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Limited Range of Genetic Variation in *Echinococcus multilocularis*

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LIMITED RANGE OF GENETIC VARIATION IN *ECHINOCOCCUS MULTILOCULARIS*

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ABSTRACT: DNA sequencing of 1.3 kb of rDNA containing both internal transcribed spacers (ITS1, ITS2) and adjoining rRNA coding regions in each of 11 *Echinococcus multilocularis* isolates from Germany, Japan, and Alaska resulted in identical nucleotide sequences except for a single polymorphic locus 54 bp upstream of the 3′ end of the 18S coding region, separating Eurasian isolates from an Alaskan isolate. The same base substitution was found in each of 2 additional isolates from Alaska. The distribution of the resulting genotypes with regard to their origin is highly significant (>99.9%) and corresponds to the traditional subspecies *Echinococcus multilocularis multilocularis* and *Echinococcus multilocularis sibiricensis.*

The existence of distinct populations, most often referred to as strains or variants, within the species *Echinococcus multilocularis* has repeatedly been claimed to explain phenotypic and genotypic differences between members of this species. The discrimination of genetically distinct strains of *E. multilocularis,* when correlated with clinical, morphological, biochemical, and immunological data, is expected to be relevant in explaining differences in susceptibility to chemotherapy, infectivity, and differences in the development of this parasite in infected intermediate hosts (Vogel et al., 1991) and to have implications for epidemiological studies and the elucidation of transmission patterns (Bretagne et al., 1996). Phenotypic and biological differences between strains of *E. multilocularis* have been described with respect to morphology (Vogel, 1957), the spectrum of intermediate hosts (Sakui et al., 1984; Pfister and Frank, 1988), the localization of larval foci in the intermediate host (Vogel, 1957), virulence (Gottstein et al., 1987; Rausch et al., 1987; Bresson-Hadni et al., 1994), antigen expression (Gottstein et al., 1991), and the prepatent period in the definite host (Thompson and Eckert, 1983).

In principle, factors affecting the parasites' morphology and biology could be contributed by the host, the parasite, or both. They could be controlled by the environment, or be genetically determined, or both. Investigations on possible host contributions have been few (Kroeze and Tanner, 1987; Gottstein et al., 1994) and divergent for susceptibility to and clinical prognosis of the infection in humans (Scherbakov, 1993; Gottstein and Bettens, 1994). With regard to the parasite, evidence for the existence of distinct strains of *E. multilocularis* has been favorably reviewed (Eckert and Thompson, 1988; Thompson and Lymbery, 1988), and their molecular characterization has been acknowledged as an urgent need (Thompson et al., 1995). Preliminary data are available for the existence of distinct *E. multilocularis* genotypes by sequencing mitochondrial genes (Bowles et al., 1992; Bowles and McManus, 1993a), Southern hybridization with an anonymous DNA probe (Vogel et al., 1991), random amplification of polymorphic DNA (Scott and McManus, 1994), and analyses of microsatellite polymorphisms (Bretagne et al., 1996). However, due to the small sample sizes (4 or less isolates, except for the latter study) and due to the choice of the target gene or the employed method to analyze it, their epidemiologic and phylogenetic implications remain unclear.

In an effort to avoid target genes and analysis methods that might represent individual features of different isolates rather than reflect taxonomically relevant phylogenetic relationships, we have chosen the ribosomal RNA gene (rDNA) as the genetic target because during evolution these genes accumulate mutations in a very steady fashion and are, therefore, also called "molecular clocks" (Olsen and Woese, 1993). Their hypervariable spacer regions have repeatedly been useful for taxonomic distinctions at and below the species level, including taeniid cestodes (Gasser and Chilton, 1995). In this report, we have amplified the first and second internal transcribed spacers (ITS1 and ITS2), the enclosed 5.8S, and adjoining portions of the 18S and 28S rRNA coding sequences from 11 *E. multilocularis* isolates from Germany, Japan, and Alaska in order to find characteristics that might qualify as genetic markers for the description of strains within this species.

MATERIALS AND METHODS

Echinococcus multilocularis isolates

All German isolates (nos. 1–9; Table I) were intestinal smears from hunted red foxes (*Vulpes vulpes*), the Japanese sample (no. 10) was isolated from a trapped vole (*Clethrionomys rufocanus bedfordiae*), the first Alaskan isolate (no. 11) originated from a human biopsy taken from a resident of St. Lawrence Island in 1982 and had been maintained in voles (*Clethrionomys rutilus*) and gerbils (*Meriones unguiculatus*) until the present sample was obtained in 1996. The other 2 samples from Alaska (nos. 12 and 13) originated from voles (*Microtus oeconomus* and *C. rutilus,* respectively) trapped in May 1996 and were maintained in gerbils (*M. unguiculatus*) between June and November of the same year. An overview of the geographical origins and hosts is given in Table I.

DNA isolation

DNA from intestinal smears was isolated by an alkaline lysis method as described previously for stool specimens (Katzwinkel-Wladarsch et al., 1994). DNA from tissue samples was prepared by proteinase K digestion and phenol/chloroform extraction following standard protocols (Sambrook et al., 1989). All DNA was finally purified and concentrated by silica gel adsorption (Geneclean; BIO 101, La Jolla, California) and eluted in 120 μl water.

Nested polymerase chain reaction (PCR)

The rDNA target was amplified by 2 consecutive (nested) PCR reactions. The upstream primer for the first PCR, MSP-3 (GGA ATT CAC ACC GCC CGT C(AG)(CT) TAT), is located in the 3′ region of the 18S coding sequence and was constructed to match several microsporidian species as described by Katzwinkel-Wladarsch et al. (1996). The downstream primer RIB-3 (CGG GAT CCT TC(AG) CTC GCC G(CT)T ACT) is targeted to a highly conserved section of the 28S 5′

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TABLE I. Geographical origins and hosts of *E. multilocularis* isolates examined.

No.	Geographic origin	Host	Collection year
1	Bavaria, Germany (south)	Fox	1994
2	Bavaria, Germany (south)	Fox	1994
3	Bavaria, Germany (south)	Fox	1995
4	Brandenburg, Germany (east)	Fox	1995
5	Brandenburg, Germany (east)	Fox	1995
6	North Rhine—Westphalia, Germany (west)	Fox	1995
7	North Rhine—Westphalia, Germany (west)	Fox	1995
8	Thuringia, Germany (southeast)	Fox	1994
9	Lower Saxony, Germany (northwest)	Fox	1995
10	Hokkaido, Japan	Vole	1996
11	St. Lawrence Island, Alaska, USA	Human	1982
12	St. Lawrence Island, Alaska, USA	Vole	1996
13	St. Lawrence Island, Alaska, USA	Vole	1996

region. MSP-3 and RIB-3 were empirically found to be capable of amplifying *E. granulosus* DNA isolated from hydatic fluid from infected sheep, yielding a 1.7-kb product, and subsequently from isolated *E. multilocularis* adults, which gave a 1.5-kb product. The inner upstream primer ECH-6 (CCA CTA GTC GCT GAG A(AG)G ACG AC), ending 73 bp before the 3′ end of the 18S coding region, and the downstream primer ECH-5 (CGG AAT TCA GCG GGT ACC CAC C), ending 19 bp from the 3′ start of the 28S coding region were constructed to match the sequences obtained with MSP-3/RIB-3 from both *Echinococcus* species but not those of mammalian, plant, bacterial, and fungal rDNA (Dams et al., 1988; Gutell and Fox, 1988) which was expected to contaminate especially the intestinal smears. The PCR reactions were done employing a "hot start" technique in which 38.2 μl DNA extract (or 2 μl of the first PCR in 37.5 μl water for the second PCR) was denatured at 96 C for 2 min after the addition of 1 μl each of 50 mM solutions of the primers, 1.8 μl (1.5 μl for the second PCR) of 50 mM $MgCl_2$, 2 μl (1 μl for the second PCR) of dimethyl sulfoxide (DMSO), and 2 drops of mineral oil. After cooling to 85 C, 6 μl of a freshly prepared mixture of 5 μl buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 0.5 μl of dNTP mix (25 mM each; United States Biochemical Corporation, Cleveland, Ohio), and 0.5 μl (5 u/μl) of *Taq* polymerase (Amersham Buchler, Braunschweig, Germany) was added; 40 cycles (30 for the second PCR) were performed with denaturation at 92 C for 60 sec, annealing for 60 sec at 58 C (62 C for the second PCR), and extension at 72 C for 90 sec. Products were visualized in a 1.3% agarose gel containing 0.2 μg/ml ethidium bromide.

Cloning and sequencing

The inner primers ECH-6 and ECH-5 included linkers with *Spe*I and *Eco*RI sites, respectively, to facilitate the subsequent cloning of the products into *Xba*I/*Eco*RI-cut pBluescript II SK⁻ vectors (Stratagene, La Jolla, California). Sequencing was performed by dideoxy nucleotide chain termination using a Sequenase II-kit (United States Biochemical Corporation). In addition to the sequencing primers T3 (ATT AAC CCT CAC TAA AG) and KS (CGA GGT CGA CGG TAT CG) located on the vector on both sides of the insert, 8 internal, facing sequencing primers, ECH-7 (GCA AAT CGC AGC AAC GC), ECH-8 (GTG GTT GTT ATC GCT GC), ECH-9 (ACGTCC ACT GAG CAG CC), ECH-10 (GTG TAA TTG GTT TAA GG), ECH-11 (ACG TGA CCA CAG GCA AG), ECH-12 (GTG CAG CCA ACT GTG TG), ECH-13 (TCC ACC ACA GCA TCC AC), and ECH-14 (TGG TGA TGC GGT TGC AG), whose locations are given in Figure 1, were used to obtain sequence information from both DNA strands in order to resolve sequencing compressions and stops. To exclude polymerase errors, the DNA sequences for isolates 1–11 were determined by sequencing 2 clones, each originating from independent PCR amplifications, except for isolate 1, for which 8 clones amplified in separate PCR reactions were cloned and sequenced once to additionally account for the possibility of intraisolate polymorphisms. All sequences originated from separate PCR amplifications, including isolate 12 and 13 for which only the genotype-discriminating locus 54 bp before the end of the 18S coding region was determined by direct sequencing of the PCR products.

RESULTS

Intraisolate variation

From 8 independent clones originating from separate PCR amplifications with DNA extracted from isolate no. 1, 1,294 bp of DNA sequence between the inner primers ECH-6 and ECH-5 were determined (Fig. 1). *Taq* polymerase errors were detected as individual mutations not found in any other sequenced clone. A total of 10 substitutions was found at different positions in 4 of the sequences. Whereas some of these mutations might be explained by rare intraisolate polymorphisms, the majority are certainly due to polymerase errors because the observed rate of 0.1% (10 bp per $8 \times 1,294$ bp = 0.00097) agrees with the usual rate observed in our laboratory under similar conditions.

rDNA classes

In several isolates, the repeated generation of a second type of rDNA clones was observed, in some cases originating from the same PCR runs as the first. These products had similar lengths, and their DNA sequences were homologous to, but clearly different from the first rDNA type. Sequencing of 0.23 kb from the 5′ end (data not shown) revealed an identity of 90% compared to the rDNA class studied in this report. The identity of this second rDNA class sequence compared to the homologous portion of an *E. multilocularis* ITS1 sequence reported by Bowles et al. (1995) was 68%. For comparison, the identities between that *E. multilocularis* and an *Echinococcus granulosus* (sheep strain) ITS1 sequence reported in the same publication was 86.9%.

Intrapopulation variation

A total of 9 isolates from 5 different German states (Table I), between 1 and 3 samples each, was characterized. After accounting for polymerase errors by characterizing independent clones from separate PCR amplifications as explained before, identical nucleotide sequences were found for each of the 9 isolates.

Intraspecific polymorphism

A single polymorphic site was found 54 bp before the 3′ end of the 18S coding region. All of the 10 isolates from Germany and Japan displayed an adenine base at this position, whereas all of the 3 isolates from Alaska possessed a guanine there (Fig. 1). For isolates 12 and 13, the identity of this nucleotide was determined by direct sequencing. It was unequivocal at this position and did not require cloning of the PCR products. The rDNA sequence presented in Figure 1 was found to be 78% identical with the corresponding 992-bp ITS1-containing sequence reported by Bowles et al. (1995), which, however, did not cover the above-described polymorphic locus.

DISCUSSION

A striking feature of the characterized *E. multilocularis* isolates is their high degree of genetic conservation within the

```
                   *
AAAACTTGATCATTTAGARG AAGTAAAAGTCGTAACAAGG TTTCCGTAGGTGAACCTGCG   60

      <18S  ITS1>
GAAGGATCATTAcacgttcc tcatgtgcctgtggccagtc ctatccaatgggtgggtagc  120
tggcctggctgcttttagcg gtaggggtgatggctgctgc tgctactgtacagtcttctt  180

    ________ECH-8______>
gtgtggtggttgttatcgct gcaatggggtgcctggtctg ccttcagcctgcaatgagag  240

                     <______ECH-7______
gtcgggtgtgcccacctcgc tgcgttgctgcgatttgcgc cacctggcggtggtgatggt  300
ggtgtgatgtacgcagctgt cgcagtggcgcgaggccggt ccataccggggcggcagagg  360
agtacatgtgcatacgcgtg tgtgcaaggcgtaagatgtt tggaccgcggcggcggtagt  420

                                                           ___
accggtgctgttgctgatgc tgagggtcgcgtgcgcgtct acgccccgtcatgtgttgtg  480
__ECH-10_____>
taattggtttaaggtgttac atgtgtggctactgcatacg ctgttgtgtcgccttgcttt  540

                       <_____ECH-9______
atgctactggtgctgttgtt gaaggctgctcagtggacgt gattgcggcctgcagcggtg  600
cagacgttgtgtttgccgcc gcctcggtggcggtggtgtt gacatgtggtgcgcagagtt  660

          <ITS1  5.8S>
gtgctctgggttttttacTA ACTGTGTGCGGTGGATCACT CGGCTCGTGCGTCGATGAAG  720
_____ECH-12______>
AGTGCAGCCAACTGTGTGAA TTGATGTAAATCGTAGACTG CTTTGAACGTTGACATCTTG  780

                     <______ECH-11______          <5.8S  ITS2>
AACGCCTATTGCGGTCACAG GCTTGCCTGTGGTCACGTCT GTCCGAGGGTCGGCttgtag  840
actatcactgcgtgcattaa gcagtggctggggagagtgc cgtgtcgttgcgtaaggtgt  900
agcgtgtgcctgcagagtgg tgcggcttctccctagggtg ctggcgccgtcgcctccggt  960
agcgtggggtttgtggtttt gctgccgcggtggccacagt ggggtaaggggtcgtactgt 1020

                                                   ________ECH-
gccgtggcgtcgatgcgtgt gtgcgccggttgtgccagta ggttggcgggtggtgatgcg 1080
14____>
gttgcagcctttgcagtcca caggccgcggcccagtgtgc gaatgggtggttgcgtgcgt 1140

                                           <______ECH-13______
gggtgcgtaggtgtgagcac gtggcgcgcgttatgtggat tgtggatgctgtggtggagg 1200
gggcgtgccttcttcttcac ccaccacaagcacttgcatc gttgtgcgtgtgacgtctag 1260

           <ITS2  28S>
cctagctttgactcgtaTTC TGACCTCGGATCGGT 1295
```

FIGURE 1. *Echinococcus multilocularis* rDNA sequence between primers ECH-6 and ECH-5. The locations of the coding sequences (capital letters) and spacers (small letters) are approximations inferred from analogies to data on other eukaryotes (Dams et al., 1988; Gutell and Fox, 1988). The locations of the internal sequencing primers is given above the target sequence. *, Polymorphic site; R, adenine (Eurasian isolates) or guanine (Alaskan isolates).

rDNA spacer regions. In contrast, these spacers have been found to be hypervariable in numerous other species of both kingdoms, allowing the differentiation of genetically defined strains within species. A possible explanation could be that *E. multilocularis* is a phylogenetically relatively young species, if speciation is completed at all yet (Bowles et al., 1995).

Second, the location of the single polymorphic site was quite unexpected because it was detected within the 18S coding region and not within 1 of the presumably more variable spacer regions of the rDNA gene. The 2 resulting genotypes, nevertheless, are highly significant in discriminating the Alaskan isolates from those of Eurasian origin. The chance of accidentally obtaining this result, i.e., assuming there was no geographic difference in the distribution of these genotypes, is $0.5^{13} \times 2 = 0.024\%$ for a 1:1 ratio between the genotypes, and $0.77^{10} \times 0.23^{3} + 0.23^{10} \times 0.77^{3} = 0.089\%$ for the most favorable ratio of 77:23. Because foxes are known to migrate over the sea ice between St. Lawrence Island and northeast Siberia as well as the mainland of Alaska during winter (Fay and Rausch, 1992), it cannot be decided at this point if this genotypic distribution actually represents a restriction of gene flow between Eurasian and Alaskan "strains," or if the allele distribution has not reached equlibrium yet.

These results are congruous with the traditional taxonomic

classifications of *E. multilocularis*. In their first description, *E. multilocularis* isolates from Alaska have been discriminated from European and Asian isolates and were initially described as a separate species, *Echinococcus sibiricensis* (Rausch and Schiller, 1954). Later, it was reduced to subspecies status (Vogel, 1957) as 1 of 3 subspecies of *E. multilocularis,* the others being *E. m. kazakhensis,* whose authenticity is, however, disputed as reviewed by Eckert and Thompson (1988), and *E. m. multilocularis* as the nominate subspecies, whose type locality is southern Germany (reviewed by Eckert and Thompson [1988] and Thompson et al. [1995]). In 1967, even the subspecies status as the lowest valid taxon was given up in favor of the arbitrariness of the term "strain" (Rausch, 1967).

This arbitrariness is in line with the ambiguity of recent genetic characterizations. The first intraspecific genomic differences were described after Southern hybridizations using an anonymous probe called pAL1 whose target gene has not been characterized (Vogel et al., 1991). Several different banding patterns were obtained that could separate some of the 10 isolates studied but did not correlate with their geographical origin. In another pattern analysis technique, the distribution of PCR products of different lengths due to different numbers of pentameric microsatellites upstream of the coding region of the repetitive U1 snRNA gene from 40 different isolates resulted in 3 pattern types (Bretagne et al., 1996). The patterns correlated well, but not completely, with the continent of origin of the samples. Because 1 of the patterns was shared equally between 3 isolates each from Alaska and Hokkaido, Japan, the authors inferred relatedness, whereas more traditional biological studies on host specificities indicate that a population different from that in Alaska is prevalent in all or some areas of Hokkaido (Okamoto et al., 1992). Likewise, seemingly out-dated studies on phenotypic characteristics, such as the sizes of the rostellar hooks, show greater differences between strains from Alaska and Hokkaido than between either of these 2 and European isolates (Rausch and Richards, 1971). Great caution must be exercised when applying microsatellite analysis or other highly discriminating techniques because of the danger of overdiscrimination. Just as a human fingerprint is highly discriminating even between individuals, it offers little information with regard to phylogeny or taxonomy, e.g., when trying to draw conclusions about a person's race. A second confounder that applies to both studies became evident when microsporidia were found in cells from a human liver lesion due to *E. multilocularis* (Furuya, Sato, et al., 1995). Both the target sequence of the anonymous probe pAL1 as well as the U1 snRNA coding region could be amplified from microsporidial DNA, with corresponding primer pairs previously thought to be genus and species specific, respectively, for *E. multilocularis* (Furuya, Nagano, and Sato, 1995). Subsequent sequencing of the products revealed almost identical DNA sequences between *E. multilocularis* and the microsporidium *Encephalitozoon cuniculi* (Nagano et al., 1996).

A third technique generating product patterns, random amplification of polymorphic DNA (RAPD), produced 2 patterns for 2 *E. multilocularis* isolates (Scott and McManus, 1994). Nevertheless, these findings must be interpreted cautiously, not only because of the danger of overdiscrimination, but also because this random technique often produces random results. They can be difficult to reproduce in other laboratories because this technique is affected by numerous factors, e.g., even by the DNA concentration or the cycling speed (Davin-Regli et al., 1995; Schweder et al., 1995). This situation can be remedied, at least in principle, by DNA sequencing that generates unambiguous as well as quantifiable data at the nucleotide level. This is doubtlessly the reason why 2 reports on sequencing 2 mitochondrial genes, cytochrome c oxidase subunit I (CO1) and NADH subunit I (ND1), in a total of 4 *E. multilocularis* isolates (Bowles et al., 1992; Bowles and McManus, 1993a), have received grateful appreciation (Thompson et al., 1995). Two different genotypes were detected although the second genotype was only found in a single isolate and the observed genetic differences did not correspond to geographic divisions (Thompson et al., 1995). Later, CO1 sequences were obtained from 5 Japanese and a second Alaskan isolate and all of them were found to possess the first of the 2 genotypes (Okamoto et al., 1995). The authors of the first 2 reports conclude that CO1 and ND1 data alone are insufficient for phylogenetic analyses and propose the characterization of an rDNA internal transcribed spacer region as a more promising target gene for that purpose (Bowles and McManus, 1993b). However, restriction fragment lengths polymorphism (RFLP) analysis of the ITS1 of 5 isolates from 3 continents (Bowles and McManus, 1993c), as well as that of the ITS2 of 5 isolates from 2 continents (Gasser and Chilton, 1995) failed to show any differences between the studied *E. multilocularis* isolates, which is in line with our observations described in this report.

In this context, mention should also be made of the possibility of different "classes" of rDNA genes, even within a single individual (Dover, 1982; Gonzalez et al., 1990; Wesson et al., 1992; Ritland et al., 1993). The existence of different types of rDNA has already been proposed for *Echinococcus oligarthrus* (Bowles and McManus, 1993c) and *E. granulosus* (Gasser and Chilton, 1995). We have detected at least 2 rDNA classes in *E. multilocularis* that could be amplified simultaneously from the same isolates and in the same PCR reactions with the same primer pair. As shown in the results, they were also different from the ITS1 sequence reported by Bowles et al. (1995), which hence could be interpreted as yet another rDNA class. This observation proves the necessity to clone uncharacterized rDNA products before sequencing rather than sequencing them directly that might result in mixed sequence information.

Further, it is an indispensable though thus far scarcely honored requirement to assess the intraisolate variation that might be amplifiable under the specific conditions and with the particular PCR primers used in a given study. To do this, several PCR reactions should be performed from at least 1 of the isolates and independently generated PCR products should be cloned and sequenced. The same is true for often neglected population genetics aspects in those studies where descriptions of strain differences are sought by characterizing only a single member from each population claimed to thus have been described. The impending pitfall is that differences in rDNA classes could be attributed to represent differences between populations (and subsequently postulated "strains"), whereas they might actually also be found in a single individual if they had been investigated.

As a consequence of the preceding discussion, the lure of rapidly generated and overinterpreted genetic data obtained with more or less appropriate techniques on more or less ap-

propriate target genes should be realized before principles, if only that of a sufficient sample size to describe a given population, which were self-evident in most "traditional" morphological and biological investigations on taxonomy and phylogeny are put aside as being out-dated. The quick description of a narrow genetic locus of unclear phylogenetic significance in just a few isolates must not be accepted as a convenient shortcut to substitute for the comprehensive and thus more painstaking methods of traditional taxonomy.

In conclusion, the distribution of genotypes described in this report supports the traditional distinction between *E. m. multilocularis* and *E. m. sibiricensis,* be they called subspecies or strains. It presents reason to caution before taxonomic revisions are proposed for *E. multilocularis* subspecies and strains based on isolated, diverging descriptions of genotypes alone.

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