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ORIGINAL PAPER

Detection of the G3 genotype of *Echinococcus granulosus* from hydatid cysts of Chilean cattle using COX1and ND1mitochondrial markers

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Abstract For a deeper understanding of the phylogenetic relationships of *Echinococcus* genotypes and species in different intermediate hosts, we analyzed samples from human and bovine hydatid cysts. For this, segments of the cytochrome oxidase (COX1) and NADH dehydrogenase (ND1) mitochondrial genes were used. To obtain sufficient amounts of the ND1 marker to be sequenced properly, a new variant of the PCR assay was implemented. Phylogenetic analysis with both markers showed that most of the analyzed samples correspond to genotype G1. However, a sample from cysts of a bovine lung (Q21), with the COX1 marker, was grouped in a node together with a sample belonging to genotype G3. In the phylogenetic tree obtained with the ND1 marker, this sample was grouped with sequences of genotypes G3, G2, and G4. Analyzing the single nucleotide polymorphic (SNP) sites of both markers, it was observed that the Q21 sequence is almost identical to the G3 sequence and differ in only one SNP from the G2 sequence, and is completely different from G4. These results are noteworthy, since neither G2 nor G3 genotypes have been described previously in Chile, raising the possibility that the G3 genotype is present in these latitudes. This information is highly relevant; it can be employed to uncover additional unknown details of transmission cycles of this important parasite.

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Introduction

Cystic echinococcosis is a disease caused by a parasite belonging to the *Echinococcus granulosus* complex; it is considered as endemic in some regions of Chile and other countries that have a large production of sheep and herbivores related to the use of dogs as pasturage animals (Thompson et al. 2006; Moro et al. 2009). This disease causes a huge social and economic impact in several countries due to poor campaigns and control and surveillance programs applied by the sanitary departments.

Since 1951, echinococcosis has been considered as an obligatory notification disease in Chile. In 2011, in humans, 256 cases were notified (Fuenzalida 2012), and according to the total population determined by the last census (16,572, 475), an incidence of 1.54 cases per 100,000 inhabitants is calculated. However, based on hospital discharge data of patients who underwent removal surgeries, several authors, including the minister of health, have suggested that sub-

notification of this disease exists (Apt et al. 2000; Minsal 2002; Noemí et al. 2003; Martínez 2011). Therefore, the actual incidence of this disease in our country may be at least three times higher than reported, that is to say 4.62 cases per 100,000 inhabitants. Chilean regions with highest incidence rate per 100,000 inhabitants in 2011 were Aysén (35.89), Los Ríos (5.52), La Araucanía (4.60), Biobío (3.37), Coquimbo (2.74), and Magallanes (2.51). In animals, the number of annual cases confirmed in Chile was 76,075 infected individuals (cattle, sheep, camelids, etc.); the regions with the largest number of cases were Biobío, La Araucanía, and Aysén, according to the biannual report of the "Oficina Internacional de Epizootias" (OIE 2011a; 2011b).

Until a few years ago, in the genus *Echinococcus* only, four species were recognized as follows: *Echinococcus granulosus*, *Echinococcus multilocularis*, *Echinococcus vogeli*, and *Echinococcus oligarthus* (Thompson and McManus 2002). From an epidemiological point of view, several strains were associated with different intermediate hosts. More recently, based on different mitochondrial sequences of the strains, ten genotypes were described (Thompson and McManus 2002; Thompson 2008). However, currently, it is accepted that the species *E. granulosus* includes only genotypes G1, G2, and G3, which are called *E. granulosus sensu stricto* (Thompson 2002; Thompson and McManus 2002; Jenkins et al. 2005; Moks et al. 2008). The other seven genotypes are different species; *E. equinus* (G4), *Echinococcus ortleppi* G5), and *Echinococcus canadensis* (G6–G10), (Thompson and McManus 2002; Thompson 2008). Nevertheless, the taxonomic status of genotypes G9 and G10 is not fully elucidated.

Mitochondrial and nuclear genes have been used to identify determined genotypes during the last two decades, allowing deeper phylogenetic studies in this cestode. The most used genes due to their variability and capacity to differentiate between the genotypes have been the mitochondrial encoding genes of subunit 1 of cytochrome oxidase C (COX1) (Bowles et al. 1992) and subunit 1 of NADH dehydrogenase (ND1) described by Bowles and McManus (1993). Other mitochondrial genes less used are the ATPase subunit 6 gene (ATP6), cytochrome B, the genes from subunits 1 to 3 of cytochrome oxidase (COX1, COX2, COX3), the genes from subunits 1 to 6 of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND5, ND6), and 4 L NADH dehydrogenase (Nakao et al. 2007; Moks et al. 2008).

Also, a nuclear gene has been described to complement the studies of genotype identification using the method of digestion of the amplification product with restriction enzymes (PCR-RFLP). The amplified gene is the internal transcribed spacer 1 (Shahnazi et al. 2011).

In Chile, studies were conducted using two genomic segments as markers called Eg9 and Eg16 by the PCR-RFLP method and then confirmation with the COX1 marker in 20 samples of hydatid cysts removed from humans. The authors found that the majority of the genotypes belong to the G1 genotype (Manterola et al. 2008); only one was G6. At the beginning, the latter genotype was associated with camels, and subsequently, it was also found in hydatid cysts from human patients in Argentina (Rosenzvit et al. 1999).

Identification of different genotypes of parasites that circulate in a specific endemic region is important not only due to the specificity with different intermediate hosts, but also to the time that the cysts take to grow inside the definitive host; thus based on which genotype is more prevalent, the period of antiparasitological treatments of dogs and the principal reservoirs of the parasite should be adjusted to the time of maturation of the platyhelminth (Guarnera et al. 2004; Thompson 2008).

The general objective of this study was to determine the phylogenetic relationships of *Echinococcus* spp. isolated from hydatid cysts of different intermediate hosts (human and cattle) from the central–southern area of Chile. The specific objectives were to identify the genotype of the parasite of each sample (humans and cattle) and to establish their phylogenetic relationships with published data sequences of hydatid cysts extirpated from different intermediate hosts coming from Chile and abroad (Bowles and McManus 1993; Le et al. 2002; Lavikainen et al. 2003; Nakao et al. 2007;; Moro et al. 2009; Soriano et al. 2010; Sharbatkhori et al. 2011; Boufana et al. 2012; Piccoli et al. 2012; Rajabloo et al. 2012).

Materials and methods

Sampling

The samples of hydatid cysts were collected from May to September, 2012. They were frozen and then transported to the laboratory. The samples of cattle were provided by several slaughterhouses from the Metropolitan and La Araucanía regions. The human sample of hydatid cyst was obtained from the Barros Luco Hospital (Table 1). All the procedures were approved by the bioethics committee of our institution

DNA extraction, amplification, and sequenciation. Genomic DNA was extracted using the EZNA® tissue DNA kit (Omega Biotech, Inc., Doraville, GA, USA) from the cyst membrane and from the hydatid liquid (obtained by puncture) as previously described (Zhang et al. 1998). The efficiency of the DNA extraction from each sample was judged by electrophoresis in agarose gels stained with ethidium bromide. In most of the samples, a large diffuse band was detected only in samples from the membrane of the cyst. However, all samples were subsequently analyzed with the implemented PCR methods for different gene markers due to their great sensitivity.

Partial sequences from the COX1 and ND1 genes that are commonly used for the identification of the species within the

Table 1 Characteristics of the samples analyzed in this study

Sample code	Species of origin[a]	Geographical origin	Organ of origin	Analyzed cyst[b]	Kind of sample[c]
Q1	Human-1	Panguipulli	Liver	A	L
Q2	Human-1	Panguipulli	Liver	A	M
Q3	Bovine-1	Osorno	Liver	B	L
Q4	Bovine-1	Osorno	Liver	B	M
Q5	Bovine-2	Osorno	Liver	C	L
Q6	Bovine-2	Osorno	Liver	C	M
Q7	Bovine-3	Osorno	Liver	D	L
Q8	Bovine-3	Osorno	Liver	D	M
Q9	Bovine-4	Nva. Imperial	Liver	E	L
Q10	Bovine-4	Nva. Imperial	Liver	E	M
Q11	Bovine-4	Nva. Imperial	Liver	F	L
Q12	Bovine-4	Nva. Imperial	Liver	F	M
Q13	Bovine-4	Nva. Imperial	Liver	G	L
Q14	Bovine-4	Nva. Imperial	Liver	G	M
Q15	Bovine-4	Nva. Imperial	Lung	H	L
Q16	Bovine-4	Nva. Imperial	Lung	H	M
Q17	Bovine-5	Nva. Imperial	Heart	I	L
Q18	Bovine-5	Nva. Imperial	Heart	I	M
Q19	Bovine-5	Nva. Imperial	Liver	J	L
Q20	Bovine-5	Nva. Imperial	Liver	J	M
Q21	Bovine-6	Pitrufquén	Lung	K	L
Q22	Bovine-6	Pitrufquén	Lung	K	M
Q23	Bovine-7	Pitrufquén	Liver	M	L
Q24	Bovine-7	Pitrufquén	Liver	M	M
Q25	Bovine-7	Pitrufquén	Liver	N	M
H-2	Human-2	Santiago	Liver	O	M

[a] Origin of the cysts: Two humans (human 1 and 2) and seven bovines (bovines 1 to7)

[b] Fourteen cysts were analyzed. Cysts E to H were from bovine-4 and cysts I–J from bovine-5

[c] Kind of sample: hydatid liquid (L) or cyst membrane (M)

genus *Echinococcus* (Pour et al. 2011; Sharbatkhori et al. 2011) were amplified with the following primers: COI-F: 5′-TTTTTTGGGCATCCTGAGGTTTAT-3′ (Bowles et al. 1992) and COI-R: 5'TAAAGAAAGAACATAA TGAAAATG-3 for the COX1 gene. In the first instance for the ND1 gene, we used primers JB11 and JB12 (Bowles and McManus 1993). After several attempts to amplify the ND1 marker with the described primers JB11 (5′-GAGTTTGCGTCTCAATGATGG-3′) and JB12 (5′-TGGTGATTGATTAAGTGAAAG-3′), in which we obtained little amplification and non-reproducible results, the primers were redesigned based on alignment with mitochondrial genomic sequences of the ND1 marker corresponding to genotypes G1, G4, G5, and G6. The new primers were called JB11-b (5′-RTYTCGTAAGGGYCCTAAYA-3′) and JB12-b (5′-ACCDCTRACCAAYTCHTCYTC-3′). The accession numbers of the mitochondrial genomes used to extract the ND1 sequences are as follows: G1 (AF297617), G4 (AF346403), G5 (AB235846), and G6 (AB208063).

For the markers COX1 and ND1, the PCR was conducted with a final volume of 25 μl with 4 ng/μl of genomic DNA, using the GoTaq® Green master mix (Promega, Wisconsin, EUA) and finally using 1.5 and 2.5 μl, respectively, of each primer. The reaction was carried out in TC-412 equipment (Teche). After an initial denaturation of 60 s at 95 °C, the reaction of 40 cycles continued with the following conditions: 60 s at 95 °C, 60 s at 50/56 °C (COX1 and ND1, respectively), and 60 s at 72 °C, concluding with a final extension of 10 min at 72 °C. The final concentrations of the reagents were 100 to 200 ng/μl of genomic DNA, 200 μM of dNTPs, 0.2 μM of each primer, 1.25 U of Platinum ® Taq DNA polymerase (Invitrogen) with the buffer provided by the manufacturer.

The PCR products of the COX1 gene were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and then were sent for sequencing (DNA Core Sequencing Facility, University of Illinois, EEUU) with the same primers.

Analysis of the data

The results of the sequencing were aligned with the known genotypes of the *Echinococcus* genus (Table 2). This information is available in the database http://www.ncbi.nlm.nih.gov/. The alignments were made with the CLUSTALX program and then removal of the gaps or single-stranded segments was performed with the BioEdit software (Hall 1999). For the reconstruction of the phylogenetic tree, we used the neighbor-joining method, employing the K2P algorithm (for genetic distance) and 1,000 replications for bootstrap support applied with the MEGA 4 program (Tamura et al. 2007).

Results

Implementation of the PCR methods with COX1 and ND1 markers

In order to identify the genotypes of parasites and to perform the phylogenetic study, PCR methods were implemented for the mitochondrial markers COX1 (Bowles et al. 1992) and ND1 (Bowles and McManus 1993). From the beginning, an expected band of 446 bp for the COX1 marker could be obtained for almost all samples of hydatid cysts, including those of hydatid fluid samples and hydatid cyst membranes (Fig. 1).

In the case of the ND1 marker, no bands were detected with the JB11/JB12 primers described by Bowles and McManus (1993). For this reason, these described primers were aligned

Table 2

Sample	COX1[a]: 465	471	474	489	494	504	543	546	549	561	591	612	666	678	686	694	695	696	705	720	744	750	752	759	783	786	793	794	798	ND1[a]: 107	493
G1	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
Q1	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
Q2	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
Q4	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
Q6	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
Q7	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
Q8	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
Q14	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	C	G	ND	ND
Q15	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	ND	ND
Q17	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	ND	ND
Q18	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	ND	ND
Q20	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
Q21	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	C	T	G	T	G	G	G	G	G	A	A	T	G	G	G
Q23	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	A	A
H-2	T	G	T	T	C	C	G	T	C	T	G	C	A	G	G	G	T	T	G	T	G	G	G	G	G	A	A	T	G	ND	ND
G2	T	G	T	T	T	T	G	T	C	T	G	C	A	G	G	G	C	T	G	T	G	G	G	G	G	A	A	T	G	G	G
G3	T	G	T	T	C	T	G	T	C	T	G	C	A	G	G	G	C	T	G	T	G	G	G	G	G	A	A	T	G	G	G
G4	A	A	G	G	T	T	A	A	T	G	A	T	G	A	A	A	A	G	T	G	T	A	A	T	A	G	G	T	A	G	G

[a] The numbers at the top of the table correspond to the SNP positions of G1 sequences for the corresponding COX1 (AB033407) and ND1 (gi:295883110). The *shaded cells* include sites SNPs that are different from the G1 sequences at the top of table. The accession numbers of the other COX1sequences are G2 (M84662), G3 (M84663), and G4 (AF346403). The accession number of the other ND1 sequences are G2 (AJ237633), G3 (DQ856469), and G4 (GQ168807). The Q1–Q23 and H-2 sequences correspond to the samples described in Table 1. *ND* not determined

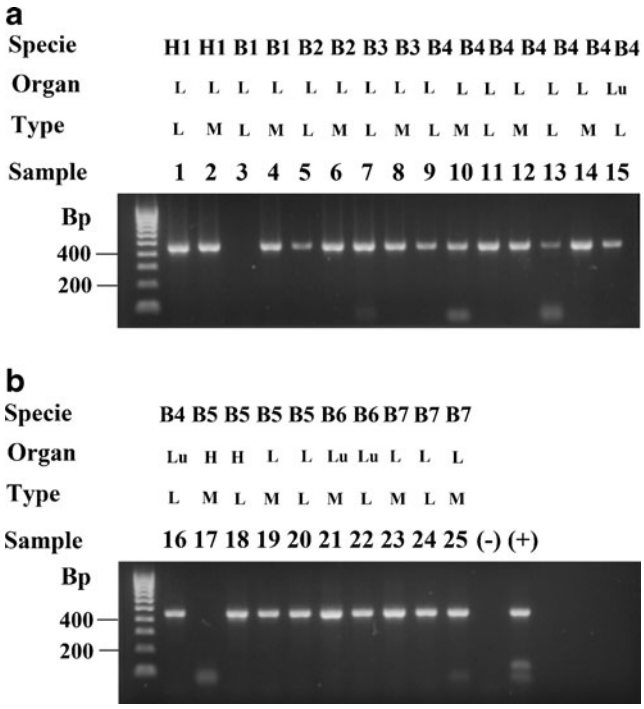

Fig. 1 Detection of the COX1 marker in DNA samples from hydatid cysts removed from cattle and a human patient. Fragments were detected by electrophoresis in agarose gels (1.5 % w/v) stained with ethidium bromide. Analysis with the COX1 marker, corresponding to a segment of 446 base pairs (bp) from subunit 1 of cytochrome oxidase. *Lane L*, ladder marker from 100 to 1,000 bp, *lane* (−) negative control, *lane* (+) positive control. *Lanes 1* to *25* correspond to different samples of DNA extracted from hydatid cysts isolated from different species (*H1* human, *B1–B7* bovine 1 to 7), different organs (*L* liver, *Lu* lung, and *H* heart), and type (*L* hydatid liquid, *M* cyst membrane)

with ND1 sequences corresponding to different genotypes (Fig. 2). The figure shows that neither the JB11 nor the JB12 primer matches perfectly with the corresponding ND1 sequences. The JB11 and JB12 primers show five (positions 1, 2, 3, 13, and 19) and seven (positions 3, 6, 9, 12, 15, 18, and 21), respectively, that do not match with all genotypes (Fig. 2). In order to improve the PCR efficiency, we designed the degenerate primers JB11-b and JB12-b, trying to cover all the single nucleotide polymorphism displayed in this figure (Fig. 2).

In order to obtain the best efficiency of the PCR assay with the new primers JB11-b and JB12-b, the amount of DNA sample, the DNA polymerase enzyme, and the annealing temperature were optimized. As Fig. 3 shows, the best results were obtained using the new primers, 12 ng of DNA samples, the Go Taq system, and 57 °C (Fig. 3).

Phylogenetic analysis of the *Echinococcus* spp. of DNA samples isolated from hydatid cysts

Fifteen DNA samples corresponding to hydatid cysts isolated from individual hosts were amplified by PCR with the respective COX1 and ND1 assays; the PCR products were purified and sent for sequencing to the DNA Core Sequencing Facility, University of Illinois, USA. All sequences were sequenced almost the entire length in both senses.

The sequences were aligned using the CLUSTALX software (Thompson et al. 1997) along with data bank sequences corresponding to different genotypes; gaps were excised with the BioEdit program (Hall 1999). This new alignment without gaps, corresponding to about 300 and 400 bp segments of the Cox1 and ND1 markers, respectively, were analyzed with the MEGA4 software (Tamura et al. 2007) to elucidate the phylogenetic relationships of the group of sequences (Fig. 4). To calculate the genetic distances for these phylogenetic reconstructions, we used the neighbor-joining method with the Kimura-2 algorithm.

The phylograms obtained with these two mitochondrial markers showed that all sequences, with the exception of samples Q21 and Q22, clustered together in the same node with a G1 sequence; in the case of the ND1 tree, with high statistical support (86 %, Fig. 4, panel b). Interestingly, in the COX1 tree, the Q21 sample was grouped in the same node with a G3 sequence (Fig. 4 panel a). However, in the ND1 tree, this Chilean sample was grouped, in addition to the G3 sequence, with G2 and G4, in this case with a high bootstrap support of 86 % (Fig. 4 panel b). As expected, all other genotypes were grouped into other nodes, indicating different genetic origins.

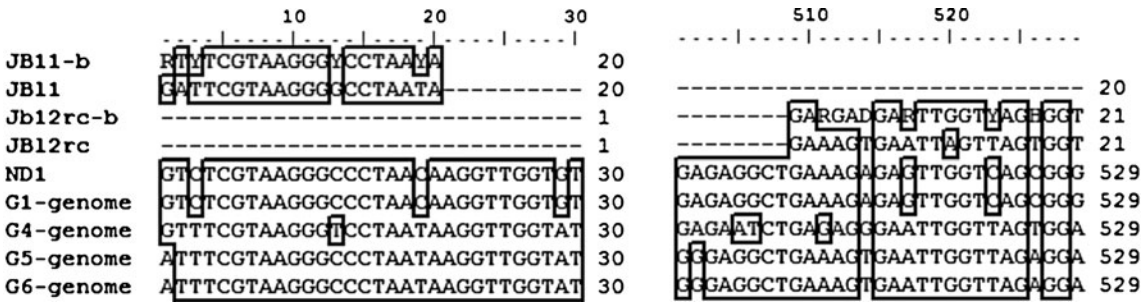

Fig. 2 Alignment of described ND1 primers JB11 and JB12 and the re-designed JB11-b and JB12-b primers. The G1–G6 sequences were extracted from the mitochondrial genomes G1 (AF297617), G4 (AF346403), G5 (AB235846), and G6 (AB208063). The accession number of the ND1 sequence is 295883110

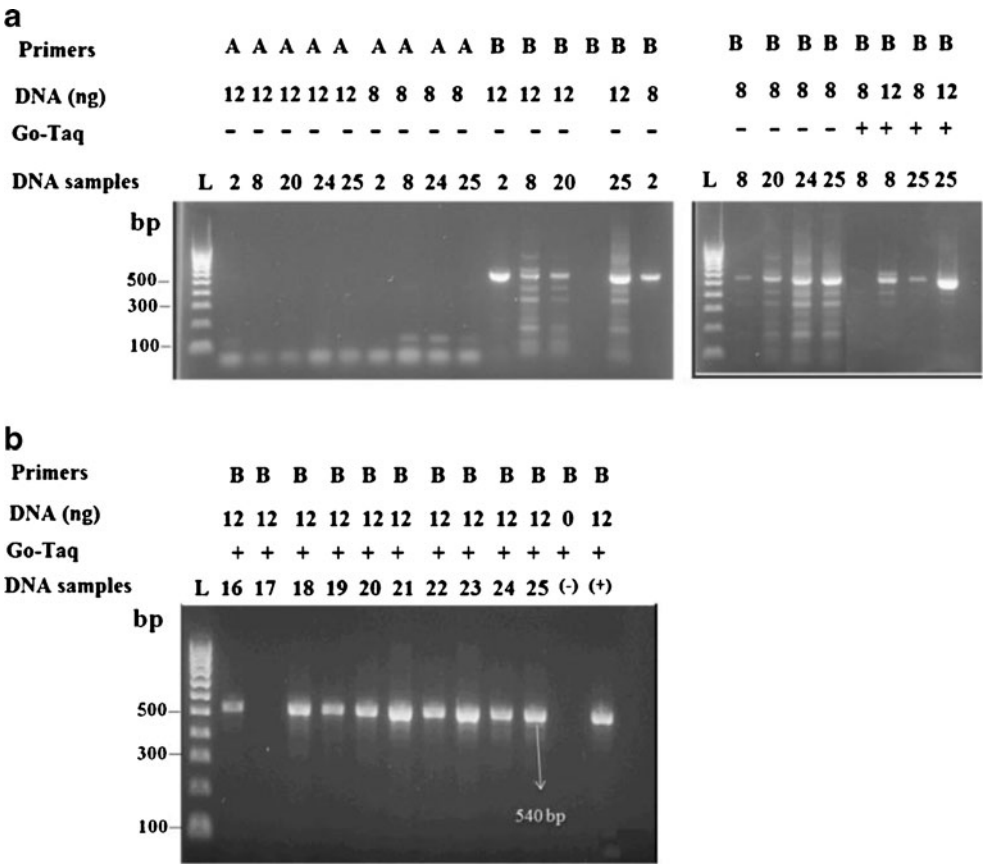

Fig. 3 Optimization of the PCR assay with new primers for the amplification of the ND1 marker using DNA samples purified from hydatid cysts. Analysis using the ND1 marker corresponding to a segment of 540 bp from subunit 1 of the NADH dehydrogenase gene with the JB11–JB12 primers previously described (Bowles and McManus 1993) and the redesigned primers JB11-b and JB12-b. *Panel* **a** and **b**, annealing temperature of 50 and 57 °C, respectively. *Lane L* ladder marker from 100 to 100 bp. The DNA samples were purified from hydatid cysts extracted from a human patient (2) and bovines (8, 16–25) (see Table 1). The primers A and B correspond to the pairs JB11/JB12 and JB11-b/JB12-b, respectively. The PCR assay was performed with the Platinum ® Taq DNA polymerase (Invitrogen) (*lanes* −) and the GoTaq® Green master mix (Promega, Wisconsin, EUA) (*lanes* +)

Single nucleotide polymorphism analysis

As shown in Table 2, with the COX1 markers, we detected 29 single nucleotide polymorphism (SNP) along G1–G4 genotypes, whereas with the ND1 marker only two. With the exception of samples Q14 and Q21, all the SNPs are identical to the G1 sequence. On the contrary, in the COX1 marker, the Q21 sequence differs from the G1 genotype in the 695 SNP and from the G2, in the 494 SNP (Table 2). With the ND1 marker, Q21 differ from the G1 sequence in positions 107 and 493 (Table 2).

Interestingly, the sequence Q14 differ from all the other ones in the 794 SNP (Table 2), despite in the phylogenetic tree, this sample was clustered in the main G1 node, together with the majority of Chilean samples (Fig. 4).

Discussion

In the present paper, the phylogenetic analyses of *Echinococcus* spp. cysts isolated from two Chilean patients and seven bovines are presented; the analysis was performed using the mitochondrial COX1 and ND1 markers. The phylogenetic reconstruction by sequence analysis and the study of SNP showed clearly that the majority of our samples correspond to the G1 genotype, as was previously found analyzing hydatid cysts extracted from Chilean human patients (Manterola et al. 2008), and in hydatid cysts from definitive and intermediate hosts from Argentina (Rozenzvit et al. 1999; Guarnera et al. 2004). It is well documented that the G1 genotype is the most prevalent *Echinococcus* genotype all over the world, in different definitive and intermediary hosts (Thompson and McManus 2002; Thompson 2008; Shahnazi et al. 2011).

Interestingly, 1 of the 15 *Echinococcus* spp. Chilean samples is more similar to the G3 sequence, which strongly suggests that the Q21 sample correspond to the G3 genotype. The Q21 sample was purified from the membrane of a pulmonary hydatid cyst isolated from a bovine individual. The results that support this hypothesis are the phylogenetic and SNP analysis; in the phylogram using the COX1 marker, the Q21 sample clustered together with the G3 sequence.

The analysis of the COX1 SNPs shows that this Chilean sequence is identical to the G3 sequence, despite the low bootstrap support observed in the corresponding node.

Although the G2 and G3 sequences used in the present study only differ in one SNP with the COX1 marker, (SNP 494) several authors consider it sufficient to differentiate G2 from G3 based only on COX1 marker and in the mentioned SNP-494 (according to sequence AB033407), which in G2 is

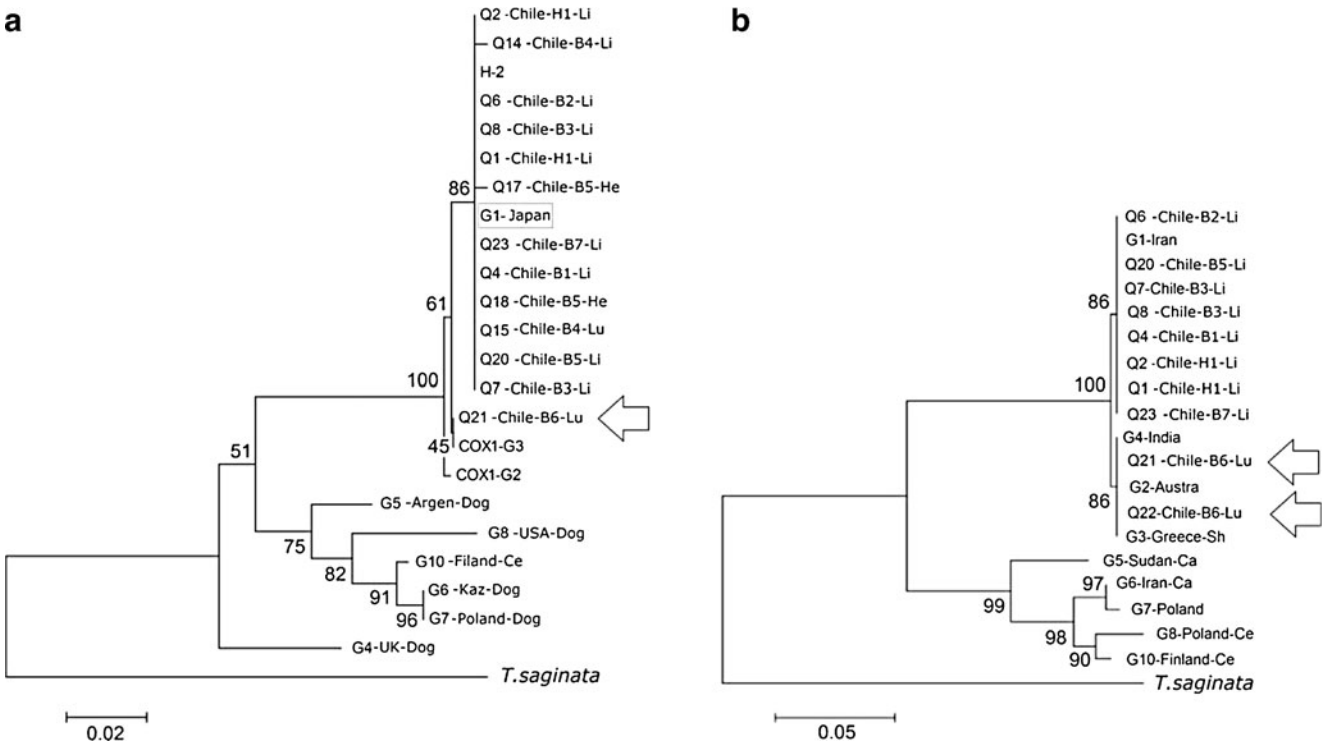

Fig. 4 Phylogenetic analysis of Chilean *Echinococcus* spp. cysts using the COX1 (**a**) and ND1 (**b**) markers. *Samples Q1* to *Q23* were isolated and sequenced in this study. *Samples G1* to *G10* were obtained from GenBank corresponding to accession numbers (*tree* **a**): G1 (AB033407), G2 (M84662), G3 (M84663), G4 (AF346403), G5 (AB235846), G6 (AB208063), G7 (AB235847), G8 (AB235848), G10 (AF525457). (*tree* **b**): G1 (295883110), G2 (AJ237633), G3 (DQ856469), G4 (GQ168807), G5 (365269012), G6 (HQ585934), G7 (AJ237638), G8 (AJ237643), G10 (AF525297). The abbreviations for countries are *Argen* Argentina, *Austra* Australia, *Kaz* Kazakhstan, *Rom* Romania, *UK* United Kingdom, *USA* United States. The abbreviations for hosts are *B* bovine, *Ce* cervid, *Dog* dog, *H* human, and *Sh* sheep. These are unrooted phylograms inferred by the neighbor-joining method, using the Kimura 2 algorithm, and the MEGA4 program. The numbers in the nodes represent the percentage of bootstrap support obtained from 1,000 replications. *T. saginata* was used as outgroup to indicate the root of the tree

T and G3 is G (Obwaller et al. 2004; Busi et al. 2007; Calderini et al. 2012). Although being a single mutation, the fact that this DNA sample was sequenced in two separate events and sequenced in both directions, assure that the SNP-494 of the Q21 are not due to an artifact introduced by the PCR assay or by a sequencing mistake. Therefore, this fact gives confidence that the Q21 sequence actually correspond to G3 genotype. However, even to confirm this observation, it would be advisable in future studies to perform further analysis with other polymorphic markers. Clearly, the Q21 does not correspond to the G1 and G4 genotype because, in the first case, they differ in four SNP in relation to the G1 sequence (COX1 SNP 504 and 695; ND1 SNP 107 and 493), and in the second case, they differ in all SNPs showed with the COX1 marker.

The fact that, in the ND1 phylogram, the Q21 sequence appears in the same node together with G2, G3, and G4 sequences is consistent with earlier observations in the literature showing that this marker cannot differentiate these three genotypes (Obwaller et al. 2004; Busi et al. 2007). This observation was also confirmed by our studies showing that this marker only has two polymorphic sites (SNP 107 and 493).

Interestingly, another sample which was not identical to the rest of all the samples was Q14. This sample showed a single SNP at the 794 position, although it was clustered in the same G1 node of the phylogenetic tree. The fact that both Q14 and Q15 were isolated from the same host but from different cysts located in different organs (liver and lung, respectively) suggests that cysts were the result of different infection events. This result suggests that, in endemic areas, intermediate hosts are permanently exposed to reinfections with parasite eggs, which do not necessarily have to be of the same lineage or genotype.

The above results strongly suggests that *Echinococcus* G3 genotype has been identified for the first time in a hydatid cyst from a Chilean bovine. This genotype, previously called the buffalo strain, the literature shows that, as well as G1 and G2, has no host specificity, and it is infective for humans (Guarnera et al. 2004; Obwaller et al. 2004; Busi et al. 2007; Thompson 2008). However, the identification of the exact genotype can provide important information about the possible routes of transmission and the localization of potential reservoirs (Rosenzvit et al. 1999; Thompson and McManus 2002; Guarnera et al. 2004; Obwaller et al. 2004; Busi et al. 2007; Thompson 2008; Calderini et al. 2012). In addition, if Q21 and

Q22 really correspond to the G3 genotype, it would be the first time this strain has been found at this latitude and in a bovine intermediate host, which could investigate how this genotype could have arrived to Chile. To confirm this finding and to know in more detail the different parasite genotypes circulating in Chile, further analysis will be necessary in future studies with a larger number of markers and to analyze other species of intermediate hosts.

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