A dual PCR-based sequencing approach for the identification and discrimination of *Echinococcus* and *Taenia* taxa

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ABSTRACT

Reliable and rapid molecular tools for the genetic identification and differentiation of *Echinococcus* species and/or genotypes are crucial for studying spatial and temporal transmission dynamics. Here, we describe a novel dual PCR targeting regions in the small (*rrnS*) and large (*rrnL*) subunits of mitochondrial ribosomal RNA (rRNA) genes, which enables (i) the specific identification of species and genotypes of *Echinococcus* (*rrnS*+*L*-PCR) and/or (ii) the identification of a range of taeniid cestodes, including different species of *Echinococcus*, *Taenia* and some others (17 species of diphyllidean helminths). This dual PCR approach was highly sensitive, with an analytical detection limit of 1 pg for genomic DNA of *Echinococcus*. Using concatenated sequence data derived from the two gene markers (1225 bp), we identified five unique and geographically informative single nucleotide polymorphisms (SNPs) that allowed genotypes (G1 and G3) of *E. granulosus* sensu stricto to be distinguished, and 25 SNPs that allowed differentiation within *E. canadensis* (G6/7/8/10). In conclusion, we propose that this dual PCR-based sequencing approach can be used for molecular epidemiological studies of *Echinococcus* and other taeniid cestodes.

Keywords: *Echinococcus* spp.; *Taenia* spp., PCR; specific and genotypic identification; 12S and 16S nuclear ribosomal RNA genes.

1. Introduction

Echinococcosis is a parasitic disease caused by the infection with tiny tapeworms of the genus *Echinococcus*, with *E. granulosus* and *E. multilocularis* being the species of principal medical importance. The life cycles of *Echinococcus* species predominantly involve a definitive host and an intermediate host, each harbouring different developmental stages of the parasite. The larval stage (metacestode) develops mainly within the liver, but also in other organs, of the intermediate host, while adult worms parasitize mostly the small intestine of canids, and produce eggs, which are infective to intermediate hosts. Humans are accidental intermediate hosts [1]. The two main and pathologically distinct forms of disease in humans are alveolar echinococcosis (AE) caused by *E. multilocularis*, and cystic echinococcosis (CE) caused by *E. granulosus* sensu lato (s.l.) [1].

Formerly, a high degree of genetic diversity was found within *E. granulosus* s.l.; thus, 10 genotypes (G1-G10) have been documented based on analyses of mitochondrial DNA (mtDNA) regions [2–4]. Subsequently, the taxonomy of the genus *Echinococcus* was reviewed to clarify the systematic status of various species and genotypes [5,6]. This revision has led to the attribution of species status to some of the *E. granulosus* genotypes. Thus, *E. granulosus* is now recognized as a complex of four cryptic species: *E. granulosus* sensu stricto (s.s.) (genotypes G1/2/3), *E. equinus* (G4), *E. ortleppi* (G5) and *E. canadensis* (G6/7/8/10) [5,6]. A large number of comprehensive analyses of mitochondrial [6,7] and nuclear genome targets [8,9] in *Echinococcus* has not assisted in fully resolving some nomenclature and taxonomic issues. For instance, there is still controversy as to whether *E. canadensis* (G6/7/8/10) comprises at least three species [9–15]. In contrast to *E. granulosus* s.l., the genetic differences among *E. multilocularis* isolates from different geographical areas are minimal [16,17].

The molecular epidemiology of the genus *Echinococcus* is of substantial importance, since it allows a better understanding of the spatio-temporal distribution patterns of individual species/genotypes, and their relative contribution to human alveolar and cystic echinococcosis. Such investigations also aid in the planning and implementation of disease control measures [18–20]. Indeed, cumulative ‘genotyping’ data for human isolates have revealed that *E. granulosus* s.s. (G1/2/3) and *E. canadensis* (G6/7) are the two commonest species responsible for human CE [19,21].

To date, molecular phylogenetic analyses have relied on DNA sequence data obtained from partial single loci (ranging from ~250 to 500 bp in length), particularly from mitochondrial DNA loci, such as cytochrome *c* oxidase subunit 1 (*cox1*) [17,22,23], NADH dehydrogenase subunit 1 (*nad1*) [22–25], ATP synthase subunit 6 (*atp6*) [26] and the small subunit (*SSU*) of the rRNA gene [27,28]. However, these relatively short sequences have insufficient discriminatory power to resolve fine scale taxonomic peculiarities (e.g. placement of strains/haplotypes into clades) within the *E. granulosus* complex, particularly in relation to *E. granulosus* s.s. (G1/2/3) and *E. canadensis* (G6/7/8/10). Thus, the selection of gene markers useful that allow the accurate identification and differentiation of species and genotypes (intraspecific variants) has been a major challenge for systematic and molecular epidemiological studies of *Echinococcus* and other taeniids.

Recently, we developed a multiplex PCR (Egc-mPCR) as a rapid and reliable test for the identification of all recognized species within the *E. granulosus* complex [29]. However, like other one-step techniques, such as high-resolution melting (HRM) [30], loop-mediated isothermal amplification (LAMP) [31] and multiplex PCR [32], the Egc-mPCR did not permit further extensive and deeper systematic analyses.

In the present study, we developed a dual PCR-coupled sequencing methods (using *rrnS* and *rrnL*) for the identification and characterization of all species and genotypes of *Echinococcus*. To this end, we designed two primer sets: (i) the primer pair *rrnS*-F and R is highly specific for the genus *Echinococcus* and (ii) the *rrnL*-F and *rrnL*-R amplifies a region within *rrnL* from taeniid cestodes, including all recognized species and genotypes of *Echinococcus*. We assessed and validated the analytical sensitivity and specificity of the dual PCR using a panel of *Echinococcus* reference DNAs (representing cysts or eggs) and DNA samples from other helminths (cestode, nematode and trematode species).

2. Materials and methods

2.1. Ethical aspects

Parasite specimens of animal origin were derived from an existing bio-bank collection of the Institute of Parasitology (Bern, Switzerland), and some of these samples had already been used in previous studies [29,33]. Samples were collected within the framework of governmentally regulated meat inspections in slaughterhouses; respective specimens were sent to the laboratory of Parasitology for parasitological and molecular identification. Parasite samples from human patients were derived from an existing collection of *Echinococcus* DNA-biobank at the EPS Fattouma Bourguiba Hospital in Monastir/Tunisia. This collection had been designed and ethically approved for basic research studies.

2.2. Biological materials

A panel of *Echinococcus* genomic DNA (gDNA) samples was used to assess the sensitivity and specificity of the two PCR assays established here. These DNAs had been individually extracted from metacestodes (hydatid cysts for *E. granulosus*, metacestode tissue for *E. multilocularis* or polycystic tissue for *E. vogeli*). Table 1 provides detailed information (including geographic origin, intermediate host and metacestode localization) about these *Echinococcus* reference gDNAs. The genetic identity of the samples (species/genotypes) representing the *E. granulosus* complex was determined based on the amplification profile of our previously published multiplex PCR [29], whereas the *E. multilocularis* and *E. vogeli* samples had been identified previously via *cox1* and *nad1* sequencing [34]. Additional taeniid and/or *E. multilocularis* egg gDNA samples were obtained from the institutional DNA-collection of the Institute of Parasitology, University of Zürich (Switzerland). Taeniid eggs (including *E. multilocularis*) were isolated using a sieving-flotation technique [35], and species identification was performed using the triplex-PCR published by Trachsel et al. [36]. Upon isolation/purification of eggs, 8 faecal samples yielded only *E. multilocularis* (3 dogs and 5 foxes), 2 samples only *E. granulosus* s.l. (1 dog and 1 wolf), and 5 faecal samples (2 wolves and 3 fox) only *Taenia* species.

A panel of gDNA samples isolated from other helminths included 1 trematode species (*Dicrocoelium dendriticum*), 7 cestode species (*Mesocostoides corti*, *Taenia hydatigena*, *T. crassiceps*, *T. polyacantha*, *T. multiceps*, *T. taeniaeformis* and *Hymenolepis diminuta*) and 3

nematode species (*Ascaris suum*, *Toxocara canis* and *Ancylostoma duodenale*). These samples were used to test for potential cross-amplification in the dual PCR established here. We used ~ 50 ng of each DNA per PCR.

2.3. Primer design and PCR conditions

All available complete mitochondrial genome sequences for *Echinococcus* (species/genotypes) and the entire mitochondrial genomes for other helminths (13 cestodes, 4 nematodes and 2 trematodes) of human or dog origin were retrieved from GenBank (National Centre for Biotechnology Information, NCBI), imported and aligned using ClustalW in the program BioEdit (version 7.0.9). Complete data on accession numbers and the species names are given in Supplementary Table A.1. Two primer pairs were designed: *rrnS*-F/*rrnS*-R for all *Echinococcus* species and genotypes, and *rrnL*-F/*rrnL*-R for all *Echinococcus* species members and 13 taeniid cestodes (corresponding to the selection that was included in the nucleotide alignment). The sequences and characteristics of the primers are shown in Table 2.

The QIAGEN Multiplex PCR Kit that contains HotStarTaq DNA Polymerase (Qiagen) was used for the amplification of partial sequences of the *rrnS* and *rrnL*. PCR reaction mix (50 µl) consisted of 25 µl of multiplex PCR master mix, 0.5 µM of each primer, and 19 µl H₂O and 5 µl of template DNA. Cycling conditions were: 95°C/15 min, 40 cycles (94°C/30 s, 56°C (*rrnS*) or 58°C (*rrnL*)/30 s, 72°C/30 s) and 72°C/10 min. The PCR products were detected by electrophoresis in 1.5% agarose gels stained with ethidium bromide and purified with the High Pure PCR Product Purification kit (Roche Applied Science) according to the manufacturer's instructions. Subsequently, they were sequenced using an automated DNA sequencer (Applied Biosystems, ABI 3130× I Genetic Analyzer Sequencer) using the same primers as use for amplification. Resultant sequences were edited using BioEdit (version 7.0.9.0). The Basic Local Alignment Search Tool (BLAST) of NCBI was used to confirm the identity of each sequence.

To verify nucleotide variations between species/genotypes, we repeated the PCR-based sequencing for a representative set of samples, but employing proofreading polymerase (*Pfu* DNA Polymerase; Promega). Here, PCRs were performed in 40 µl reaction mixes containing 0.5 µM each forward and reverse primers, 100 µM dNTPs mix (10 mM), 0.05 units µl⁻¹ *Pfu* DNA polymerase in 1× *Pfu* DNA polymerase Buffer and 2 µl of DNA. Cycling conditions were: initial denaturation 95°C/2 min, 30 cycles of 95°C/30 s, 56°C (*rrnS*) or 58°C (*rrnL*)/30 s and 72°C/30 s) followed by a final extension step of 72°C/5 min.

2.4 Analytical sensitivity of the assay

To determine the analytical sensitivity of the dual PCR, different amounts of *E. granulosus* s.s. (G1) DNA (5 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg) was tested in the PCRs described in subsection 2.3.

2.5. Phylogenetic analysis and tree construction

All partial *rrnS* and *rrnL* sequences obtained from amplicons for *Echinococcus* reference gDNA samples (see Table 1) were edited and aligned using BioEdit software (version 7.0.9.0.). The sequences were also mapped to reference mitochondrial genomes representing *Echinococcus* (all accession numbers given in Supplementary Table 1). Phylogenetic analyses were performed on individual partial gene sequences as well as on the concatenated (*rrnS* + *rrnL*) data set employing MEGA software (Molecular Evolutionary Genetics Analysis 3.1; <http://www.megasoftware.net>). To reconstruct phylogenetic trees, we used tree-building methods: neighbour joining (NJ) [37] [38], maximum parsimony (MP) [39–43], and maximum likelihood (ML) [38,41,44]. To evaluate the robustness of each tree, a Bootstrap (BS) values were determined by bootstrap resampling (BS) [45] (1000 random repetitions (NJ), 100 replications (MP and ML), and 100% and 70% were considered as high and moderate BS values.

3. Results

3.1 Sensitivity and specificity of PCRs

The analytical sensitivity of the two PCRs was assessed by using various concentrations of *E. granulosus* s.s. (G1) DNA template. As shown in Figure 1, the sensitivity analysis revealed a similar detection limit of 1 pg of gDNA template for both primer pairs (Fig. 1).

The specificity of the PCRs was assessed using 11 gDNA control samples derived from trematodes, nematodes and non-*Echinococcus* cestodes. Results showed complete agreement between predicted and observed specificity of the *rrnS*-PCR. Indeed, the *rrnS*-F/*rrnS*-R primer pair did not amplify from DNA from heterologous helminth species tested (n = 11), indicating the specificity of these primers for the specific amplification from DNA of *Echinococcus* species and genotypes. As shown in Fig. 2, the primer pair *rrnL*-F/*rrnL*-R is

cestode-specific, amplifying only the target sequences (648 bp) from DNA from all 7 cestode species tested here. The dual PCR did not show any cross-amplification from any of the DNA samples from nematodes or trematodes.

3.2. Sequencing of amplicons for *Echinococcus* genotyping

Both *rrnS* and *rrnL* gene fragments of expected lengths were amplified from all *Echinococcus* reference DNA samples (Table 1; Fig. 3). Sequence analyses of these fragments allowed the identification of and differentiation among all *Echinococcus* species and genotypes, including *E. granulosus* s.s. (G1) and *E. granulosus* s.s. (G3) and members of the *E. canadensis* (G6-G10) clade. Three (positions 152, transition A/G; position 183, transition G/A and position 533, transversion T/G) and two (position 338, transition C/T; position 580, transition A/G) stable and unique discriminatory single nucleotide polymorphisms (SNPs) were detected in the *rrnS* and *rrnL* regions, respectively. This subset of 5 unique SNPs allowed the accurate distinction between *E. granulosus* s.s. (G1) and *E. granulosus* s.s. (G3) from distinct geographical areas (Tunisia, Algeria, Italy and Greece). Four out of seven *E. granulosus* s.s. (G1/2/3) isolates were identified as *E. granulosus* s.s. (G3) (Table 1). For differentiation within *E. canadensis* (G6/7/8/10), we found 25 SNPs; 12 are present in *rrnS* and 13 in the *rrnL* markers (Table 3). Phylogenetic trees constructed based on the partial *rrnS*, *rrnL* and on concatenated (*rrnL* + *rrnS*) partial sequences are displayed in Fig 4. Three isolates (2 from Tunisia and 1 from Algeria) clustered within *E. granulosus* s.s (G1), and four sequences (1 Tunisian, 1 Algerian, 1 Italian and 1 Greek) grouped within *E. granulosus* s.s (G3) with high bootstrap support of 100% (NJ/MP/ML)

Genomic DNA samples that were extracted from taeniid eggs were tested using the dual PCR assay. All samples were positive in the *rrnL*-PCR and yielded a band of the expected size (648 bp), indicating the presence of taeniid cestode DNA in these samples. Sequencing revealed *E. multilocularis* in 8 samples (5 from foxes and 3 from dogs); *E. canadensis* (G7) was found in 1 dog, *E. granulosus* s.s. (G1) in 1 wolf, *T. taeniaeformis* DNA in 1 fox, *T. hydatigena* in 2 foxes and *T. multiceps* in 2 wolves. Using the *rrnS*-PCR, only samples containing *E. multilocularis* or *E. granulosus* s.l. DNA were test positive. For these samples, DNA sequencing results was in complete agreement with those obtained by sequencing the *rrnL* gene region.

4. Discussion

Several molecular tools have been developed for the genetic characterization of different species from the genus *Echinococcus*. The aim of the present study was to enhance the utility of a diagnostic PCR developed previously [29] to enable inter- and intra-species discrimination among all important members of the genus *Echinococcus*, and the detection of other non-*Echinococcus* cestodes. Although the previously described PCR [29] was inexpensive and easy to perform, the following limitations became apparent: it could not distinguish between *E. granulosus* s.s. genotypes (G1/3); it worked reliably only on DNA extracted from cysts; it was not sensitive enough to be used for the specific identification of eggs when these were present in low numbers [29]; extensive phylogenetic analysis was not possible because of limited informative nucleotide sites; and, finally, taeniids other than *Echinococcus* could not be detected. Similar issues might hamper other one-step or single species-specific detection systems [30–32].

Since it was shown that mitochondrial gene markers (sensitivities varying between 73.7% and 100%) provide a better performance for the detection and identification of *Echinococcus* species than nuclear targets (sensitivity of 52.6%) [46], we focused here on the identification of mitochondrial genome-derived markers. Furthermore, it is generally well-accepted that mitochondrial DNA evolves faster than the nuclear genome and appeared to render mitochondrial DNA more appropriate for genetic analyses of the genus *Echinococcus* [22,23,25–28]. In this study, we established a dual PCR assay for the specific detection of taeniids as well as *Echinococcus*. The *rrnS*-PCR detected all species and genotypes of *Echinococcus* included here, and the *rrnL*-PCR theoretically detected 17 cestode species (only 7 species were experimentally tested), including *Echinococcus*.

For the analysis of specificity, we did not test all helminth species that were included in the alignment of the mitochondrial genome sequences (32 species). However, based on aligned mitochondrial DNA sequence data, (i) the *rrnS*-F/*rrnS*-R primer pair should also detect *E. shiquicus*, *E. oligarthrus* and *E. felidis*, and (ii) the *rrnL*-F/*rrnL*-R primer pair detected other cyclophyllidean species included here (*Mesocestoides corti*, *Taenia hydatigena*, *T. crassiceps*, *T. polyacantha*, *T. multiceps*, *T. taeniaeformis* and *Hymenolepis diminuta*), and are thus proposed to amplify from other cestodes (*T. serialis*, *T. solium*, *T. ovis*, *T. madoquae*, *T. regis*, *T. twitchelli*, *T. arctos*, *T. martis*, *Dipylidium caninum* and *Versteria mustelae*). Since large differences in the primer binding sites are present between cestodes, on one hand, and trematodes or nematodes, on the other, no cross-amplification is expected. However, further experiments need to be performed to test these assumptions.

The two PCRs were highly sensitive and showed a very low detection-limit in the range of 1pg *Echinococcus* DNA. This result is comparable to previously described PCR-based approaches for molecular diagnosis of *E. granulosus* s.l. [47,48] and/or *E. multilocularis* [28,36] in the definitive hosts, where the presence of one egg was sufficient to give a specific signal (one egg contains approximately 8 pg of DNA [49]).

The PCRs allowed the discrimination between all *Echinococcus* species and genotypes included in this study, and phylogenetic trees constructed using either *rrnS*, *rrnL* or concatenated sequence data produced similar topologies, in particular the close relationships between *E. granulosus* s.s. or *E. canadensis* genotype members [5]. Since the discrimination between G1 and G3 has been a controversial issue and has largely relied on the use of mitochondrial gene markers [26], many studies have allowed only *E. granulosus* s.s. to be defined, without a discrimination among genotypes G1, G2 and G3 [50,51]. Consequently, the real contribution of *E. granulosus* s.s. (G3) to human CE might be underestimated. On the other hand, one may raise the question as to how far discriminating among these conventional genotypes (i.e. G1-G3) makes sense in the context of large haplotype diversity. Future studies should help to better address these questions. The complete mitochondrial genome of *E. granulosus* s.s. (G3) was sequenced only a year ago [52] and was used in our study for primer design. Consequently, the PCRs established here should improve the genotyping by clarifying the specific and genotypic identity of isolates.

In most cases, molecular studies of *Echinococcus* from definitive and/or intermediate hosts have been carried out using different molecular tests, depending on the origin of the biological matrix used for analysis. However, it was recently shown that the genetic diversity of *E. granulosus* s.s. in intermediate animal hosts is greater than in definitive hosts [53]. This highlights the importance of including samples derived from both hosts in a given area to elucidate the population structures and the genetic complexity of *Echinococcus*, and to use an appropriate methodology. In our experiments, besides the high performance of the present PCRs on DNA extracted from *Echinococcus* larval stage, we also obtained successful and specific amplification of the partial *rrnS* and *rrnL* sequences from the 16 *Echinococcus* DNA samples derived from eggs. However, despite this promising potential of these PCRs to be applied for comparative assessments of the genetic variability of *Echinococcus* in intermediate and definitive hosts, future research should be conducted using a larger and more diverse panel of samples. The PCRs should also be tested on DNA isolated directly from faeces from *Echinococcus*-infected definitive hosts [54], or even from environmental samples, such as soil or water [55]. In this regard, the sensitivity of the present PCRs might be

302 challenged, possibly requiring an enrichment of parasite DNA prior to performing molecular
303 analysis, as indicated recently by other workers [54].

304 In conclusion, we have established two effective PCRs, targeting regions of the
305 mitochondrial *rrnS* and *rrnL* genes. These tests can be applied for a high-resolution
306 characterization of all *Echinococcus* species and genotypes. In addition, the *rrnL*-PCR can be
307 used for the detection and identification of at least 17 cestode species other than
308 *Echinococcus*.

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Figure legends:

Fig. 1. Sensitivity of the *rrnS*- and *rrnL*-PCRs. Analytical sensitivity determined using different amounts of *E. granulosus* s.s. (G1) DNA; Lane M: 100 bp DNA ladder; lanes 1–8, amplicons produced from DNA quantities of 5 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg and 1 fg in a 20 µl PCR reaction volume; lane N: negative control (2 µl sterile water as a template).

Fig. 2. Specificity of the *rrnL*-PCR
PCR product of the partial *rrnL* sequence from 7 different cestode species Lane 1: 1 trematode species (*Dicrocoelium dendriticum*), lane 2-4: 3 nematode species (*Ascaris suum*, *Toxocara canis*, and *Ancylostoma duodenale*), lane 5-11: 7 cestode species (*Mesocostoides corti*, *T. hydatigena*, *T. crassiceps*, *T. polyacantha*, *T. multiceps*, *T. taeniaeformis* and *Hymenolepis diminuta*); Lane M: 100 bp DNA ladder.

Fig. 3. Performance of the *rrnS*- and *rrnL*-PCRs using *Echinococcus* DNAs
Agarose gels (1.5%) displaying PCR products derived from partial mitochondrial *rrnS* and *rrnL* gene sequences from *Echinococcus* reference DNA(s). Lane M: 100 bp DNA ladder; lane 1: *E. granulosus* s.s. (G1), lane 2: *E. granulosus* s.s. (G3), lane 3: *E. equinus* (G4), lane 4: *E. ortleppi* (G5), lane 5: *E. canadensis* (G6), lane 6: *E. canadensis* (G7), lane 7: *E. canadensis* (G8), lane 8: *E. canadensis* (G10), lane 9: *E. multilocularis*, lane 10: *E. vogeli* and lane N: negative control (2 µl sterile water as a template).

Fig. 4. Phylogenetic trees inferred with the Neighbour-Joining (NJ) and based on partial *rrnS* (A), partial *rrnL* (B) and concatenated partial gene sequences *rrnL*+ *rrnS* (C). Neighbor-joining (NJ, 1000 replicates), maximum parsimony (MP, 100 replicates) and maximum likelihood (ML, 100 replicates) methods were used to estimate the bootstrap values (shown in the order of NJ/MP/ML). Only bootstrap values higher than 70% are shown.

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510 Table 1

511 *Echinococcus* reference gDNA samples

Sample (gDNA)	Country	I. host	Cyst localization	<i>Echinococcus</i> spp. ^{a/b}	<i>Echinococcus</i> spp. (This study)
Tun_SL321	Tunisia	sheep	lung	^a <i>E. granulosus</i> s.s (G1/2/3)	<i>E. granulosus</i> s.s. (G1)
Tun_SLv346	Tunisia	sheep	liver	^a <i>E. granulosus</i> s.s (G1/2/3)	<i>E. granulosus</i> s.s. (G3)
Tun_HL315	Tunisia	human origin	lung	^a <i>E. granulosus</i> s.s. (G1/2/3)	<i>E. granulosus</i> s.s. (G1)
Alg_SL533	Algeria	sheep	lung	^a <i>E. granulosus</i> s.s.(G1/2/3)	<i>E. granulosus</i> s.s. (G1)
Alg_SLv312	Algeria	sheep	liver	^a <i>E. granulosus</i> s.s. (G1/2/3)	<i>E. granulosus</i> s.s. (G3)
Gre_SL276	Greece	sheep	lung	^a <i>E. granulosus</i> s.s. (G1/2/3)	<i>E. granulosus</i> s.s. (G3)
Ita_S1	Italy	–	–	^a <i>E. granulosus</i> s.s. (G1/2/3)	<i>E. granulosus</i> s.s. (G3)
Spa_S1	Spain	donkey	liver	^a <i>E. equinus</i> (G4)	<i>E. equinus</i> (G4)
Arg_S1	Argentina	cattle	lung	^a <i>E. ortleppi</i> (G5)	<i>E. ortleppi</i> (G5)
Alg_Cl12	Algeria	camel	lung	^a <i>E. canadensis</i> (G6/7)	<i>E. canadensis</i> (G6)
Aut_PLv	Austria	pig	liver	^a <i>E. canadensis</i> (G6/7)	<i>E. canadensis</i> (G7)
Est_CS1	Estonia	cervid	-	^a <i>E. canadensis</i> (G8/10)	<i>E. canadensis</i> (G8)
Est_CS2	Estonia	cervid	-	^a <i>E. canadensis</i> (G8/10)	<i>E. canadensis</i> (G10)
Swi_Em	Switzerland	-	-	^b <i>E. multilocularis</i>	<i>E. multilocularis</i>
Cnd_Em	Canada	-	-	^b <i>E. multilocularis</i>	<i>E. multilocularis</i>
Bra_Ev	Brazil	-	-	^b <i>E. vogeli</i>	<i>E. vogeli</i>

512 ^a: *Echinococcus* species was determined by multiplex PCR (Egc-mPCR) performed according to [29].

513 ^b: *Echinococcus* species was determined via *cox1* and *nad1* sequencing [34].

514

515 Table 2

516 Details of primers used in this study

Gene	Primer	Primer sequence (5' to 3')	length h (bp)	Amplified length (bp)	*T _m (°C)
<i>rrnS</i>	<i>rrnS</i> -F	GCAAAAGCTGATTAGGG	17	577	56
	<i>rrnS</i> -R	TAACACACAAAAACTC	16		
<i>rrnL</i>	<i>rrnL</i> -F	TTATTTGCCTTTTGCATCA	19	648	58
	<i>rrnL</i> -R	AAAAGATCCTAGGGTCTTCCGT	23		

517 (*) annealing temperature

518

519 Table 3

520 Single nucleotide polymorphisms (SNPs) in partial gene sequences of the *rrnS* and *rrnL*
 521 genes within *E. canadensis* (G6/7/8/10)

<i>rrnS</i> (577 bp)	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>11</i>	<i>12</i>	
Position	66	92	102	106	107	110	189	224	255	411	422	495	
<i>E. canadensis</i> (G6)	A	A	T	G	A	A	G	G	C	A	C	G	
<i>E. canadensis</i> (G7)	A	A	T	A	A	G	G	G	C	A	C	G	
<i>E. canadensis</i> (G8)	G	G	T	G	G	T	G	A	C	G	C	A	
<i>E. canadensis</i> (G10)	G	A	C	G	A	A	A	G	T	A	T	G	
<i>rrnL</i> (648 bp)	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>11</i>	<i>12</i>	<i>13</i>
Position	41	62	79	109	127	208	350	396	441	509	582	591	603
<i>E. canadensis</i> (G6)	G	C	A	A	C	G	G	T	A	A	T	A	C
<i>E. canadensis</i> (G7)	G	C	A	A	C	G	G	T	A	A	T	A	C
<i>E. canadensis</i> (G8)	G	T	A	A	T	G	A	C	A	G	C	A	T
<i>E. canadensis</i> (G10)	T	T	G	G	T	A	A	T	C	A	T	G	C

522