Echinococcus granulosus: intraspecific genetic variation assessed by a DNA repetitive element

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SUMMARY

A 186 bp *Echinococcus granulosus*-specific repetitive element, TREg, was used to assess genetic variation between strains. In G7 genotype (pig strain) it has the characteristics of a satellite DNA element with a copy number of 23000 per haploid genome. Analysis, by sequencing of TREg monomers, showed a great degree of identity within them. In the G1 genotype (common sheep strain) TREg-like repetitive elements were found in an interspersed distribution throughout the genome and in only 120 copies. The sequences of these monomers showed a great degree of variation between them and with TREg of G7 origin. The G6 genotype (camel strain) showed a pattern of distribution and copy number similar to the G7 genotype, and the G2 genotype (Tasmanian sheep strain) similar to the G1 genotype. Isolates from the G5 (cattle strain) and G4 (horse strain) genotypes also showed unique hybridization patterns in Southern blot experiments. The genomic plasticity of *E. granulosus*, which may have important consequences in the epidemiology and control of cystic hydatid disease is reflected in the results of this work.

Key words: *Echinococcus granulosus*, genetic variation, repetitive elements, cystic hydatid disease, strain differentiation.

INTRODUCTION

Cystic hydatid disease (CHD) is a zoonosis that affects man and livestock from many countries around the world. Its aetiological agent, the cestode *Echinococcus granulosus*, requires 2 mammalian hosts for completion of its life-cycle: a definitive and an intermediate host. The definitive host is always a carnivore, the dog or other canid. Numerous species of herbivores and omnivores like sheep, pig, goat, horse, cattle, camel and eventually man can serve as intermediate hosts. An important factor that affects the control of CHD is the high level of intraspecific variation in *E. granulosus*. Several genetic variants or strains adapted to different intermediate hosts were described. These strains differ in biological characters that affect the epidemiology, pathology and control of CHD (Thompson & Lymbery, 1988; Thompson, 1995). These differences showed correlation with genetic data obtained by molecular techniques like ribosomal DNA (rDNA) restriction fragment length polymorphism (RFLP) after polymerase chain reaction (PCR) (PCR–RFLP) analysis (Bowles & McManus, 1993*a*), the sequencing of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) (Bowles, Blair & McManus, 1992) and NADH dehydrogenase 1 (ND1) (Bowles & McManus, 1993*b*) genes and single strand conformation polymorphism (SSCP) (Haag *et al.* 1999). To date, 9 distinct genotypes (G1–G9) have been identified within *E. granulosus* (Bowles, Blair & McManus, 1992; Bowles & McManus, 1993*b*; Bowles, Blair & McManus, 1994; Scott *et al.* 1997).

Repetitive DNA elements have been classified in clustered and dispersed repeats. Both of them have been considered to generate variations in genome structure and in species evolution (Rose & Doolittle, 1983; Kidwel & Lisch, 1997). Although these DNA elements have been widely used to assess inter- and intraspecific genetic variation in parasites (Ellis & Crampton, 1988; Zimmerman, Toe & Unnasch, 1993; Grenier, Castagnone-Sereno & Abad, 1997), very little is known about their usefulness to characterize *E. granulosus* species and strains (McManus & Rishi, 1989).

We have previously cloned and characterized a repetitive element from *E. granulosus* DNA of porcine origin. This element, named TREg, has the characteristics of a satellite sequence in *E. granulosus* G7 genotype DNA (pig strain). It is 186 bp long, is organized as a tandem array and has a copy number of 23000 per haploid genome, representing between 2 and 3% of the parasite genome (Rosenzvit *et al.* 1997). The objective of the present work is to assess the genetic variability of *E. granulosus* by analysing the genomic organization, the nucleotide sequence and the number of copies of this repetitive element in the genome of some of the parasite strains.

MATERIALS AND METHODS

Parasite materials

Total *E. granulosus* genomic DNA was prepared from fresh, frozen in liquid nitrogen or 70% ethanol-

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preserved isolates of *E. granulosus* by conventional techniques (Maniatis, Fristch & Sambrook, 1989) or, in the case of cyst layers, by a method developed in our laboratory (Kamenetzky *et al*. 2000). In this study, an *E. granulosus* isolate refers to the protoscoleces, or germinal layer obtained from a single hydatid cyst.

E. granulosus *genotype determination*

E. granulosus genotype was determined for each isolate by mitochondrial CO1 gene sequencing (Bowles, Blair & McManus, 1992) and alignment with published sequences (366 or 391 bp) for the G1–G8 genotypes (Bowles *et al*. 1992, 1994; Okamoto *et al*. 1995). We used this technique since, in a previous study (Rosenzvit *et al*. 1999), consistent results were obtained by this and by 2 other methods, based on restriction fragment length polymorphism (RFLP) of nuclear ribosomal DNA and the sequencing of the mitochondrial NADH dehydrogenase 1 (ND1) gene. G1 isolates were obtained from sheep and human hydatid cysts. G2 and G6 isolates were of human origin and all the G7 samples were obtained from pigs. All the above-mentioned samples were from Argentina. G4 and G5 isolates, of donkey origin from Spain and cattle origin from Brazil respectively, were kindly provided by Karen L. Haag (Universidad Federal de Rio Grande do Sul, Porto Alegre, Brazil). The sequences obtained from our isolates were identical to the corresponding sequences published for the G1–G8 genotypes (Bowles *et al*. 1992, 1994; Okamoto *et al*. 1995).

Analysis by Southern blot

The genomic DNA obtained from each isolate was digested with different restriction enzymes, size fractionated on agarose gels and transferred to Z probe membranes (Bio-Rad, Richmond, CA, USA). Hybridizations were carried out at 55 °C overnight with [α^{32}P] dCTP random-labelled DNA probe in 6 × SSC (1 × SSC is 0·15 M NaCl, 0·015 M sodium citrate, pH 7), 0·1 % SDS, 0·25 % skimmed milk. Post-hybridization washes were performed either in 0·3 × SSC, 0·1 % SDS at 50 °C (low stringency) or in 0·1 × SSC, 0·1 % SDS at 65 °C (high stringency) as indicated in each experiment. Alternatively, low stringency washes in 6 × SSC, 0·1 % SDS at 60 °C were done. The probe used was iE7, the insert of a plasmid containing 2 repetitive units of TREg (Rosenzvit *et al*. 1997).

Copy number determination

Known amounts of *E. granulosus* G1 and G7 DNAs were subjected to Southern blot technique as described before and the intensities of the hybridization signals were measured by scanning with a GS-250 Molecular Imager (Bio-Rad).

Analysis by the polymerase chain reaction

For the design of the polymerase chain reaction (PCR) primers, 12 repeat units previously cloned from G7 *E. granulosus* genomic DNA, were aligned using the Pôle Bio-Informatique Lyonnais CLUSTALW multiple alignment (Thompson *et al*. 1994). The sequence identity found (over 96 %) facilitated the design of the primers. The sequences of the primers used were 5′ TGGGGCACTCTC-AGCTTCGC 3′ (primer forward) and 5′ TCGTT-GATGGCCCATTTCGT 3′ (primer reverse). The PCR reaction was performed in a final 50 μl volume containing sample DNA, 100 μM of each dNTP (Pharmacia LKB, Uppsala, Sweden), 1·5 mM $MgCl_2$, 10 pmol each of primers forward and reverse, and 1 unit of *Thermus aquaticus* DNA polymerase in reaction buffer (Promega, Madison, WI). The PCR conditions were as follows: an initial denaturing step (95 °C for 180 s) followed by 25 cycles, 95 °C for 60 s (denaturation), 60 °C for 60 s (annealing), 72 °C for 90 s (extension), and a final extension step (72 °C for 180 s). The specificity and size of the amplification products were assessed by electrophoresis in 1·2 % (w/v) Tris-acetate/EDTA (TAE) agarose gels and stained with ethidium bromide.

Sequencing analysis

The PCR reaction was performed as above but a 10 °C lower annealing temperature was used, so that many TREG-like repeats were represented in the PCR product. Amplification products were extracted from the agarose gel (Sephaglas™ Band Prep Kit Pharmacia) and cloned in T vector (pGem-T Easy Vector System 1 Promega). The sequences of the inserts were obtained automatically using an Applied Biosystems Big Dye Terminator Kit (Applied Biosystems, Foster City, CA) and an Eppendorf Mastercycler gradient 5331 version 1.2 DNA Thermal Cycler. DNA sequences were analysed using the Pole Bio-lnformatique Lyonnais CLUSTALW multiple alignment (Thompson *et al*. 1994). Clustering of G1 and G7 repetitive units was done by distance, parsimony and maximum likelihood methods using the program PAUP* version 4.0b4a.

RESULTS

Southern blot analysis

Genomic DNA obtained from *E. granulosus* G1 (common sheep strain), G2 (Tasmanian sheep strain) and G7 (pig strain) isolates was digested with *Xba*I, transferred to a nylon filter and probed with ^{32}P labelled TREg dimer. A ladder-like hybridization pattern was evident in G7 isolates under high or low stringent conditions. G1 and G2 DNAs showed a different hybridization pattern, typical of an interspersed repeat element, under low-stringency

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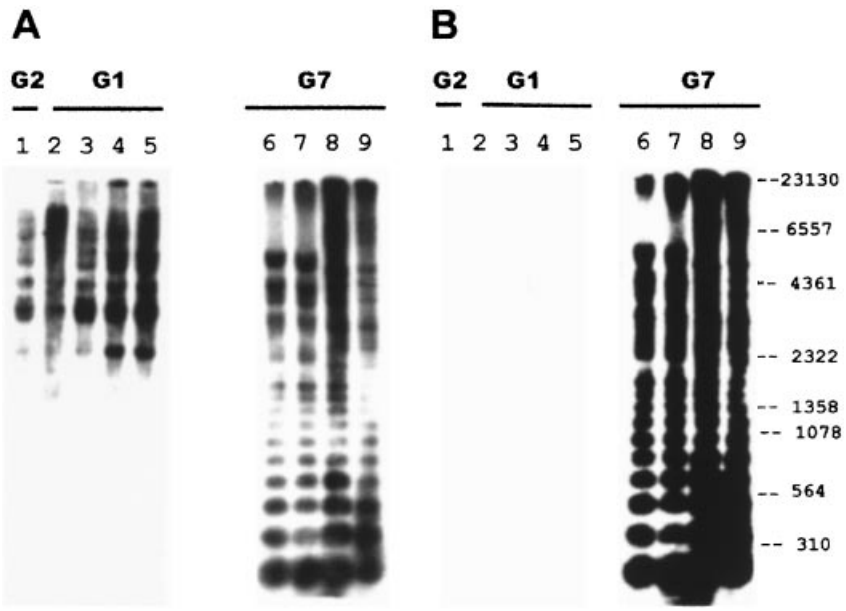

Fig. 1. Southern blot hybridization of 5 μg of genomic DNA from *Echinococcus granulosus* digested with *Xba*I hybridized to ^{32}P labelled TREg dimer. (A) Low-stringency washes (50 °C, 0·3 × SSC). (B) High-stringency washes (65 °C, 0·1 × SSC). Lane 1, G2 genotype (Tasmanian sheep strain); lanes 2–5, isolates from G1 genotypes (common sheep strain); lanes 6–9, isolates from G7 genotype (pig strain). The filters were exposed to autoradiography for 2 h (G7 genotype, A) or 24 h (G1 and G2 genotypes, A) or for 21 days (B). The numbers on the right side of the panels correspond to the sizes in bp of the molecular weight marker bands.

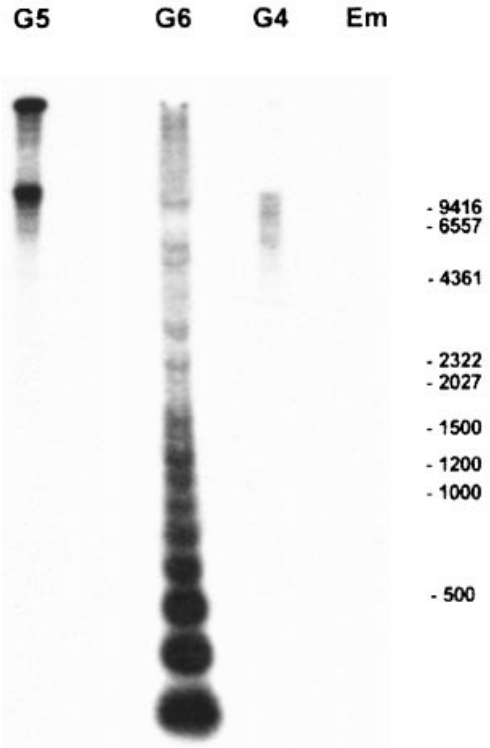

Fig. 2. Southern blot of genomic DNA from *Echinococcus granulosus* digested with *Xba*I hybridized to ^{32}P labelled TREg dimer and washed in 6 × SSC at 60 °C (low stringency). G5: 1·3 μg of DNA from G5 genotype (cattle strain), G6: 100 ng of DNA from G6 genotype (camel strain), G4: 1·3 μg of DNA from G4 genotype (horse strain), Em: 2·8 μg of DNA from *E. multilocularis*. The numbers on the right side of the panels correspond to the sizes in bp of the molecular weight marker bands.

conditions and longer exposure times, but no hybridization signal was observed when more stringent washes were done (Fig. 1). These results suggest differences in sequences, copy number and genomic organization of the repetitive element between strains. Both filters were also hybridized with ^{32}P labelled DNA fragment cloned in T vector after PCR amplification of the repetitive element from G1 genomic DNA (sh9, in Fig. 5) and identical results were observed (data not shown). Variations in the banding pattern of different isolates from the same strain could also be observed, specially at high molecular weight (Fig. 1A), and were better observed in Southern blot experiments with higher band resolution (data not shown). Genomic DNAs obtained from G5 (cattle strain), G6 (camel strain) and G4 (horse strain) isolates were also analysed by Southern blot (Fig. 2). G6 showed the same hybridization pattern as G7 and G5 showed a different intense hybridization signal. Both patterns were not removed after high-stringency washes. G4 displayed a very faint hybridization signal that was completely eliminated by high-stringency washes (data not shown), suggesting that no or very few copies of TREg-like elements are present in the G4 genome. Genomic DNA from pig, *Taenia hydatigena* and *Echinococcus multilocularis* did not hybridize with TREg (Figs 2 and 3).

Copy number determination

Copy number of TREg-related sequences in *E. granulosus* G1 genotype were estimated by com-

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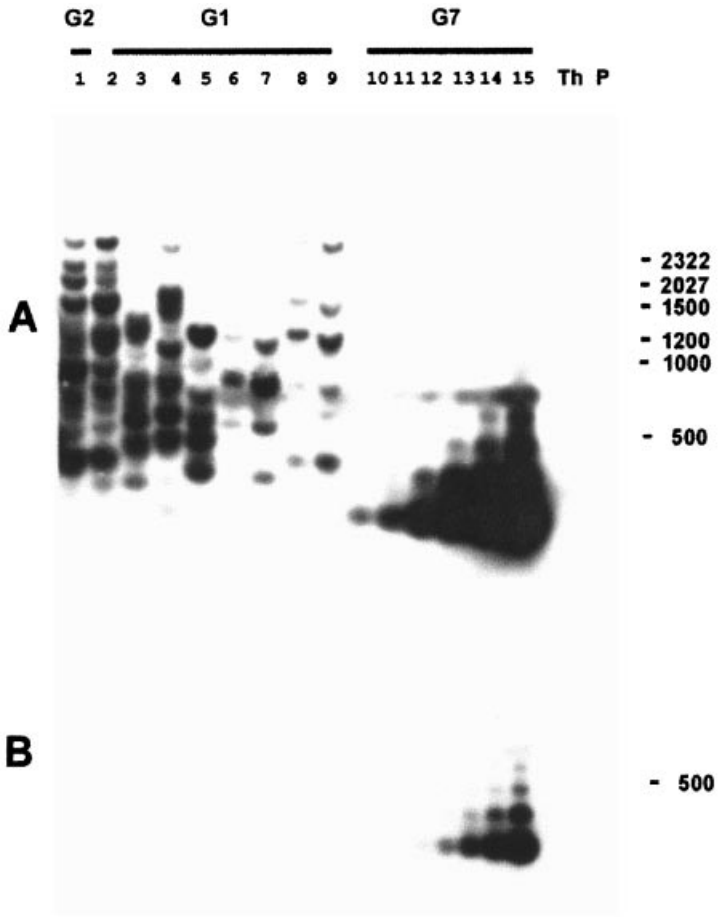

Fig. 3. Southern blot of genomic DNA from *Echinococcus granulosus*, *Taenia hydatigena* and pig hybridized to ^{32}P labelled TREg dimer. Five μg of genomic DNA from *E. granulosus* G2 genotype digested with *Hae*III (1), 5 μg of genomic DNA from *E. granulosus* G1 genotype digested with *Hae*III (2), *Rsa*I (3), *Hha*I (4) and *Alu*I (5); 1·5 μg of genomic DNA from other isolate of G1 genotype digested with *Mbo*I (6) and *Hae*III (8); 4·5 μg of genomic DNA from the same isolate digested *Mbo*I (7) and *Hae*III (9), genomic DNA from G7 genotype digested with *Hae*III, 4 ng (10), 8 ng (11), 25 ng (12), 70 ng (13), 200 ng (14), 600 ng (15), 5 μg of genomic DNA from *T. hydatigena* digested with *Xba*I (Th) and 16·5 μg of genomic DNA from pig digested with *Xba*I (P). (A) Low-stringency washes (6 × SSC, 60 °C). (B) High-stringency washes (0·1 × SSC, 65 °C). The numbers on the right side of the panels correspond to the sizes in bp of the molecular weight marker bands.

parison of hybridization intensities between this strain and the G7 genotype, for which 23000 copies of the TREg monomer per haploid genome had previously been calculated (Rosenzvit *et al.* 1997). The intensities of the bands obtained with 1500 ng of G1 genotype DNA digested with *Hae*III (Fig. 3A, lane 8) were compared with the corresponding intensities of either 4 ng (Fig. 3A, lane 10) or 8 ng (Fig. 3A, lane 11) of G7 genotype DNA. Taking into account the quantity of loaded DNA, 120 copies of repetitive elements sharing at least 75% identity (washes in 60 × SSC at 60 °C) with TREg per haploid genome were estimated in G1 DNA. Once again, after high stringency washings, hybridization signals were only observed in G7 DNA (Fig. 3B) corroborating that there are differences in copy number and nucleotide sequences of the repeats in these groups of strains.

Polymerase chain reaction and sequencing analysis

G1 and G7 genomic DNAs were used as templates for PCR with primers designed from TREg units of G7 origin. Surprisingly, the same amplification pattern was obtained for both strains. Bands of approximately 190, 380, 570 bp, corresponding to the monomer, dimer, trimer etc. of the repetitive element were observed (Fig. 4). However, more intense amplification bands were obtained with G7 DNA, indicating a higher number of copies of TREg in this genome or a more efficient annealing of the primers. In fact, when PCR was performed with a gradient annealing temperature, no amplification bands were observed with G1 genotype DNA above 65 °C, in contrast with G7 genotype DNA where the characteristic PCR products were obtained at an annealing temperature of 70 °C (data not shown).

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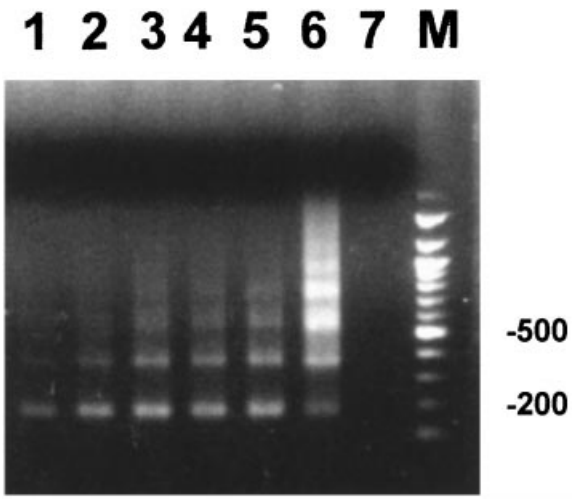

Fig. 4. PCR amplification with primers designed from TREg units of G7 origin, using template DNAs from *Echinococcus granulosus* G1 genotype (1–3) or G7 genotype (4–6). The quantity of DNA in each reaction was 0·1 pg (1 and 4), 1 pg (2 and 5) and 10 pg (3 and 6). Lane 7, control without DNA. Lane M, DNA size marker (100 bp DNA ladder; BioLabs New England).

When G2, G4, G5 and G6 DNAs were used as templates, similar amplification patterns were observed but no amplification products were obtained with pig, *Taenia hydatigena*, or *E. multilocularis* DNA (data not shown), according with the absence of hybridization signals in Southern blots (Figs 2 and 3).

In a first attempt, the monomers of G1 and G7 were extracted from the agarose gel and directly sequenced. Superimposed peaks were obtained, indicating that more than 1 amplification product was present in the lower molecular weight band. For this reason, the band of each genotype was cloned in T vector and the sequences of a number of clones were determined. As can be seen in Fig. 5 numerous differences were observed between the units of TREg-like sequences in G1 genotype but a great sequence homogeneity was evident in G7 genotype. Distance, parsimony and maximum likelihood analysis identified 2 major groups of TREg sequences. In one group were included all the sequences from the G7 genotype and in the other 6 sequences from the G1 genotype. The remaining G1 sequences (sh1, sh4 and sh5) were placed outside of these groups.

G1 repetitive sequences were also compared to a consensus sequence of p2–p6 and, as expected, numerous differences were found (Table 1).

Analysis of restriction sites showed that there were no *Xba*I recognition sites in the repetitive units of G1 but there was a site for this enzyme in one of the G7 repetitive units, in concordance with the results of Southern blot experiments (Fig. 1).

DISCUSSION

The results of this work have shown that the *E. granulosus* repetitive DNA element TREg allows the differentiation of 2 groups of strains: G1/G2 cluster from G6/G7 cluster. By mitochondrial DNA sequencing and RFLP–PCR of the ITS1 rDNA, the strains were grouped in the same way (Bowles & McManus, 1993*a*; Bowles, Blair & McManus, 1992; Bowles & McManus, 1993*b*). It is noteworthy that significant differences in copy number, nucleotide sequences and genomic organization of the repetitive DNA element were found between these groups. TREg is represented in 23000 copies per haploid genome in the G7 genotype but only approximately 120 copies are present in the G1 genome. Nine and 6 repetitive units from G1 and G7 respectively were analysed allowing the detection of sequence variability between strains. Although some of the repetitive units of G1 were grouped together in distance, parsimony and maximum likelihood analysis, a higher level of sequence variation was observed than in G7 where all the sequences analysed were clustered in the same group. It is very likely, however, that there are many more sequences yet to be found and sequenced. It is also likely, especially in G1 where a high level of intra-individual sequence variation was detected, that there are elements of the TREg family that were not amplified with the primers used, although the annealing temperature was low. TREg is organized as a long tandem repeat in G6 and G7 genomes, sharing some characteristics with satellite sequences, but it seems to have a different organization in G1 and G2 DNAs, since no ladder-like pattern was observed in Southern blot experiments with the restriction enzymes tested, except for *Alu*I (Fig. 3, lane 5) where bands with the same molecular weight of the dimer and the trimer of the repetitive element were observed. However, PCR results suggest that at least some copies are arranged in direct tandem. Although more experiments are needed to address this question, differences in the genomic organization between these groups of strains are evident.

G5 as well as G4 patterns were clearly distinguishable by Southern blot from the other strains analysed. Although the hybridization signal of the G4 isolate was faint, amplification bands were obtained by PCR, suggesting the presence of sequences related with TREg in this strain, perhaps in a very low copy number. However, more isolates of both strains should be analysed to confirm these results.

In Fig. 6 we show a comparison of the results obtained by analysis of TREg sequences from the clusters G1/G2 and G6/G7 and the most parsimonious phylogenetic tree constructed with combined CO1 and ND1 mitochondrial DNA sequences (Bowles, Blair & McManus, 1995;

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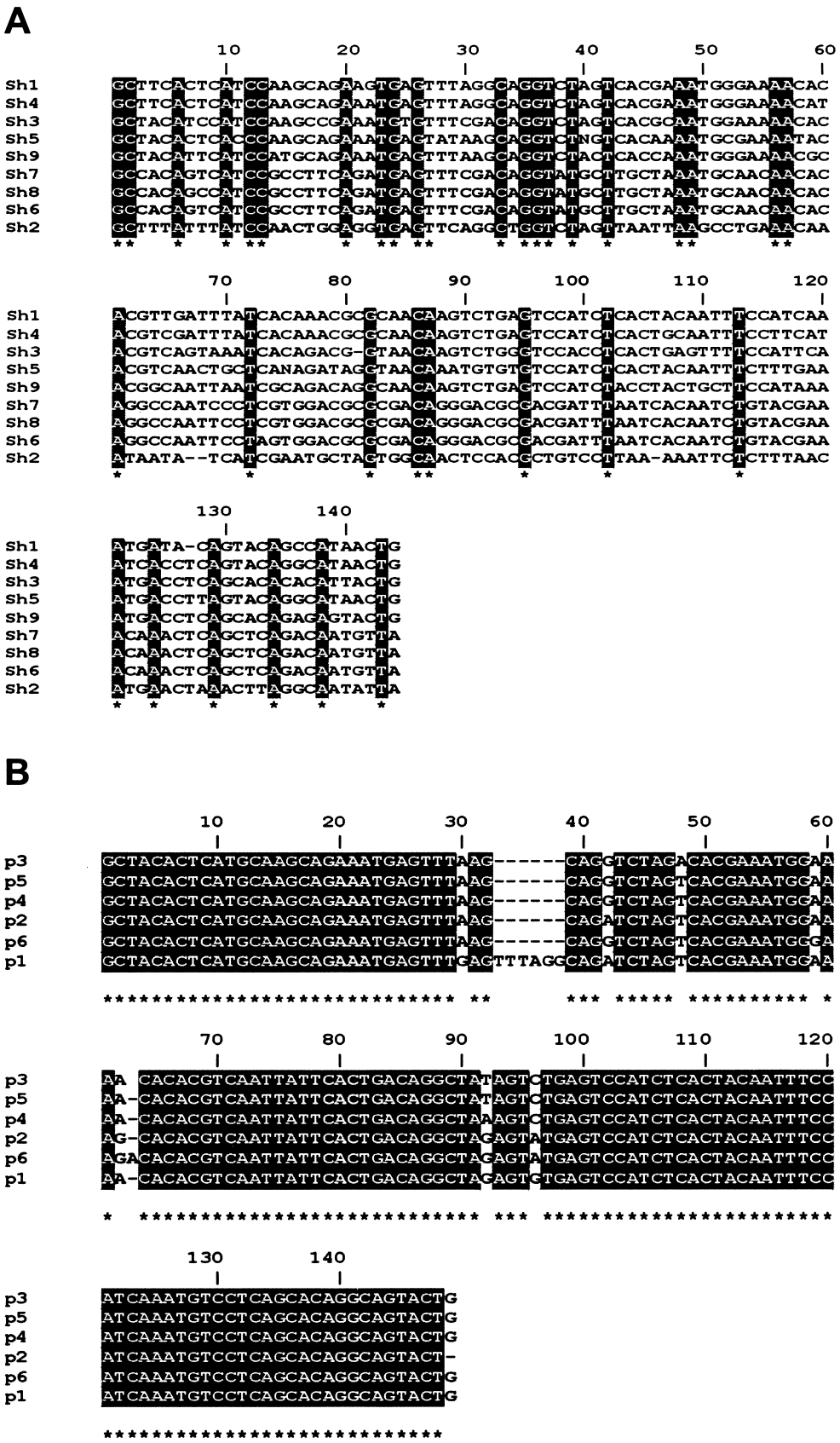

Fig. 5. Alignment of nucleotide sequences of the 186 bp PCR products cloned in T vector. Template DNA was obtained from 1 G1 isolate (A) and from 1 G7 isolate (B). An asterisk indicates a nucleotide that is conserved in all the aligned sequences.

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Table 1. Sequence identity of the 186 bp TREg-G1 PCR products with a TREg-G7 consensus sequence

G1-TREg subunit	Identity with a consensus of G7-TREg (%)
Sh1	83·33
Sh2	59·06
Sh3	78·32
Sh4	84·03
Sh5	79·86
Sh6	59·03
Sh7	59·03
Sh8	59·03
Sh9	84·03

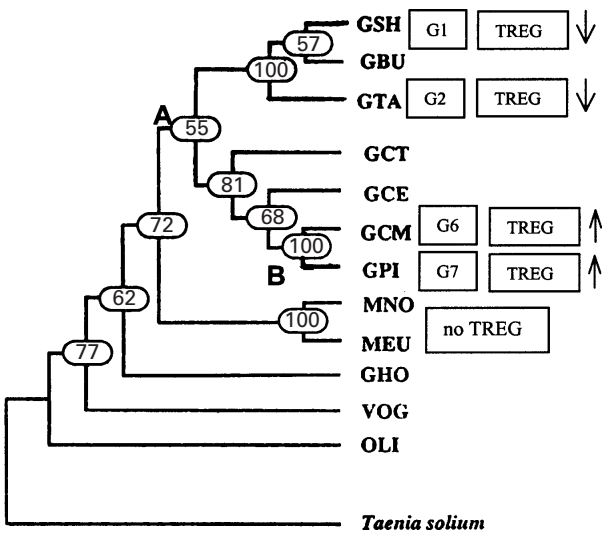

Fig. 6. Phylogenetic tree obtained using the maximum parsimony method on sequence data from regions of the mitochondrial cytochrome *c* oxidase subunit 1 and NADH dehydrogenase 1 genes taken from Lymbery & Thompson (1996). Numbers at nodes represent percentage occurrence of clades in 100 bootstrap replications of the data. The represented taxa are: common *Echinococcus granulosus* sheep strain (GSH), *E. granulosus* Tasmanian sheep strain (GTA), *E. granulosus* buffalo strain (GBU), *E. granulosus* cattle strain (GCT), *E. granulosus* camel strain (GCM), *E. granulosus* pig strain (GPI), *E. granulosus* cervid strain (GCE) and *E. granulosus* horse strain (GHO), *E. multilocularis* North American strain (MNO) and European strain (MEU), *E. vogeli* (VOG) and *E. oligarthrus* (OLI). The results of TREg are indicated. TREg copy number low (TREg ↓), high (TREg ↑) and absence (no TREG). The nomenclature used in the text (G1, G2, G6 and G7) is also indicated.

Lymbery & Thompson, 1996). We used this tree, although it has some minor differences with the one we obtained using CO1 sequences (not shown), because it is based on combined mitochondrial sequences and thus it should provide a more accurate estimate of phylogeny than an analysis of only 1 of these sequences. The repetitive element could have arisen in the common ancestor of the 2 clusters of strains and one of the differing units could have amplified in the common ancestor of G6 and G7 by mechanisms like unequal crossing over or rolling circle amplification. The lack of sequence homogeneity and expansion in G1 genotype suggests that TREg underwent different evolutionary pathways in both groups of strains. The presence in the horse strain of TREg-like subunits is more difficult to explain if we assume that the true phylogeny of *Echinococcus* is that shown in Fig. 6. However, the situation of the horse strain is the most uncertain within *E. granulosus* strains, having different locations in the different phylogenetic trees published so far (Bowles *et al.* 1995; Thompson, Lymbery & Constantine, 1995; Lymbery & Thompson, 1996). Also, we should analyse a greater number of samples and further characterize the repetitive element in this strain. In contrast with the horse strain, all phylogenetic trees published clustered G1 and G2 in 1 group, and G6 and G7 in another.

Since genetic differences found between *E. granulosus* strains were equal or even greater than those between *Echinococcus* species and, as can be seen in Fig. 6, the *E. granulosus* genetic variants are not a monophyletic group, Thompson, Lymbery & Constantine (1995) postulated that *E. granulosus* should be split into 4 species: *E. granulosus* pig strain or G7 genotype (*E.* sp), *E. granulosus* cattle strain or G5 genotype (*E. ortleppi*), *E. granulosus* horse strain or G4 genotype (*E. equinus*) and *E. granulosus* common sheep, Tasmanian sheep and buffalo strains or G1, G2 and G3 genotypes (*E. granulosus*). The camel strain or G6 genotype was not assigned to any of these postulated species. The results of this work, showing a great genomic plasticity in *E. granulosus*, reinforce the hypothesis of Thompson *et al.* (1995) and suggest the inclusion of the camel strain (G6 genotype) together with pig strain in the first mentioned species. The hybridization pattern of TREg was clearly distinguishable in the G1/G2, G5, G4 and G6/G7 genotypes according with this classification.

The variations of hybridization patterns found between isolates from the same strain suggests that TREg can be used to detect intrastrain polymorphism. It was possible to amplify the repetitive element from genomic DNA of all the strains tested, but no signal was obtained with *Escherichia coli*, *Toxocara canis*, *Taenia hydatigena* and *E. multilocularis* DNAs. PCR amplification was possible even with low DNA masses, 0·1 pg of the G1 genotype DNA and even less of the G6 or G7 genotypes (not shown). This suggests that this element can also be useful for direct parasite detection in natural samples. Also, the differences in nucleotide sequence found between G1 and G7

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strains may allow the design of specific primers for each strain.

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