Reduced genetic variability within coding and non-coding regions of the *Echinococcus multilocularis* genome

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**SUMMARY**

*Echinococcus multilocularis*, a vulpine intestinal tapeworm, is the causative agent of alveolar echinococcosis in humans, one of the most severe and lethal parasitic infections in man. To date, there is very little knowledge about the genetical polymorphism of this parasite. To assess sequence polymorphism, we analysed a sample of 33 *E. multilocularis* isolates from Europe, North America and Asia by PCR-SSCP followed by nucleotide sequencing. This assessment was performed comparatively to sheep, cattle and pig *E. granulosus* strains. Coding (nuclear antigen B and mitochondrial NADH dehydrogenase genes) and non-coding (introns of actin and homeobox-containing genes) regions of the parasite genome were chosen as targets. Since the estimated nucleotide diversity among genotypes of *E. multilocularis* were, in general, 10 times lower than among the recognized different strains of *E. granulosus*, we suggest that the conventional classification of the former species in 2 separated strains (European and North American) should be reviewed.

Key words: *Echinococcus multilocularis*, SSCP, strain, genetic variability.

**INTRODUCTION**

*Echinococcus* is a small endoparasitic flatworm belonging to the Class Cestoda. *E. multilocularis* has a medical significance by causing alveolar hydatid disease, an infection characterized by an infiltrating and metastatic larval development (Thompson, 1995). The parasite has an extensive geographical distribution in the Northern Hemisphere. Despite affecting predominantly wild animal hosts (foxes as final hosts; arvicolid and cricetid rodents as intermediate hosts), the potential of human exposure is becoming increasingly common, specially in endemic regions where domesticated carnivores such as dogs and cats may become infected (Schantz et al. 1995; Gottstein et al. 1996). In certain areas of Switzerland, the prevalence in fox and rodent populations may reach 47–56% (Gottstein et al. 1996). In China the prevalences in the final host may range from 10 to 60%; and in North America even as high as 90–100% (Schantz et al. 1995).

Rausch (1985) postulated an Eurasian Pleistocene origin for *Echinococcus* species, in cospeciation with their hosts. Within the most well-studied species, *E. granulosus* and *E. multilocularis*, several strains from different geographical areas or hosts have been described. In *E. granulosus*, only the cervid strain is supposed to have retained the ancestral life-cycle, while the other strains arose from the domestication and subsequent dispersal of wolves and ungulates. *E. multilocularis*, on the other hand, is considered to have largely retained the ancestral life-cycle and geographical distribution, with Eurasian and North American strains being separated by the flooding of the Bering bridge (Lymbery, 1995).

Although there is some evidence of variation in morphology, pathogenicity, developmental characteristics and host specificity between *E. multilocularis* isolates from Europe, Alaska and Central North America, few comparative data are available and the existence of different strains remains unconfirmed. RFLPs have been detected among *E. multilocularis* isolates originated form different endemic areas using the pAL1 DNA probe (Vogel et al. 1991), but criteria discriminating between geographical origin were not elucidated. Sequencing of the mitochondrial COI and ND1 coding genes also showed some nucleotide variation among isolates from China, North America and Europe (Bowles, Blair & McManus, 1992; Bowles & McManus, 1993). Unfortunately, the sample sizes used have been too small for drawing conclusions about strain differentiation. A broader study on *E. multilocularis* microsatellites (Bretagne et al. 1996) showed some agreement between the polymorphism in U1 snRNA genes and
the geographical distribution of the parasite isolates, which could indicate that the European and North American foci may harbour parasite populations exhibiting minor genetic discrepancies, but the authors did not provide any comparative data to support actual strain differentiation.

In the present study we address the question of strain differentiation in *E. multilocularis* by analysing two introns, one nuclear and one mitochondrial coding region by the PCR-SSCP (Polymerase Chain Reaction-Single Strand Conformation Polymorphism) method followed by sequencing.

**MATERIALS AND METHODS**

**Molecular analyses**

Thirty-three *E. multilocularis* (Em) isolates from different countries, hosts and life-cycle stages (Table 1) were used for genomic DNA extraction and further analyses. DNA extraction was done by standard procedures (McManus, Knight & Simpson, 1985).

For each isolate, 4 different targets were amplified, using 1 of the 2 different PCR programs (see Table 2), on a Perkin Elmer (Cetus) thermocycler: (1) TD 65–55: 20 cycles of 1 min denaturation at 94 °C, 30 sec annealing at 65 °C and 2 min extension at 72 °C, with a touch down of 1 °C every 2 cycles, followed by 20 more cycles with 55 °C annealing and a final 10 min extension. (2) TD 55–45: the same program, with a touch down from 55 to 45 °C annealing.

The first 2 targets (ActII and Hbx2) are non-coding introns from the respective genes characterized in *E. granulosus* (Silva et al. 1993; Vispo & Ehrlich, 1994). The other 2 targets are coding regions: part of the nuclear antigen B gene (AgB) and neutrophil chemotaxis activity (Shepherd, Ait-which codes for a protein with inhibition of elastase amplification only the case of ND1, for example, primers were designed to smaller portion of the published sequences. In the McManus, 1993). Note that our primers amplified a

denaturant of 10 % formamide; 100 mM NaOH; 0-25 % bromophenol blue; 0-25 % xylene-cyanol). The denatured fragments were maintained on ice until they were gel loaded for separation under non-denaturing conditions in 10 % polyacrylamide gels containing 10 % glycerol at 10 °C (ActII and Hbx2), or 12 % polyacrylamide gels containing 7 % glycerol at 15 °C (AgB/1 and ND1). Electrophoresis was performed at 200 V, during 1-5 (AgB/1) to 3 h (ActII, Hbx2 and ND1), to separate the single strands according their secondary structures. For visualization of the SSCP electrophoretic resolution, we used conventional silver-staining techniques.

The double-stranded PCR products derived from re-naturation before or during gel loading migrate faster than the single strands. Each secondary structure of single-stranded DNA is represented by a clear silver-stained band, but some sequences can show more than 1 equally stable secondary structure, resulting in more than 1 band per single strand in the SSCP pattern (see Figs 1 and 2). Differences in banding pattern due to nucleotide substitutions were confirmed by sequencing each SSCP band. For this, single-stranded DNA bands were cut out from the fresh, stained SSCP gels, washed several times in 1 ml of distilled water and eluted in 50 µl of 1 × PCR buffer (Gibco) containing 0-1 % Triton X–100. One µl of the eluted single strands was used for re-amplification with the corresponding primers, following the same procedures described above. The purified PCR products (Qiagen) were used for direct fluorescence sequencing of double-stranded PCR products using a 373A system (Applied Biosystems).

At least 2 isolates (1 North American and 1 European) from each SSCP pattern were chosen for sequencing. Isolate numbers 2 and 20 (Table 1) were sequenced for all targets. Isolates numbers 5, 6, 8, 11, 19, 21 and 24 were sequenced for two or more targets, to confirm the sequence identity among isolates showing the same SSCP pattern. Indeed, no sequencing differences among identical SSCP bands were found. We are aware that the sensitivity of the technique for point mutations in fragments with less than 350 bp is around 95–100 % (Lessa & Applebaum, 1993; Sheffield et al. 1993), but for simplicity we assumed that phenotype identity was due to genotype identity.

**Statistical analyses**

*E. multilocularis* sequences were aligned with homologous sequences obtained for *E. granulosus* in from each test run were used for the SSCP screening. SSCP analysis of the amplified DNA fragments as described by Orita *et al.* (1989) was modified and optimized for gel composition, electrophoresis conditions and staining procedures (Liechti-Gallati, Neeser & Giusti, 1995). Briefly, 2 µl of the products were denatured for 3 min at 94 °C in 3 µl of denaturing buffer (95 % formamide; 100 mM NaOH; 0.25 % bromophenol blue; 0.25 % xylene-cyanol). The denatured fragments were maintained on ice until they were gel loaded for separation under non-denaturing conditions in 10 % polyacrylamide gels containing 10 % glycerol at 10 °C (ActII and Hbx2), or 12 % polyacrylamide gels containing 7 % glycerol at 15 °C (AgB/1 and ND1). Electrophoresis was performed at 200 V, during 1-5 (AgB/1) to 3 h (ActII, Hbx2 and ND1), to separate the single strands according their secondary structures. For visualization of the SSCP electrophoretic resolution, we used conventional silver-staining techniques.

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**Statistical analyses**

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Table 1. Life-cycle stage, host, genotype and geographical distribution of the *Echinococcus multilocularis* isolates analysed in this study

(The metacestode is the larval stage of the parasite, usually encountered in the liver of intermediate host such as rodents and humans. The adult tapeworms are found in the intestine of definitive hosts, usually foxes, occasionally dogs and cats. For explanation of genotypes A and B, refer to the text (Results section).)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Stage</th>
<th>Host</th>
<th>Origin</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>6</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>8</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>9</td>
<td>Metacestode</td>
<td>Human</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>10</td>
<td>Metacestode</td>
<td>Human</td>
<td>Canada</td>
<td>A</td>
</tr>
<tr>
<td>11</td>
<td>Metacestode</td>
<td>Human</td>
<td>Alaska*</td>
<td>A</td>
</tr>
<tr>
<td>12</td>
<td>Metacestode</td>
<td>Human</td>
<td>Japan</td>
<td>A</td>
</tr>
<tr>
<td>13</td>
<td>Metacestode</td>
<td>Human</td>
<td>France</td>
<td>A</td>
</tr>
<tr>
<td>14</td>
<td>Metacestode</td>
<td>Monkey</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>15</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>16</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>17</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>18</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>19</td>
<td>Metacestode</td>
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<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>20</td>
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<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>21</td>
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<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>22</td>
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<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>23</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>24</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>25</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>26</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>St Lawrence</td>
<td>B</td>
</tr>
<tr>
<td>27</td>
<td>Adult</td>
<td>Fox</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>28</td>
<td>Adult</td>
<td>Fox</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>29</td>
<td>Adult</td>
<td>Fox</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>30</td>
<td>Adult</td>
<td>Fox</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>31</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>Germany</td>
<td>A</td>
</tr>
<tr>
<td>32</td>
<td>Metacestode</td>
<td>Monkey</td>
<td>Switzerland</td>
<td>A</td>
</tr>
<tr>
<td>33</td>
<td>Metacestode</td>
<td>Rodent</td>
<td>Canada</td>
<td>A</td>
</tr>
</tbody>
</table>

* Continent.
† Alaska.

Table 2. Primer sequences and PCR conditions for amplification of the 4 *Echinococcus* genomic targets analysed in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Size*</th>
<th>Reference</th>
<th>Primers</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>EmActII</td>
<td>268</td>
<td>Silva <em>et al.</em> (1993)</td>
<td>5’-GTCTTTCCCTCTCTATCGTGGG-3’ 5’-CTAATGAAATAGTGCTTGTGCG-3’</td>
<td>TD 65–55</td>
</tr>
<tr>
<td>EmAgB/1</td>
<td>102</td>
<td>Frosch (1994)</td>
<td>5’-CGTGATCCGTGTTGGTCAG-3’ 5’-GGCACCTCTATTCACCTTCA-3’</td>
<td>TD 65–55</td>
</tr>
<tr>
<td>EmHbx2</td>
<td>330–331</td>
<td>Vispo &amp; Ehrlich (1994)</td>
<td>5’-TTCTCCCTCTCAGCCAGGTCCTCG-3’ 5’-TATACGCGCGATTCGTTGGAA-3’</td>
<td>TD 65–55</td>
</tr>
<tr>
<td>EmND1</td>
<td>141</td>
<td>Bowles &amp; McManus (1993)</td>
<td>5’-TTCTAGGTATTCTTGTGGTG-3’ 5’-CAAGCTTTCATCAACAATCTATAA-3’</td>
<td>TD 55–45</td>
</tr>
</tbody>
</table>

* In base pairs (bp).

another study (Haag *et al.*, manuscript in preparation). In that study, we used sheep, cattle and pig strain isolates characterized by Siles-Lucas, Benito & Cuesta-Bandera (1996) using RAPD and isoenzyme markers as references for strain identification using PCR-SSCP followed by sequencing. The
Fig. 1. SSCP patterns of ActII (A) and Hbx2 (B) introns. Upper bands are single-stranded DNA secondary structures, while lower bands are the renaturated double strands. (A and B) Lanes 1–9 and 10–13 are *Echinococcus multilocularis* metacestode isolates from humans and rodents respectively, Switzerland; Lane 14 is a metacestode isolate from a monkey, Switzerland; Lanes 15–18 are human metacestode isolates from Canada, Alaska, Japan and France, respectively; Lanes 19–30 are rodent metacestode isolates from St Lawrence Island. M is marker VIII (Boehringer) and the numbers indicated on the right side correspond to the molecular weight of marker bands above and below the *Echinococcus* double strands. (A) Lanes 31–34 are *E. granulosus* metacestode isolates from sheep, horse, pig and cattle strains, respectively. (B) Lanes 31 and 32 are *E. granulosus* isolates from sheep and cattle.

**RESULTS**

The *E. multilocularis* (Em) SSCP patterns obtained for ActII, Hbx2, AgB/1 and ND1 are shown in Figs 1 and 2. The sequencing revealed that all patterns were homozygous for the Em alleles shown in Fig. 3. Only a few nucleotide differences were found within the *E. multilocularis* sample: deletion of a T and an A–C transversion in the Hbx2 intron (see alleles EmHbx2-1 and EmHbx2-2 in Fig. 3) discriminated the Em isolates in 2 groups. These mutations can be readily identified by a cautious inspection of the SSCP gel shown in Fig. 1B (see differences among isolates 1–18, EmHbx2-1 and 19–30, EmHbx2-2). Thus, genotype A (Table 1) was assigned to the isolates homozygous for alleles EmActII-1, EmHbx2-1, EmAgB/1-1 and EmND1-1; genotype B was assigned to isolates homozygous for alleles EmActII-1, EmHbx2-2, EmAgb/1-1 and EmND1-1.
Genotype A is distributed worldwide, while genotype B occurs only in St Lawrence Island, Alaska (see Table 1). The molecular diversity parameters estimated from *E. multilocularis* sequences indicate that the genetic variability within the species is extremely low. Table 3 shows the values of 3 parameters calculated from our SSCP and sequencing data in *E. multilocularis* and *E. granulosus*. Molecular diversity indices normally have high standard deviations due to stochastic and sample bias, which makes them useless for significance tests (Kreitman, 1991), but a comparison of the estimates obtained for both species, suggests that *E. granulosus* has at least 10 times more variability than *E. multilocularis*.

We used \( \pi \) and \( p_i \) as indicators of strain differentiation, taking them as measures of divergence between populations. The parameters were calculated for each pair of *E. granulosus* recognized strains and also between each pair of *E. granulosus* \( \times E. multilocularis \) haplotypes. The results are shown in Table 4. As expected, divergence estimates were higher when comparing haplotypes from different species than those obtained from strains of the same species. Parameters calculated between *E. multilocularis* A and B genotypes were at least 5 times lower than those obtained from *E. granulosus* strains.

Assuming a constant rate of substitution and that parasite populations expanded after the colonization of a new host, \( \pi \) can also be used as an estimator of
Fig. 3. Alignment of *Echinococcus granulosus* (*Eg*) and *E. multilocularis* (*Em*) alleles. The *E. granulosus* isolates were all homozygous for the following haplotypes: (1) sheep strain: *EgActII*-1, *EgHbx2*-1, *EgAgB*{1}-1, ND1-1; (2) cattle strain: *EgActII*-2, *EgHbx2*-2, *EgAgB*{1}-2, ND1-2; and pig strain: *EgActII*-3, *EgHbx2*-2, *EgAgB*{1}-2, ND1-3.

For *E. multilocularis* genotypes, see text. Points represent homologous nucleotides; dashes represent deletions. Genbank accession numbers of the sequences are: L07774, AF003748, AF003749 and AF003750 (*Act II intron*); X66818, AF003976, AF003977 and AF003978 (*Hbx2 intron*); Z26481, Z26482 and Z26483 (*AgB*{1}); and U65748 [ND1—authors did not provide information about variant sequences published by Bowles & McManus (1993)].
Genetic variation in *E. multilocularis*

Table 3. Molecular diversity estimates for *Echinococcus multilocularis* and *E. granulosus* ActII, Hbx2, AgB/1 and ND1 sequences

<table>
<thead>
<tr>
<th>Parameter*</th>
<th><em>E. multilocularis (n = 33)</em></th>
<th><em>E. granulosus (n = 78)</em>†</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of polymorphic sites</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>Mean no. of pairwise differences (p&lt;sub&gt;i&lt;/sub&gt;)</td>
<td>0.4785</td>
<td>7.6020</td>
</tr>
<tr>
<td>s.d.</td>
<td>0.4252</td>
<td>s.d. 3.5911</td>
</tr>
<tr>
<td>Nucleotide diversity (π)</td>
<td>0.0005</td>
<td>0.0090</td>
</tr>
<tr>
<td>s.d.</td>
<td>0.0005</td>
<td>s.d. 0.0040</td>
</tr>
</tbody>
</table>

* Nei (1987).
† Includes sheep, cattle and pig strains.

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Our results show that, conversely to *E. granulosus*, the genetic variability within *E. multilocularis* is quite low. Except for 2 nucleotide differences in the Hbx2 intron between genotypes A and B, all isolates had the same nucleotide sequences in coding and non-coding regions. Indeed, in a preliminary analysis we also found identical SSCP patterns for the same isolates in 2 other sequences (unpublished data), mitochondrial CO1 and nuclear BG1/BG3 (Gottstein & Mowatt, 1991; Bowles et al. 1992). Nevertheless, the value of the present findings is to be found in the direct comparison between the obvious variability within *E. granulosus* and the corresponding conserved status within *E. multilocularis*.

In *E. multilocularis* the markedly lower genetic variability is putatively due to the fact that *E. granulosus* adapted historically to a number of different host species in different geographical areas, while *E. multilocularis* retained the ancestral cycle with a conserved host spectrum. An almost exclusive self-fertilizing system, associated with asexual reproduction in the metacestode and strong selection by the host (Smyth & Smyth, 1964) might have led to highly homogeneous evolutionary units. If strains are taken as evolutionary independent lines, in the way to originate new species, the variability of a species showing slower rates of evolution would be similar to that of a well-defined strain. Analogous to *E. multilocularis*, the intra-strain variability in *E. granulosus* is also quite low (Haag et al., manuscript in preparation). However, inter-strain variability appears so high, that it even resulted in the postulation of a taxonomic revision in this group (Thompson, Lymbery & Constantine, 1995).

The geographical distribution of genotypes A and B does not follow the pattern of the conventionally accepted North American and European *E. multilocularis* strains (Rausch, 1985). While genotype A is distributed world-wide, genotype B is restricted to St Lawrence Island in the Bering Sea between Alaska and Siberia. They appear to have diverged in a very recent past, much later than the *E. granulosus* strains did. The rather simple and isolated tundra biome of the island, in which the fox feeds almost exclusively on microtine rodents, and rates of infection are high (Schantz et al. 1995), could be related to the appearance of a slightly different genotype. It remains to be elucidated by more detailed analyses if this situation is unique to St Lawrence Island or if it may also appear in other...
geographically disparate endemic areas, such as on the East or South West Asian continent or even within the isolated affected states of the lower United States of America.

The decision as to whether or not these genotypes belong to different strains, depends on the definition of the nebulous term 'strain' (Thompson et al. 1995). Lymbery & Thompson (1988) proposed that it should be used in a 'practical context', in which 2 conditions should be satisfied by populations can be regarded as different strains: (1) they should be genetically differentiated and (2) they should differ in 1 or more characters of epidemiological significance. However, it is not clear how great the degree of that genetic differentiation should be. Furthermore, Thompson (1995), referring to a situation in which populations of another parasite fail to show genetic variability in the presence of phenotypic differentiation, argues that in some circumstances these conditions cannot be met.

From our point of view, a strain must have a clear and distinctive biological profile, involving a number of genetic, ecological, developmental and epidemiological characters. It is clear from our results and from those of other authors (Bretagne et al. 1996) that, using sensitive genetic markers, it is possible to find polymorphism among populations of E. multilocularis from North America. However, the degree of variability seems to be too low for strain differentiation. For this reason, we suggest that speciation in E. multilocularis may only be justified if supplemented with additional criteria to the minor genetic findings described in this paper and previously by other authors (Bowles et al. 1992; Bowles & McManus, 1993; Bretagne et al. 1996). These criteria should predominantly include biological and pathogenic aspects to support, in a relevant way, the clear characterization of different E. multilocularis strains.

Thanks to Dr Maria del Mar Siles-Lucas for the genomic DNAs of E. granulosus isolates; to Mrs Corin Müller for her collaboration with the SSCP-s; and to Dr Sandro Bonatto for the helpful discussions and critical review of the manuscript. We are indebted to Professor R. Rausch for his invaluable support to obtain appropriate material from St Lawrence Island. The support by the Swiss National Science Foundation (project no. 31-45575.95), PADC/T/CNPq (Proc. 620081/95-3), EEC (DG XII Cl 10284-0), the Jubiläumstiftung der Schweizerischen Lebensversicherungs- und Rentenanstalt für Volksge-sundheit und Medizinische Forschung and the Sandoz-Stiftung zur Förderung der medizinisch-biologischen Wissenschaften is gratefully acknowledged.

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