The presence of mixed sheep and goat farms in Calabria has allowed *E. granulosus* s.s. to spread to goats.

Overall, our results confirmed the role of sheep as intermediate hosts of *E. granulosus* s.s. G1 in CE epidemiology.

Table 3. Molecular characterisation of parasites isolated from different animal species using PCR method and Sanger DNA sequencing.

Isolates_ID	Animal species	PCR Amplicon (+/-)			Sequence-based genotyping
		<i>cox1</i>	<i>cal</i>	<i>nad1</i>	
ECHI_isolate 1	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 2	wild boar	+	-	+	<i>Echinococcus canadensis</i> (G7)
ECHI_isolate 3	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 5	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 6	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 8	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 9	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 10	wild boar	+	-	+	<i>E. canadensis</i> (G7)
ECHI_isolate 11	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 12	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 13	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 17	pig	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 18	pig	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 22	pig	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 32	sheep	+	+	-	<i>Echinococcus granulosus</i> sensu stricto (G1/G3)
ECHI_isolate 35	sheep	+	+	-	<i>Echinococcus granulosus</i> sensu stricto (G1/G3)
ECHI_isolate 36	sheep	+	+	-	<i>Echinococcus granulosus</i> sensu stricto (G1/G3)
ECHI_isolate 37	sheep	+	+	-	<i>Echinococcus granulosus</i> sensu stricto (G1/G3)
ECHI_isolate 45	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 54	sheep	+	+	-	<i>Echinococcus granulosus</i> sensu stricto (G1/G3)
ECHI_isolate 55	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 56	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 57	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 61	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 62	wild boar	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 65	pig	+	-	-	<i>Taenia hydatigena</i>
ECHI_isolate 72	goat	+	+	-	<i>Echinococcus granulosus</i> sensu stricto (G1/G3)
ECHI_isolate 73	goat	+	+	-	<i>Echinococcus granulosus</i> sensu stricto (G1/G3)

Moreover, our isolation of *Echinococcus canadensis* (G7) from hydatid cysts in wild boars, and our characterisation of *E. granulosus* s.s. G1 microvariant in goats helps to better understand of the dispersal of various genotypes of the *E. granulosus* complex in southern Italy. These findings should prompt further and more comprehensive molecular

and epidemiological studies, in order to investigate regional transmission patterns and to guide control programs.

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