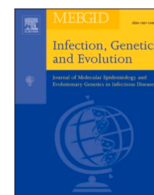
Infection, Genetics and Evolution 137 (2026) 105868

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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



A panel of five microsatellites as an alternative to mitogenome sequencing for investigating the genetic diversity of *Echinococcus granulosus sensu stricto* from global to farm scale

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ARTICLE INFO

Keywords:
Echinococcus granulosus sensu stricto
Microsatellite target
Population genetics
Infection event

ABSTRACT

While the molecular identification of *Echinococcus granulosus* species is essential for surveillance, a detailed investigation of the parasite's genetic diversity would offer a more accurate understanding of its transmission. This may be done by partial or whole mitochondrial genome sequencing, but both cost and time requirements limit routine application. Microsatellites are an alternative approach as they are a rapid, simple, highly discriminative, and low-cost tool. Our research project was designed to obtain a panel of microsatellite targets for population genetic analyses to describe the genetic diversity of *E. granulosus sensu stricto* (s.s.) at the global to farm scale. A panel of five informative microsatellite loci for population genetic analysis of *E. granulosus s.s.* was developed and validated. A total of 145 isolates from 11 countries were used to evaluate the panel at the international level. Additionally, 131 samples from sheep and cattle across six regions in southern Italy were analyzed to assess the panel's usefulness at both the regional and farm scale. Population genetics showed high genotypic diversity. Simpson's index gave high values (0.94–0.98) for the expected heterozygosity (0.80 to 0.87). Discriminant analysis of principal components (DAPC) revealed the structure of populations for each country, with Greece distinctly distant from Algeria, Italy, and Tunisia. This was also confirmed by FST values. DAPC

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https://doi.org/10.1016/j.meegid.2025.105868
Received 8 October 2025; Received in revised form 13 December 2025; Accepted 16 December 2025
Available online 18 December 2025
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further revealed a genetic structure among the farms from the Italian regional sample set, with three different clusters explained by different farming practices. This microsatellite panel can provide data relevant for *E. granulosus s.s.* population genetics from global to farm scale, making it a low-cost, simple alternative to full mitochondrial genome sequencing for analyzing numerous samples and obtaining a detailed overview of genetic diversity, an input that is useful for cystic echinococcosis control programs.

1. Introduction

Cystic echinococcosis (CE), caused by species of the *Echinococcus granulosus sensu lato* (*s.l.*) complex, is a parasitic disease with a worldwide distribution and a human burden estimated at 1,009,662 disability-adjusted life years (DALYs) or monetary losses of US $763,980,979 when taking into account underreporting (Budke et al., 2006). Although CE has a low fatality rate, it remains a chronic and debilitating disease (Casulli et al., 2023). The parasite mainly affects the liver and lungs, with a long asymptomatic period of several years before the onset of clinical signs associated with pressure from the enlarging cyst(s) or tissue fibrosis/necrosis (Craig et al., 2007). Humans are infected after ingestion of microscopic eggs and are considered aberrant hosts in the parasite's lifecycle. The intermediate hosts are mostly livestock (*e.g.* sheep, goats, cattle, pigs, and camels) infected by the larval stage of the parasite, which develops as cysts in the lungs and/or liver. Protoscoleces present in fertile cysts will develop into worms in the intestines of definitive hosts (mainly the dog) after consumption of infected viscera. After 37–45 days post-infection, eggs produced by the worms begin to be released into the environment *via* feces, potentially causing accidental infection of intermediate animal hosts and humans.

The taxonomy of *E. granulosus s.l.* has been revised during the last decades, and currently numbers five species: *E. granulosus sensu stricto* (*s. s.*), *E. equinus*, *E. ortleppi*, *E. canadensis,* and *E. felidis* (McManus, 2013; Romig et al., 2017). Among these species, *E. granulosus s.s.* is the most common, with a worldwide distribution. It is responsible for the large majority of human cases in Europe (76.8 %) and in the world (88.4 %) (Alvarez Rojas et al., 2014; Casulli et al., 2022). The lifecycle of *E. granulosus s.s.* is essentially based on the consumption of sheep viscera by dogs, particularly affecting pastoral and poor rural communities where people raise sheep or goats in close contact with dogs. The molecular identification of *E. granulosus* species is important for identifying the intermediate host species involved in the lifecycle, which allows control measures to be targeted. The surveillance of *E. granulosus s.l.* is generally based on the parasite's detection at slaughterhouses on the liver and lungs of livestock. Cysts—which are a tissue matrix very suitable for molecular diagnosis of the *E. granulosus* species involved—can thus be easily sampled. However, more accurate data are needed to describe the genetic diversity among the *E. granulosus* species and gain a deeper understanding of the maintenance of the parasite. Sanger sequencing (400–800 bp) of fragments of one or more mitochondrial genes (*e.g. cox1*, *nad1*) has commonly been used in various geographic regions, but suffers from a low discrimination power (Romig et al., 2015). Complete mitochondrial genes, especially full *cox1* (1609 bp), have also been sequenced in some studies (Alvarez Rojas et al., 2016; Alvarez Rojas et al., 2017; Yanagida et al., 2012). More recently, the near-complete mitogenome has been sequenced, providing a very high resolution (Kinkar et al., 2016; Kinkar et al., 2018). The latter approach is highly recommended for phylogenetic and especially phylogeographic studies, but is usually not necessary for species identification (Biedermann et al., 2025; Laurimäe et al., 2018). In this context, an alternative approach for studying genetic diversity with a high resolution has become possible through the use of microsatellites, which have the advantage of being potentially highly discriminant while also offering a quick, simple, and inexpensive solution.

Unlike mitochondrial genes, microsatellites correspond to non-coding sequences composed of small motifs of one to twelve nucleotides repeated in tandem with a tandem repeat of less than 100 bp. The high mutation rate (10^{-2}–10^{-6}) compared with point mutations in coding genes is due to two mechanisms: DNA slippage during DNA replication, and recombination between DNA strands (Li et al., 2002). Microsatellite targets EgSca6 and EgSca11, designed from the *E. granulosus* genome, have proved to be highly discriminant and can be used to investigate genetic diversity at the intra-individual level when testing CE samples from sheep and cattle originating from Tunisia and France, but also from Tunisian children (M'rad et al., 2020; Umhang et al., 2018). The EgSca11 microsatellite is present in two copies in the genome of *E. granulosus s.s.*, resulting in one to four alleles and a high polymorphism, though there is some difficulty in interpreting the profiles, which requires taking into account not only the presence of the peaks representing different fragment sizes but also their heights (Umhang et al., 2018). Globally, even if it is useful for intra-individual genetic diversity investigation, the complexity of the EgSca11 target prevents it from being routinely used for population genetic analyses required for epidemiological studies from global to local scale. A classical panel of microsatellites would be of great interest for this purpose, as this approach could provide relevant genetic information rapidly, at low cost, and from a large number of samples, thus facilitating its use in numerous epidemiological studies.

In this study, we aimed to obtain a panel of relevant microsatellite targets to rapidly and easily perform population genetic analyses and structure inferences to describe the genetic diversity of *E. granulosus s.s.* from global to farm scale so as to better understand the epidemiology of the parasite in endemic areas.

2. Materials and methods

2.1. Sampling

2.1.1. International sampling for microsatellite evaluation

Samples were collected from North Africa (Algeria, Morocco and Tunisia), Europe (France, Greece, Italy, Spain and Ukraine) and Asia (Armenia, Pakistan and Türkiye) in order to confirm both the polymorphism heterozygosity of the microsatellite markers with *E. granulosus s.s.* samples and evaluate the use of the microsatellite panel for population genetic analyses at national and regional scales. A total of 145 CE samples were collected from livestock or humans across 11

Table 1
List of the *E. granulosus sensu stricto* cyst samples forming the international sample set used to evaluate the microsatellite targets regarding country of origin and host species.

Country	Host Species						
	Sheep	Cattle	Camel	Goat	Pig	Human	Total
Morocco	5	2					7
Algeria	9	8	2				19
Tunisia	14			1		10	25
South Italy	56	4					60
Greece	16						16
France	5						5
Spain					1	1	2
Italy (Sardinia)		2					2
Ukraine				2			2
Armenia	1					1	2
Türkiye		2					2
Pakistan	1			2			3
Total	108	17	2	5	1	12	145

countries (Table 1). Most of them ($n = 127$) were collected directly at the slaughterhouses using FTA cards (Boué et al., 2017), while the remaining samples were obtained from cyst membranes. They were collected in the context of previous studies concerning North Africa (Benchikh El Fegoun et al., 2023; El Berbri et al., 2020; M'rad et al., 2020; Umhang et al., 2018), the national surveillance of animal CE in France (Umhang et al., 2020), and the ECHINO-SAFE-MED project (Nocerino et al., 2024b).

The sample size per country ranged from 2 to 62 animals. Eight of the countries are located in the Mediterranean basin, covering parts of southern Europe (Italy, France, Greece, and Spain), North Africa (Algeria, Morocco, and Tunisia), and Asia (Türkiye). The remaining three countries were Armenia, Pakistan and Ukraine. The samples also included two isolates from Sardinia, which in this study was considered as a distinct geographic location from Southern Italy.

2.1.2. Regional and intra-host sampling

A second set of samples was designed to evaluate the relevance of the microsatellite panel at the regional to farm scale (Table 2). In all, 131 CE samples corresponding to 30 sheep and 21 cattle were collected, with one to six cysts obtained from the liver and/or lungs of each individual at the slaughterhouses, as part of the ECHINO-SAFE-MED project (Nocerino et al., 2024a). The sampling was based on six Italian regions (Basilicata, Calabria, Campania, Lazio, Puglia, and Sicilia), with one to four farms represented in each region for a total of 25 farms sampled (Fig. 1). Twenty-one of these samples (one per animal for 18 sheep and 3 cattle) were already included in the 60 South Italian samples for the international sample set. The additional samples from the same animals were used to investigate intra-host diversity and assess the applicability of the microsatellite panel on a more granular spatial scale. Each sample was identified by the farm number (ITAXX) followed by the number of the animal from the farm and then a letter from "a" to "f" denoting the specific cyst isolated from the animal.

2.1.3. Sample collection and DNA extraction

Parasitic DNA was extracted from FTA cards or a piece of tissue using an automated nucleic acid purification platform, the iPrep purification instrument (Invitrogen, iPrep Charge Switch gDNATissue Kit) or the Maxwell48 (Promega, DNA Blood Extraction kit) according to the manufacturer's instructions for processing tissue samples. DNA was already available for samples received from Pakistan, Spain, Türkiye, Ukraine, and some from Tunisia. Additionally, samples for three others species of the *E. granulosus* complex were also used in order to evaluate the potential amplification and polymorphism consisting of *E. equinus* ($n = 4$), *E. ortleppi* ($n = 5$) and *E. canadensis* ($n = 9$).

Table 2
List of the *E. granulosus sensu stricto* cyst samples forming the Italian sample set used to evaluate the microsatellite targets at regional to intra-host level regarding host species, regions, and farms of origin.

Animal species	Region	City	No of farms (farm ID)	No of animals	No of samples
Sheep	Campania	Ricigliano	4 (ITA07, 08, 10, 18)	16	43
		Eboli	1 (ITA11)	4	17
	Basilicata	Ruvo del Monte	4 (ITA32, 91, 92, 100)	8	16
		Potenza	2 (ITA71, 72)	2	8
Cattle	Lazio	Ferentino	1 (ITA03)	1	3
		Sora	1 (ITA19)	1	2
		Castelliri	1 (ITA42)	1	5
		Valmontone	1 (ITA50)	1	3
	Puglia	Manfredonia	1 (ITA28)	2	2
		Rignano Garganico	1 (ITA45)	3	5
		San'Eramo in Colle	1 (ITA51)	1	1
	Calabria	Lungro	2 (ITA53, 59)	4	11
	Sicilia	Cesaro	1 (ITA38)	2	3
		Francavilla di Sicilia	1 (ITA39)	2	3
		Sclafani Bagni	3 (ITA57, 78, 89)	3	9

2.2. Identification and validation of microsatellite targets

The genome sequences of *E. granulosus* (v2 03232012) had already been screened for microsatellites using the Tandem Repeats Finder software (https://tandem.bu.edu/trf/trf.html) (Benson, 1999) in a previous study (Umhang et al., 2018). Among the two microsatellite targets previously selected, EgSca6-GAA was retained, while Egsca11 was excluded due to the complexity arising from having to manage the alleles from the two copies in the nuclear genome. New targets were selected from microsatellites with at least ten repeat units and a motif length of 3 to 10 nucleotides. Six other microsatellites were then selected for inclusion in a new *E. granulosus s.s.* microsatellite panel: the tri-nucleotide repeat markers Sca1-TTG, Sca2-GTT, Sca6-GAA, Sca6-TGG, and Sca6-CAT and the tetra-nucleotide repeat markers Sca3-ATCC and Sca7-GGAT (Table 3). Chromosome localization of each microsatellite target was determined using *E. granulosus s.s.* G1 chromosome scale (GenBank accession no. JAIKUZ000000000) from (Korhonen et al., 2022).

The seven microsatellites were first individually amplified by simplex PCR using fluorescently-labeled forward primers and unlabeled reverse primers. The PCR program was as follows: initial denaturation at 95 °C for 15 min; 30 cycles of 94 °C for 30 s, 60 °C for 90 s, 72 °C for 60 s; and a final extension at 60 °C for 30 min. The final primers concentrations are 0.1 μM for Sca2-GTT, 0.2 μM for Sca1-TTG, Sca3-ATCC and Sca6-GAA; and 0.4 μM for Sca7-GGAT. Each PCR product (1 μl) was mixed with 0.5 μl of a dye-labeled size standard (500LIZ; Applied Biosystems) and 9.5 μl of deionized formamide (Hi-Di formamide; ThermoFisher Scientific). This mixture was electrophoresed using a genetic analyzer (GA3500; Applied Biosystems). The fragment size was determined and allele assignment estimated using GeneMapper analysis software (Applied Biosystems).

2.3. Multiplex PCR

Microsatellite amplification was optimized by modifying dye choices to better cover allelic size ranges and by adjusting primer concentrations to produce uniform signal intensities across markers. The cycling conditions were modified as follows in order amplify all the targets in a single multiplex PCR: 5 min of denaturation at 95 °C, 30 cycles of 30 s at 95 °C, 1 min and 30 s at 60 °C, and 30 s at 72 °C, followed by a final extension of 30 min at 63 °C using a Type-it kit (Qiagen).

2.4. Population genetic analyses

The R packages poppR (Kamvar et al., 2014), adegenet (Jombart, 2008) and hierfstat (Goudet, 2005) were used for data analysis. Figures were constructed with the ggplot2 R package (Wickham, 2011). Microsatellite summary statistics, such as the number of observed alleles, Simpson index (1-D), evenness, and missing data by locus were computed. The minimum number of microsatellite markers to enable discrimination between each individual was assessed by a genotype accumulation curve, as described on the poppR website (https://grunwaldlab.github.io/Population_Genetics_in_R/First_Steps.html). Population (country) statistics were conducted for the whole panel. Diversity by country was assessed by computing the number of multilocus genotypes and private alleles.

Fig. 1. Geographic location of the 25 farms from the six Italian regions from which CE samples were obtained for the Italian regional sample set.

Table 3
List of the primers for the seven microsatellite targets selected for evaluation.

Target	Primer (5′–3′) Forward and Reverse	Size range (bp)	Fluorescent dye	Localization
Sca1_TTG	F: ACACCGCTTGCGTCAGTAAA R: TTTTCCAGGGATTCGCTTGC	222–354	NED	chromosome 1
Sca2_GTT	F: TTGAGGTGAAGCGTGGCATT R: GCGGCTAATCATGTGTGCTT	139–199	FAM	chromosome 3
Sca3_ATCC	F: GGGCACTCACTCACTCATCC R: GCGGGTAAGAAAATGGGTGA	246–310	PET	chromosome 4
Sca6_TGG	F: AGTCGCTTCAAAGGCGCTC R: ATTGTGTTCAGCGTTGGTGC	209–356	VIC	chromosome 2
Sca6_CAT	F: TCGGATCTAGAGGCGTCGAT R: ACTGACCATGTCCTCCGTTAG	198–329	VIC	chromosome 9
Sca6_GAA	F: TCTGGATTCTGTCCAGCTTGT R: TGGCTGTGAGCTATTTGGAA	169–217	VIC	chromosome 9
Sca7_GGAT	F: AGGATATGGGATGGATGCACG R: CCTCCATTCATGCCCTATCCA	299–509	FAM	chromosome 9

A preliminary study of the population structure of *E. granulosus s.s.* was conducted by country. For these analyses, countries with more than 10 samples and a comparable sampling protocol were considered (*i.e.* Algeria, Italy, Greece, and Tunisia). The Simpson index, evenness and unbiased Nei's diversity were estimated for each population. Population structure was studied by a discriminant analysis of principal components (DAPC), a multivariate statistical approach used to infer the number of clusters of genetically-related individuals. DAPC is commonly used to visualize the most striking structures of a data set in a reduced dimensional space without assuming an evolutionary model (Jombart et al., 2010). The DAPC was based on Bruvo's distance (Bruvo et al., 2004). The level of genetic differentiation between populations was estimated by calculating the F_{ST} (fixation index), used to estimate the genetic differentiation between two populations as inferred from allele frequencies.

Based on DAPC, a structure-like analysis was conducted to investigate the group membership information of samples. Group memberships are indicators of how clear-cut genetic clusters found in DAPC are. Indeed, the membership probabilities analysis in this kind of approach is based on the retained discriminant functions generated by DAPC. The generated figure shows each individual represented by a vertical line broken into differently-colored genetic clusters (Algeria, Greece, Italy or Tunisia) whose length is proportional to the probability of assignment to each cluster.

Finally, farm and individual diversities were assessed. A DACP was performed for farms as previously described. Individual diversity was assessed by generating a dendrogram based on Bruvo's distance (Bruvo et al., 2004). The interactive tree of life (iTOL) online tool (Letunic and Bork, 2016) was used to visualize and annotate the dendrogram.

3. Results

3.1. Characteristics of the microsatellite marker panel

A total of 15 microsatellites targets were initially tested according to the criteria's previously mentioned. Those with multiple loci and those that seemed to amplify at a low intensity were eliminated. The seven microsatellite markers selected were highly polymorphic (Table 4). The number of alleles by locus varied between 13 and 50 (mean of 34). Simpson's index, defined as the probability that two randomly selected genotypes are different, ranged from 0.44 to 0.97. Most of the loci (5/7) had a Simpson's index higher than 0.90. Evenness, which reflects equitability in the distribution of alleles per locus, ranged between 0.38 and 0.84. An evenness value of zero indicates a locus dominated by a

Table 4
Summary statistics of the microsatellite panel evaluated using the international sample set of *E. granulosus s.s.* cysts. The asterisk refers to the five microstellite markers SCA1_TTG, Sca7_GGAT, Sca3_ATCC, Sca2_GTT, and Sca6_GAA.

Locus	No of observed alleles	Simpson's index	Evenness	Missing alleles (%)
Sca1_TTG	44	0.96	0.78	0.69
Sca7_GGAT	49	0.97	0.84	–
Sca3_ATCC	13	0.44	0.38	0.69
Sca2_GTT	19	0.91	0.84	0.69
Sca6_GAA	16	0.76	0.55	2.07
Sca6_CAT	47	0.95	0.73	12.41
Sca6_TGG	50	0.96	0.78	6.21
mean with 7 microsatellite markers	34.0	0.85	0.70	3.25
mean with 5 microsatellite markers*	28.2	0.81	0.68	1.23

single allele, and a value of one indicates a locus with equally abundant alleles. The percentage of missing data ranged from 0.69 to 12.41 (mean of 3.25). The power of discrimination between each individual, estimated by the genotype accumulation curve, reached 100 % for five microsatellite markers (Fig. 2). However, the power of discrimination was already high (close to 100 %) for three markers. Notwithstanding the optimization of PCR conditions, a considerable proportion of missing data were observed for Sca6-CAT (12.4 %) and Sca6-TGG (6.2 %) markers compared to a very low rate for the others five (average of 0.3 %) but also some difficulties in the interpretation of profiles notably due to numerous numbers of peaks for some samples. They were consequently excluded from the panel. Given their sufficient discriminatory power, five microsatellite markers—Sca1-TTG, Sca2-GTT, Sca3-ATCC, Sca6-GAA, and Sca7-GGAT—were retained to establish the final panel. All five targets were able to be amplified in a single multiplex PCR and in the same injection, thanks to the choice of different fluorescent dyes for those with overlapping base pair ranges (Table 3 and Fig. 3).

Regarding the other species of the *E. granulosus* complex, four of the five microsatellites (Sca1-TTG, Sca2-GTT, Sca3-ATCC, and Sca6-GAA) amplify for *E. equinus*, *E. canadensis*, and *E. ortleppi*. The samples tested have demonstrated an absence of polymorphism for both Sca-3 and Sca-6. The results indicate that Sca2-GTT amplifies only very weakly for 10 of the 18 samples that were tested. For Sca2-GTT, Sca3-ATCC, and Sca6-GAA, the size range of the amplicons differs from that of *E. granulosus s.s.* The Sca7-GGAT marker demonstrated no amplification for any of the samples that were tested.

3.2. Population genetics-based statistics

High multilocus genotype diversity was observed in all the countries included in this study. An analysis of all the isolates ($n = 145$) with the five-marker panel resulted in 143 multilocus genotypes (MLGs) (Table 5). Shared MLGs were observed within South Italy, Tunisia, Ukraine, and Sardinia (Supplementary Table 1). No shared MLGs were observed among countries/locations. Seven to 35 private alleles were found in the four countries with more than 10 samples available. Regarding the others, private alleles were found in six of the nine countries, even with seven to two samples available. For the three countries (Armenia, Spain, and Ukraine) without private alleles, only two samples were available.

Population genetics applied to populations with more than 10 individuals (Algeria, Italy, Greece, and Tunisia) resulted in high genotypic diversity. The Simpson's index was high, ranging from 0.94 to 0.98 (Table 5). Nei's unbiased gene diversity (expected heterozygosity) was also high, ranging per population from 0.80 to 0.87. DAPC revealed a population structure for each country (Fig. 4). Although Tunisian and Italian populations showed overlapping, Greek and Algerian populations formed distinct clusters. Axes 1 and 2 of the DAPC explained 95.3 % of this variability. Pairwise Fst between populations was consistent with DAPC results. It revealed low differentiation between Italy and Algeria (Fst = 0.008), and between Italy and Tunisia (Fst = 0.007). A higher level of differentiation, based on the allele frequencies, was observed between Tunisia and Algeria (Fst = 0.02). The Greek population was different from the other three, presenting the following Fst values: 0.08, 0.07, and 0.08 for Greece-Algeria, Greece-Tunisia, and Greece-Italy, respectively. The structure-like analysis was consistent with DACP and Fst results (Fig. 5). Most of the samples from the Greek populations formed their own cluster, although some samples appear to be related to the Italian population. While samples from Italy, Algeria, and Tunisia showed considerable levels of admixture, the gene flow appears strongest between Italian and Algerian populations.

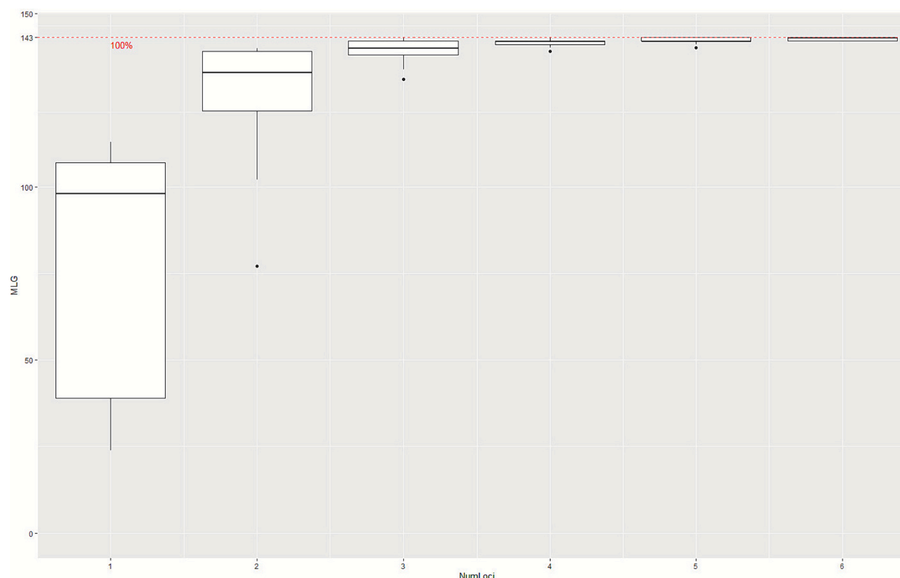

Fig. 2. Genotype accumulation curves to estimate the power of discrimination for the seven microsatellite loci obtained using the international set of 145 CE samples.

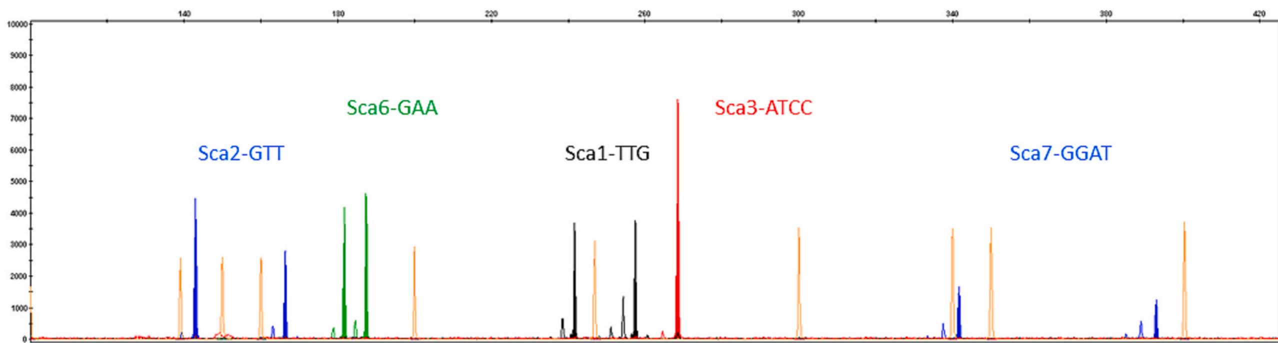

Fig. 3. Capillary electrophoresis pattern of the 5-plex microsatellite assay. The x-axis represents DNA fragment size in base pairs, and the y-axis represents fluorescence units. Sca2-GTT and Sca7-GGAT are labeled with Fam, Sca6-GAA with Vic, Sca1-TTG with Ned and Sca3-ATCC with Pet.

Table 5
Simpson's index and Nei's unbiased gene diversity were only computed for populations subject to the same sampling protocol and when there were more than 10 samples. The five microstellite markers concern the targets SCA1_TTG, Sca7_GGAT, Sca3_ATCC, Sca2_GTT, and Sca6_GAA.

Population	Nb of samples	Statistics computed with 7 microsatellite markers				Statistics computed with 5 microsatellite markers			
		MLG	No of private alleles	Simpson's index	Nei's unbiased gene diversity	MLG	No of private alleles	Simpson's index	Nei's unbiased gene diversity
Algeria	19	19	7	0.947	0.798	19	3	0.947	0.739
Tunisia	25	25	26	0.960	0.853	23	7	0.954	0.806
Italy	60	60	35	0.983	0.835	59	17	0.983	0.785
Greece	16	16	9	0.938	0.868	16	6	0.938	0.856
Armenia	2	2	–	–	–	2	–	–	–
France	5	5	1	–	–	5	–	–	–
Morocco	7	7	3	–	–	7	3	–	–
Pakistan	3	3	3	–	–	3	3	–	–
Sardinia (Italy)	2	1	1	–	–	1	–	–	–
Spain	2	2	–	–	–	2	–	–	–
Türkiye	2	2	1	–	–	2	1	–	–
Ukraine	2	1	–	–	–	1	–	–	–
Total	145	143	–	0.992*	0.855*	140	–	0.991*	0.812*

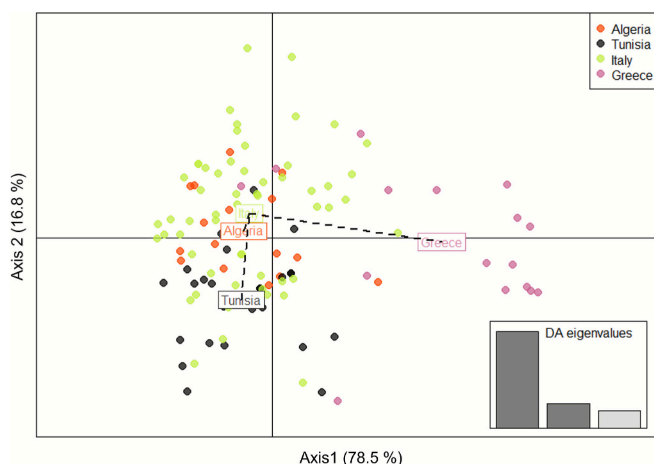

Fig. 4. Results of discriminant analysis of principal components (DAPC) on the CE samples collected in Algeria, Italy, Greece, and Tunisia and included in the international sample set. Each sample is represented by a point. The color of each point corresponds to the country where the sample was collected. The eigenvalues reflect the amount of variance in the input variables, which is explained by each principal component. Labels correspond to group centroids, which is the average position of individuals of each group along the discriminant axes. The X-axis and the Y-axis account for 95.3 % of the variance.

The Italian regional sample set aimed to assess genetic structure among farms in the same country through DAPC (Fig. 6, Supplementary Table 2). An initial single cluster was made up of all the samples from the four sheep collected on the same farm in Eboli, Salerno (Campania region, farm ITA11). Two more clusters were observed. One of them included most of the samples from the other four farms in the Campania region located in Ricigliano, Salerno (ITA07, ITA08, ITA10, ITA18) but also a more distant farm (ITA59) from the Calabria region in Lungro, Cosenza. A third cluster, which included most of the other samples, was made up of samples from the farms in Basilicata, Calabria, Lazio, Puglia, and Sicility.

The dendrogram of all the Italian samples (Fig. 7) showed that in most cases, cysts collected from the same animal clustered together. This is similar to those from animals on the same farm, though to a lesser extent. Some exceptions to this main pattern were nonetheless observed. Furthermore, in six cases, the same MLG was obtained for two cysts from the same animal, corresponding exclusively to sheep for a proportion of 20 % of the sheep sampled here. Additionally, the same MLG as the one from two samples from the same sheep was obtained for a sample from another sheep from the same region (Campania), but from another farm. The two samples concerned by infection with the same MLG were from the same organ, with both lungs ($n = 3$) and the liver ($n = 2$) concerned. In only one case was the same MLG found in both the liver and lungs from the same sheep.

4. Discussion

The Mediterranean basin has long been described as a hotspot for CE (Dakkak, 2010; Nocerino et al., 2024b). While human incidence appears to remain high in North Africa (*i.e.* Morocco, Algeria, and Tunisia), it seems to be decreasing in the south of Europe according to reported cases (i.e France, Spain, Italy, and Greece) (Casulli et al., 2023; Nocerino

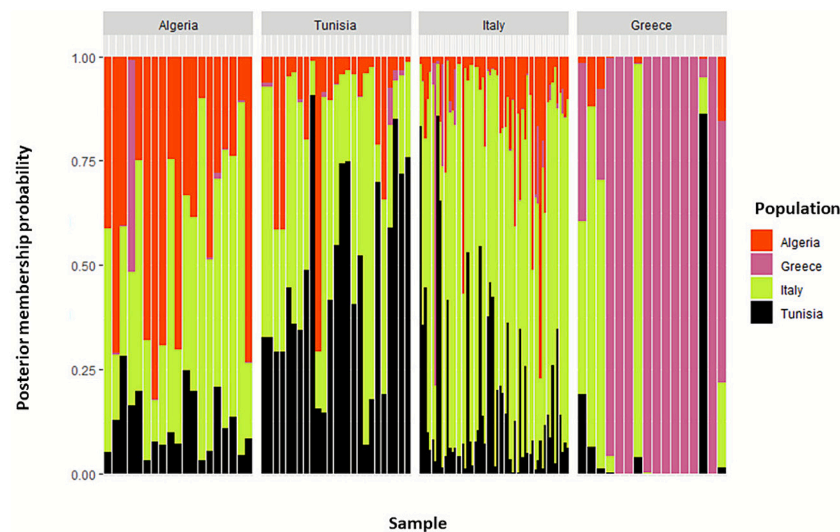

Fig. 5. Structure-like analysis for investigating the group membership information of CE samples collected in Algeria, Italy, Greece, and Tunisia and included in the international sample set. Each sample corresponds to a vertical bar broken into different colored genetic clusters (4 sampled populations), with length proportional to the probability of assignment to each cluster (posterior membership probability).

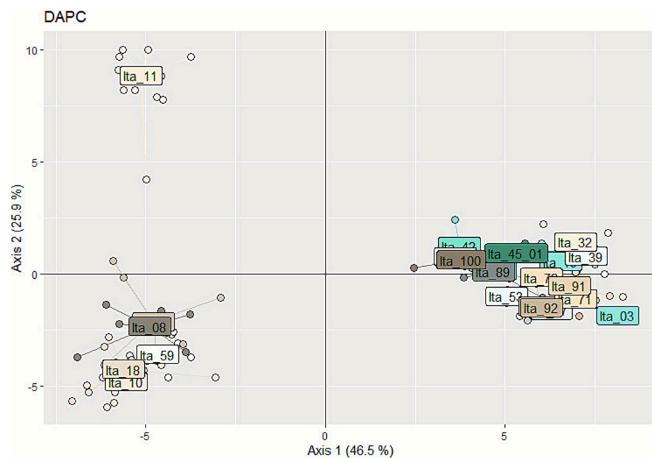

Fig. 6. Results of discriminant analysis of principal components (DAPC) on the CE samples collected for the Italian regional sample set. The farm code is indicated in the box, while each region is represented by a different color. Each point corresponds to a sample. Labels correspond to group centroids, which is the average position of individuals of each group along the discriminant axes. The X-axis and the Y-axis account for 72.4 % of the variance.

et al., 2024a). Nevertheless, control measures for CE are still needed throughout this entire region. The application of population genetics to *E. granulosus s.s.* in livestock would greatly help to obtain an accurate picture of how the domestic lifecycle is maintained. In this context, the development of a panel of microsatellites should considerably facilitate population genetic analyses compared with classical sequencing of mitochondrial genes.

The microsatellite panel developed in this study has proved to efficiently reveal the high genetic diversity found in CE livestock samples, which was already unveiled previously using the two EgSca6 and EgSca11 targets (Umhang et al., 2018). The high polymorphism of the seven targets initially selected has finally been streamlined into an efficient and relevant panel of five microsatellite targets. The exclusion of two microsatellites was based not only on technical considerations but also because of the genotype accumulation curves, and their very low impact on population genetics-based statistics using the five remaining targets. Regarding the international sample set, only two of the 145 MLGs were not unique, attesting to a very high genetic diversity. This is similar to that observed after sequencing the nearly complete mitogenome (11,682 bp) of 221 *E. granulosus s.s.* samples collected from 22 countries on different continents, which resulted in 171 haplotypes (Kinkar et al., 2017). Furthermore, the high number of private alleles identified here using the international sample set was already promising regarding the ability of the microsatellites to distinguish geographic clusters from national to local scale, which was the main aim in the development of this microsatellite panel.

Most of the CE samples tested corresponded to genotype G1 of *E. granulosus sensu stricto,* while eight samples among those from Armenia, France, Pakistan, Spain, and Türkiye were identified as G3. There were too few samples from each of these countries analyzed in our study to identify whether private alleles corresponded to this genotype or a geographic area. Nevertheless, as the genotype classification is based on the mitochondrial genome, it is to be expected that microsatellites could not distinguish between genotypes as they correspond to the non-coding nuclear genome. Moreover, the sequencing of three nuclear loci (2984 bp) has demonstrated no genetic separation of G1 and G3 genotypes, suggesting that these genotypes can be regarded as a single species (Kinkar et al., 2017). Additionally, the use of the different microsatellite markers for others *E. granulosus* species tested appears to be not relevant due to absence of amplification or of polymorphism, with a potential exception for Sca2-GTT. Nevertheless, these results confirm the necessity to initially confirm the *E. granulosus s.s.* species according to the knowledge of the epidemiological situation or using molecular methods trough sequencing of mitochondrial genes (Bowles et al. 1992) but also more directly by real-time PCR (Maksimov et al. 2020) or RFLP methods (Hidalgo et al., 2019; Santolamazza et al., 2020).

Due to the low number of samples available in each country (*i.e.* <7) for Morocco, France, Greece, Spain, Italy (Sardinia), Ukraine, Areminai, Turkyie and Pakistan, no other genetic or epidemiological conclusions can be obtained. The relevance of these samples was rather in order to obtain a broader overview of the genetic diversity at global scale. The use of DAPC for the subsampling of the international sample set (Algeria, Italy, Greece, and Tunisia) revealed a clear distinction between countries, with Greece more distant from the three others, as also confirmed by Fst values. The distinct genetic position of Greek *Echinococcus* isolates likely reflects ecological and structural factors beyond CE prevalence. Limited gene flow due to geographically isolated sheep and goat populations, together with traditional transhumance practices, may

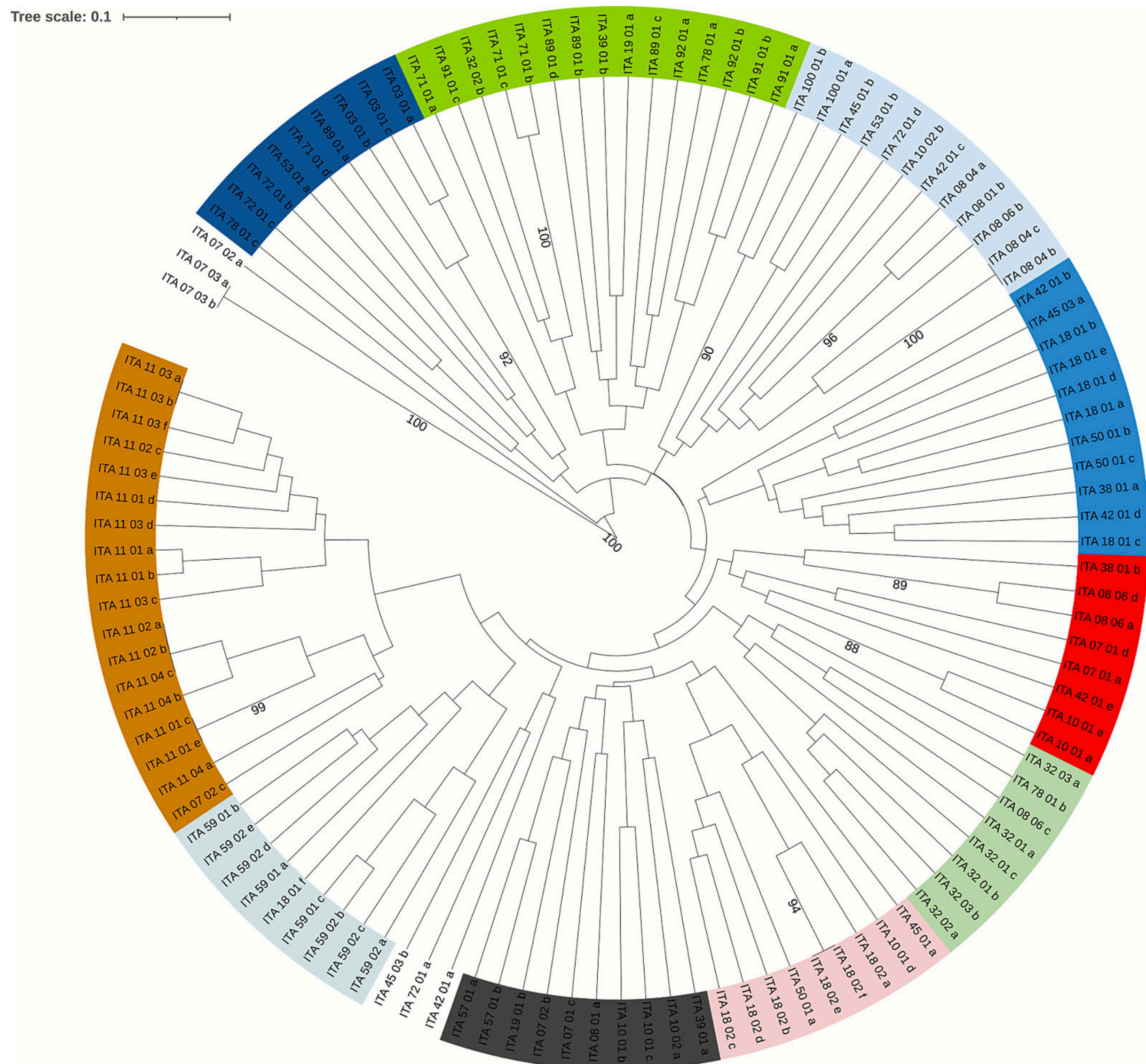

Fig. 7. Dendrogram based on Bruvo's distance to assess individual diversity from the 131 CE samples of the Italian regional sample set. Each sample is coded by the name of the country (ITA) followed by the number of the farm, the number of the animal from this farm and a letter corresponding to the cysts sampled from this animal (a to e for 1 to cysts).

maintain localized parasite genotypes (FAO, 2019). Animal trade is a major driver of parasite dispersal (Romig et al., 2017), and in Greece, live sheep trade is primarily Balkan-based, mostly from Romania, in contrast to other countries such as Italy or North African nations included in this study (Chiurciu et al., 2023). Variability in dog population management across the Balkans (WOAH, 2023) and the recent expansion of wild canids, particularly the golden jackal, may further shape transmission by introducing or sustaining distinct lineages (Karamanlidis et al., 2023). Integrated research approaches considering livestock movements, dog ecology, and wildlife reservoirs are required to fully understand parasite population structure (Hassell et al., 2025). Surprisingly, admixture between Italian and Algerian populations is slightly higher than between Algeria and Tunisia despite their geographic proximity. This situation may be explained by sampling bias, but could be linked to the historic relational past and current intensity of livestock trading between these countries, requiring specific future investigations.

The six different regions in the Italian sample set were clearly divided into three clusters, even as far down as the farm level. The first cluster, composed exclusively of the sheep samples from the farm in Eboli, Salerno (ITA11, Campania region), is explained by the absence of sheep trading with other farms in Italy. The flock is renewed either by self-replacement or through sheep purchased in France. The active CE lifecycle on this farm is maintained exclusively by micro-local transmission at the farm level. The second cluster concerns all the farms from Ricigliano (Salerno) in the Campania region except the one from Eboli, but also one from Lungro, Cosenza (ITA59, Calabria region). In addition to self-replacement, farmers purchase sheep only from Ricigliano, Salerno (Campania), resulting in the close genetic proximity between the CE samples from these farms. The same explanation applies to the farm from Lungro, as farmers often purchase sheep from the Ricigliano area. The third cluster is composed mainly of cattle samples (with only sheep samples from the Basilicata region) from farms in five different regions. As cyst fertility in cattle is very low in Italy, as in the rest of Europe, transmission is essentially based on a lifecycle between dogs and sheep (Poglayen et al., 2017). The sheep also present on these cattle farms are usually purchased in the central/southern regions of Italy, including Basilicata, in addition to self-replacement and the purchase of animals in the same areas. Therefore, cattle are infected as a result of dogs, themselves infected after eating sheep offal.

Overall, the microsatellite panel has been able to reveal the genetic structure of the parasite populations in these six regions, confirming the farming practices based on local maintenance of the parasite's lifecycle on each farm and trading of sheep between Italian farms from the same or other regions. This kind of information would be of great help in controlling CE, especially by providing additional arguments for banning dogs' access to offal during home slaughtering and for regular deworming of not only farm dogs, but also stray dogs (Ciccone et al., 2024; Nocerino et al., 2024a).

Intra-individual genetic diversity was assessed by analyzing several cysts from the same animal for both sheep and cattle in order to estimate the minimal number of infection events using the number of different MLGs as a proxy (Umhang et al., 2018). According to the samples analyzed in this study, it can be considered that most of the CE cysts in Italy are due to different infection events in sheep, and even more so in cattle. There is also confirmation that the development of cysts in the lungs and liver after ingestion of several eggs from a single infection event is rare. Both observations have previously been reported in the south of France and Tunisia (M'rad et al., 2020). Given that this tool has proven its usefulness for investigating infection events, a dedicated study with a larger total sample population would be necessary to confirm the trends observed here. As previously highlighted with EgSca6 and EgSca11 targets, it would be useful to analyze samples from dogs, as well as worms and eggs from feces, in order to evaluate genetic diversity at the origin of the environmental contamination to which livestock is exposed.

By performing one multiplex PCR followed by one capillary electrophoresis, this panel of five microsatellites can provide relevant data for a population genetic study of *E. granulosus s.s.* from global to farm scale. The simplicity and low cost of this method makes it a practical alternative to full mitochondrial genome sequencing, as it can be used to analyze a large number of samples to obtain a detailed overview of genetic diversity, which is useful input for a CE control program. Furthermore, mitochondrial sequences (especially when derived from the whole mitochondrial genome or at least a large part of it) are informative for phylogenetic and phylogeographical studies, helping to reveal deeper events, when nuclear data derived from highly variable microsatellites are especially useful for detecting more recent population events (as their mutation rate is usually much faster). Microsatellites are also better markers when one has to identify the source of infection, as they are more powerful in identifying individuals. It is notably expected that the panel can be used to track the source of human infection, especially in the case of a suspicion of imported cases, by providing genetic arguments in addition to other epidemiological data. The use of nuclear targets may also be useful to investigate the relative frequency of self-fertilization *versus* cross-fertilization notably focusing on the heterozygosity rate of the different markers. More specific studies using this microsatellite panel approach are now needed to confirm the promising results obtained.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2025.105868.

CRediT authorship contribution statement

Gérald Umhang: Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Vanessa Bastid:** Writing – review & editing, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Rinaldi:** Writing – review & editing, Resources, Funding acquisition. **Paola Pepe:** Writing – review & editing, Resources, Funding acquisition. **Elena Ciccone:** Writing – review & editing, Resources. **Smaragda Sotiraki:** Writing – review & editing, Resources. **Panagiota Ligda:** Writing – review & editing, Resources. **Myriam Oudni-M'rad:** Writing – review & editing, Resources. **Selim M'rad:** Writing – review & editing, Resources. **Samia Lahmar:** Writing – review & editing, Resources. **Yousra Said:** Writing – review & editing, Resources. **Kefiya Elmehatli:** Writing – review & editing, Resources. **Haroon Ahmed:** Writing – review & editing, Resources. **Tetiana Kuzmina:** Writing – review & editing, Resources. **Valentyna Yevstafieva:** Writing – review & editing, Resources. **Sargis A. Aghayan:** Writing – review & editing, Resources. **Hasmik Gevorgyan:** Writing – review & editing, Resources. **Sami Simsek:** Writing – review & editing, Resources. **Francesco Ponce-Gordo:** Writing – review & editing, Resources. **M.C. Benchikh El Fegoun:** Writing – review & editing, Resources. **Ikhlass El Berbri:** Writing – review & editing, Resources. **Ouafaa Fassi Fihri:** Writing – review & editing, Resources. **Urmas Saarma:** Writing – review & editing, Resources. **Frédéric Grenouillet:** Writing – review & editing, Conceptualization. **Franck Boué:** Writing – review & editing, Project administration, Funding acquisition. **Jaime Aguayo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Investigation, Formal analysis, Conceptualization.

Funding

This research was funded by the project "New sustainable tools and innovative actions to control cystic ECHINOcoccosis in sheep farms in the MEDiterranean area: improvement of diagnosis and SAFEty in response to climatic changes-ECHINO-SAFE-MED," supported by PRIMA (Partnership for Research and Innovation in the Mediterranean Area) and the European Union's Horizon 2020 Research and Innovation programme under grant number 773830: One Health European Joint Programme (MEME project; https://onehealthejp.eu/jrp-meme/). The Estonian Ministry of Education and Research also contributed to funding (grants PRG1209 and TK215).

Declaration of competing interest

The authors declare no conflict of interest

Data availability

Data will be made available on request.

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