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 echinococosis 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 Pleistocenic 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

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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/1), which codes for a protein with inhibition of elastase and neutrophil chemotaxis activity (Shepherd, Aitken & McManus, 1991), and the other is part of the mitochondrial NADH dehydrogenase 1 gene (ND1). Both sequences were described in *E. granulosus* and *E. multilocularis* (Frosch *et al*. 1994; Bowles & McManus, 1993). Note that our primers amplified a smaller portion of the published sequences. In the case of ND1, for example, primers were designed to amplify only the *E. multilocularis* polymorphic region. The designed primers were shown to be specific for *Echinococcus* DNA, since no amplification occurred using host DNA as template. The primer sequences, amplicate size and corresponding genomic sequences are shown in Table 2 and Fig. 3. All PCR reactions were performed in 50 μl containing 10 ng genomic DNA, 20 pmol of each primer (MWG, Germany), 0·5U Taq polymerase (Gibco), 1 × PCR buffer containing 1·5 mM $MgCl_2$ (Gibco) and 20 μM dNTP (Perkin Elmer).

Subsequent to PCR, the denatured PCR products 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% xylencyanol). 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

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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).)

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

* Continent.
† Alaska.

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

Target	Size*	Reference	Primers	PCR
EmActII	268	Silva *et al.* (1993)	5′-GTCTTCCCCTCTATCGTGGG-3′ 5′-CTAATGAAATTAGTGCTTGTGCGC-3′	TD 65–55
EmAgB/1	102	Frosch (1994)	5′-CGTGATCCGTTGGGTCAG-3′ 5′-GGCACCTCTATTCACCTTCA-3′	TD 65–55
EmHbx2	330–331	Vispo & Ehrlich (1994)	5′-TTCTCCTCTAGCCAGGTCCA-3′ 5′-TATAGCGCCGATTCTGGAAC-3′	TD 65–55
EmND1	141	Bowles & McManus (1993)	5′-TTCTAGGTATTCTTTGTTGTG-3′ 5′-CAAGCTTCATCAACAACTATAA-3′	TD 55–45

* 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

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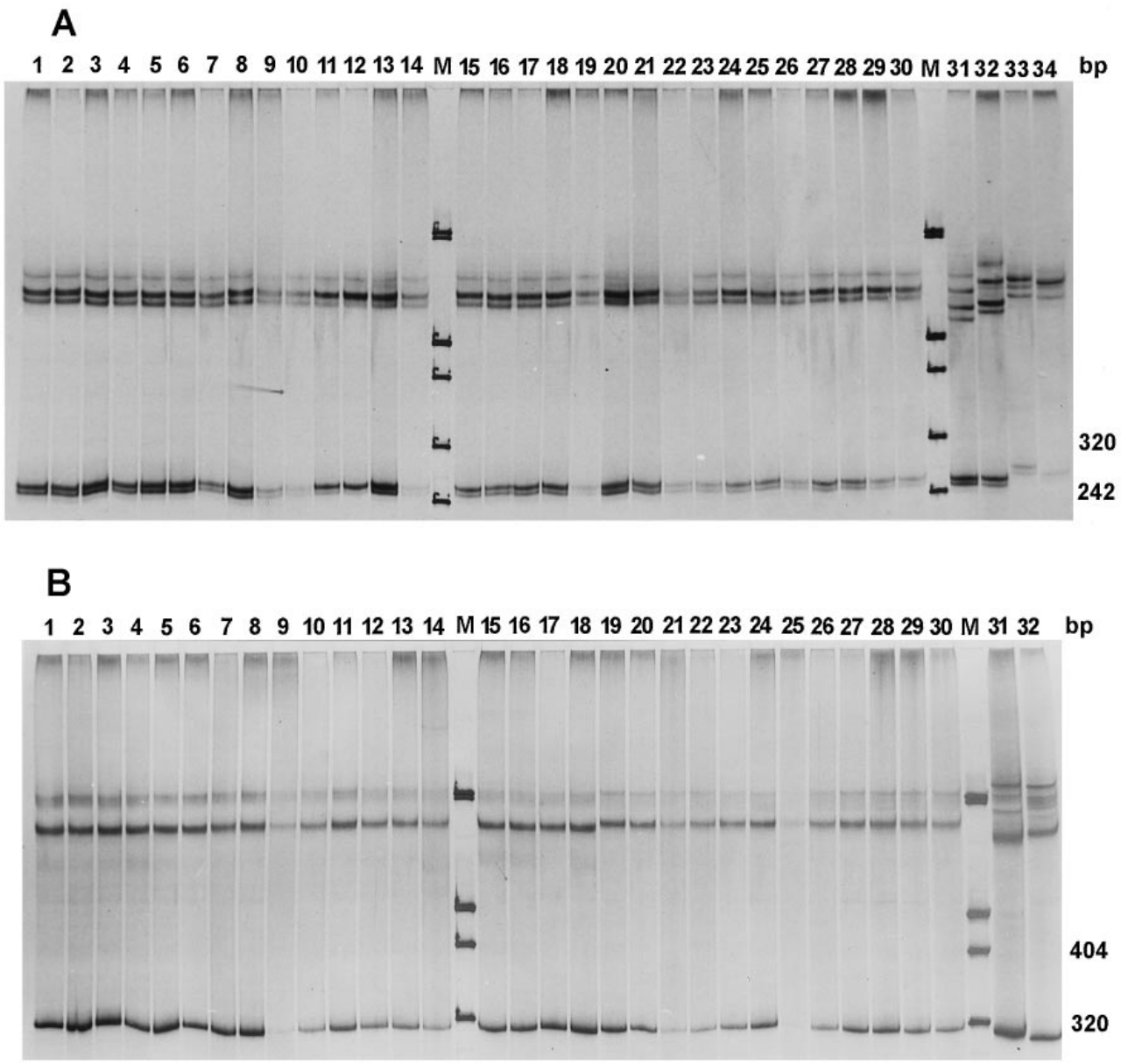

Fig. 1. SSCP patterns of ActII (A) and Hbx2 (B) introns. Upper bands are single-stranded DNA secondary structures, while lower bands are the renaturated double strands. (A and B) Lanes 1–9 and 10–13 are *Echinococcus multilocularis* metacestode isolates from humans and rodents respectively, Switzerland; 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.

alignments were performed with the GCG Package (version 8, 1994). Molecular diversity indices were estimated by Arlequin (version 1.0b5).

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.

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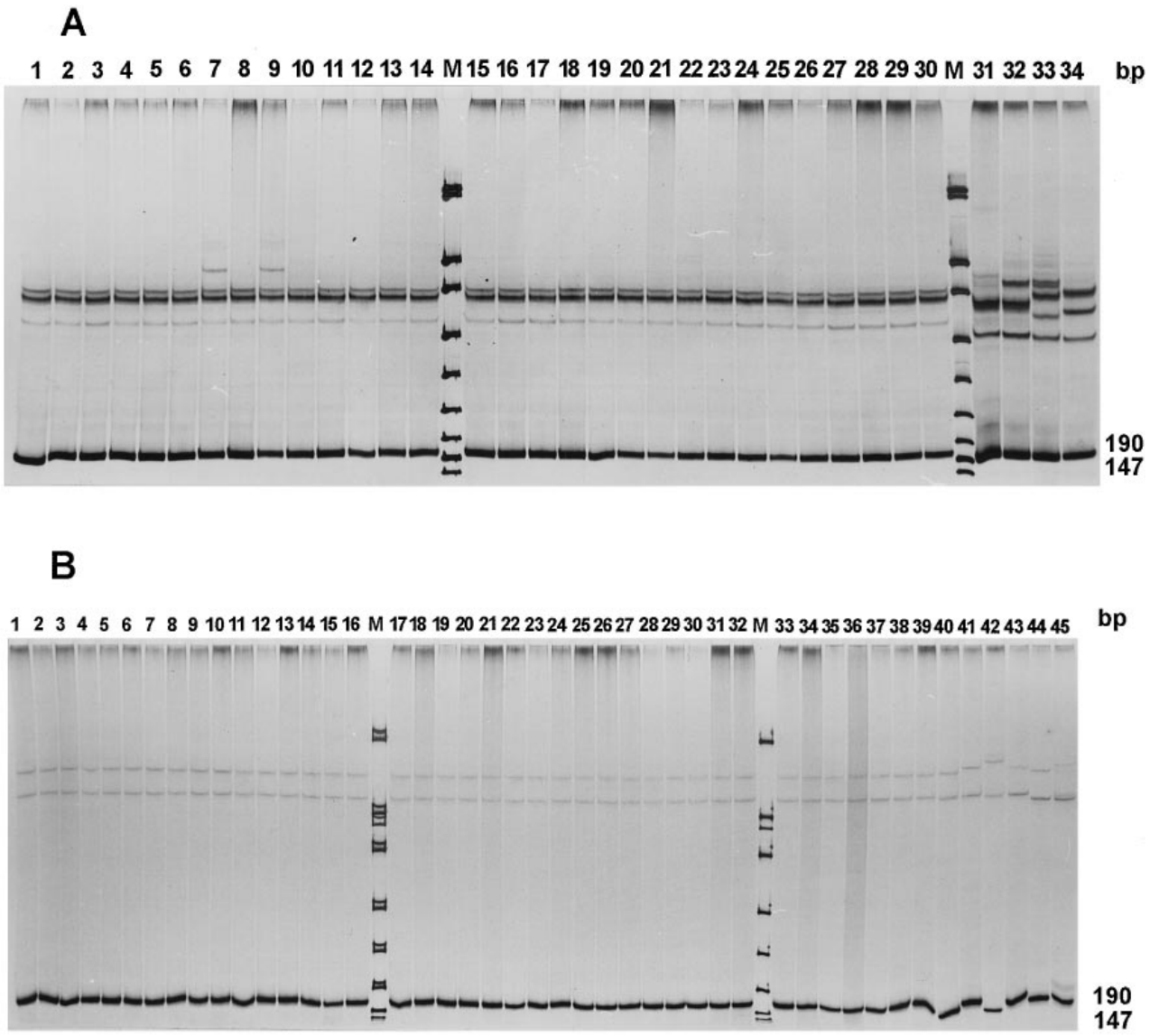

Fig. 2. SSCP patterns of AgB/1 (nuclear, A) and ND1 (mitochondrial, B) coding sequences. The isolates in Lanes 1–30 of both pictures and the markers are the same as described for Fig. 1. (A) Lanes 31–34 are *Echinococcus granulosus* metacestode isolates from sheep (31 and 32), horse and pig strains, respectively. (B) Lanes 31–35 and 36–40 correspond to the same set of isolates shown in Lanes 14–18 analysed by 2 additional and independent PCR experiments; Lanes 41–44 are *E. granulosus* metacestode isolates from sheep, cattle, horse and pig strains; 45 is an *E. vogeli* isolate.

Genotype A is distributed world-wide, 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 π 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* × *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, π can also be used as an estimator of

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a) ACTII (intron):

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                10                  30                  50                  70                  90                  110
EgActII-1 TCGTCCAAGACATCAGGTTAGTTGGATAGGTAGGCAGTGTTTCAGCCGCACCGGAACTGGTACCAACTAGTGGACCAATTTTCTCAAATAAGAGACAGAAATGGTTTGCTTTCATGCACT
EgActII-2 ..........................................T.........G.............C.........C.......A.............CA.......C.........
EgActII-3 ..........................................T.........G.............C.........C.......A.............CA.......C.........
EmActII-1 ..................................................T.........C...T.....CTG..T..........A...CA.......C.........

              130                 150                 170                 190                 210                 230                 250
EgActII-1 AAATGTATGGTGAAGAAGTCGGCTTTTCATCTAACTAGATAGGCATGATTAGTGTGGAGATCAAGTGCTCTCTTGTAGAGTCGCCATCTGAGGGCAGTCTTTCTATTTTCGCCCTGTGACAACGTACCTATTCCGAAATAATCTTT
EgActII-2 ........................................G.....................................................T...T.....................................A..
EgActII-3 ......................T.....................................................................T.........GG.C.....................................A..
EmActII-1 ........................................A.....................................................................................................
```

b) HBX2 (intron):

```
                10                  30                  50                  70                  90
EgHbx2-1 CGTCTTAGAAGAGCGATTTGATCGACAAAAGTACCTCAGCAGTGCTGAACGCGCCGAGATGTCACGAGACCTGGGGCTCTCTGAAACCCA
EgHbx2-2 ..................................................................................................
EmHbx2-1 ..................................................................................................
EmHbx2-2 ..................................................................................................

                    110                 130                 150                 170
EgHbx2-1 GGTATGTCACAGCCGATGTCATTAAACATGGGAAGGGGTGAGAGTAGTTGGAGCGTCACGAAGTGCCAAATTGGGCGCTTGTCAAGCTGC
EgHbx2-2 ................C.............................T.....................................
EmHbx2-1 ................C.....................................................................
EmHbx2-2 ................C....-....A.....................................................

              190                 210                 230                 250                 270                 290                 310                 330
EgHbx2-1 GCCTTTATAACTGTTGAGTGCATCATCACCCATAAAAAATTGGGAGAGAGGGGGGCGGGAGCGGGTCAAAAGGGTCATCACGGCTCATGCATTAGTAAGATCGTAAAAGGCATGCCTCTAATTATGACCCCCACCACTAGGTGAAAATATG
EgHbx2-2 ..................................................................................................................................................
EmHbx2-1 ..........................................................T.....................................................T.....................
EmHbx2-2 ..........................................................T.....................................................T.....................
```

c) AGB/1 (nuclear coding region):

```
                10                  30                  50                  70                  90
EgAgB/1-1 AGTGGTTGACCTCTTAAAGGAACTGGAAGAAGTGTTCCAGTTGTTGAGGAAGAAGCTACGCATGGCACTCAGGTCCCACCTCAGAGGGTTGATTGCTGAAGG
EgAgB/1-2 ..................................................C......T.A..............A....G.........
EmAgB/1-1 .T.........A.....................A....A..............C........A..............A....G.........
```

d) ND1 (mitochondrial coding region):

```
                10                  30                  50                  70                  90                  110                 130
EgND1-1 CTGGTTGGGGTGGTTACAACAATTATTCATTTTTAAGGTCGGTTCGATGTGCTTTTGGATCTGTTAGGTTTGAGGCTTGTTTTATGTGTGTGGTGATTTTTTGTGCTTTGTGTAGTTGTAGGTATAATTTAATTGATTTTT
EgND1-2 .A.........A....T..T.G......T.....................................................T.....................T.....................
EgND1-3 ...........A.......T.G......T.....................................................T.........C.....A..CT.....G.....................
EmND1-1 ...........A.......T..A.....G.........T..T.................G..............A.....................A.........T.........TAC..............G.........
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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: EgActIII-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)].

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

Parameter*	*E. multilocularis* ($n = 33$)	*E. granulosus* ($n = 78$)†
No. of polymorphic sites	2	35
Mean no. of pairwise differences (p_i)	0·4785	7·6020
	S.D. 0·4252	S.D. 3·5911
Nucleotide diversity (π)	0·0005	0·0090
	S.D. 0·0005	S.D. 0·0040

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

Table 4. Mean number of pairwise differences (above the diagonal) and nucleotide diversities (below the diagonal) among *Echinococcus granulosus* strains and *E. multilocularis* genotypes A and B

	Sheep	Cattle	Pig	Gen A	Gen B
Sheep		5·34	2·99	14·75	11·08
		(2·61)*	(1·59)	(6·67)	(5·09)
Cattle	0·0063		5·60	14·00	18·18
	(0·0034)		(2·94)	(6·48)	(8·44)
Pig	0·0035	0·0066		9·13	14·06
	(0·0021)	(0·0039)		(4·35)	(6·68)
Gen A	0·0174	0·0165	0·0108		0·48
	(0·0088)	(0·0085)	(0·0057)		(0·42)
Gen B	0·0131	0·0215	0·0166	0·0005	
	(0·0067)	(0·0112)	(0·0089)	(0·0005)	

divergence times (Rogers & Jorde, 1995), where $T = \pi/2\lambda$ and λ is the average substitution rate per million years (MY) (Nei, 1987). If $\lambda = 3{\cdot}7 \times 10^{-9}$ (average substitution rate in introns (Nei, 1987)), the divergence times among sheep, cattle and pig strains would be around 0·6 MY, based on ActII and Hbx2 intron sequences only. The times of divergence among strains and genotypes of the different species would range from 1 to 2 MY. *E. multilocularis* genotypes A and B, on the other hand, were estimated to have diverged at just 0·1 MY ago.

DISCUSSION

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 before 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.

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