

Phylogenetic relationships of three hymenolepidid species inferred from nuclear ribosomal and mitochondrial DNA sequences

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SUMMARY

Three hymenolepidid tapeworms, *Hymenolepis diminuta*, *H. nana* and *H. microstoma*, are commonly maintained in laboratory rodents and used in many experimental model systems of tapeworm infections. We examined partial sequences from the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene and nuclear ribosomal internal transcribed spacer 2 (ITS2) sequences to infer phylogenetic relationships of the 3 hymenolepidid species. Parts of the CO1 gene and ITS2 were amplified by PCR and sequenced directly. The CO1 gene sequence obtained was the same in length (391 bp) among all specimens. In the case of ITS2, however, several insertions and deletions were detected (671–741 bp) not only among species but also between an American isolate and a Japanese isolate of *H. diminuta*. Percentage nucleotide differences between *H. diminuta* and *H. microstoma*, or *H. diminuta* and *H. nana* were 16.6–18.2% for the CO1 gene and 21.3–22.9% for ITS2. The differences in both sequences between *H. microstoma* and *H. nana* were about 14%. Phylogenetic trees inferred from both of the nucleotide sequences showed similar topology, and suggest that *H. diminuta* may have diverged from the common ancestral line the earliest, and that *H. nana* is closer to *H. microstoma* than to *H. diminuta*.

Key words: molecular phylogeny, *Hymenolepis diminuta*, *Hymenolepis nana*, *Hymenolepis microstoma*, CO1 gene, ITS2.

INTRODUCTION

Three hymenolepidid tapeworms, *H. diminuta*, *H. nana* and *H. microstoma*, are commonly maintained in laboratory rodents and used in many experimental model systems of tapeworm infections (Smyth & McManus, 1989). In addition, *H. nana* is the most common tapeworm of humans in the tropics and subtropics. The genus *Hymenolepis* contains a large number of species which occur chiefly in wild birds and mammals. In the past, the genus *Hymenolepis* became so large as to be meaningless to deal with a single genus, because most species in the subfamily Hymenolepidinae with 3 testes used to be placed in this genus. Since the work of Spasski & Spasskaja (1954), taxonomists have subdivided the genus *Hymenolepis* into many new genera, and finally Schmidt (1986) consolidated these genera in his hand-book of tapeworm identification. Schmidt (1986) classified *H. nana* and *H. microstoma* into the different genus *Vampirolepis* due to the presence of an armed rostellum.

Some biochemical techniques have recently been advanced as alternatives to the traditional morphological means for identification and classification

of cestodes. Isoenzyme analysis is one of the useful techniques for characterization of species or strains of tapeworms (McManus & Smyth, 1979; Macpherson & McManus, 1982). Novak, Taylor & Pip (1989) investigated isoenzyme band patterns from 4 hymenolepidid species, *H. diminuta*, *H. nana*, *H. microstoma* and *H. citelli*, and concluded that *H. microstoma* was the most dissimilar of the 4 species and that *H. diminuta* was closer to *H. citelli* and *H. nana* than to *H. microstoma*. They also asserted that those results were consistent with biological features of *H. microstoma* reported in previous studies (Evans, Gray & Novak, 1979; Arai, 1980; Novak, Hardy & Evans, 1982). Their conclusions however, conflict with the classification of hymenolepidid tapeworms proposed by Schmidt (1986). Besides, certain studies of immunobiological features of *H. microstoma*, *H. diminuta* and *H. nana* implied that *H. nana* and *H. microstoma* had more similarity and differed from *H. diminuta* (Ito, Onitake & Andreassen, 1988). Furthermore, observations of cysticeroid development from eggs of these 3 hymenolepidid cestodes revealed that cysticeroid development in *H. microstoma* was more comparable to that of *H. nana* than that of *H. diminuta* (Voge, 1964; Caley, 1974).

With the development of molecular biology, sequences of various genes have been used for molecular phylogenetic study. Mitochondrial DNA (mtDNA) is considered one of the best neutral

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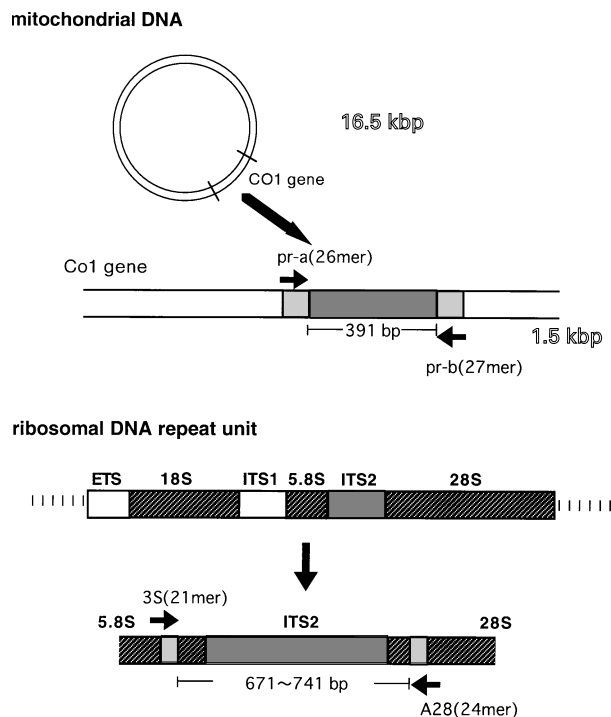


Fig. 1. Schematic representation of an amplified region and primer locations for PCR and cycle sequencing. pr-a: 5'-TGGTTT'TTTGTGTCATCCTGAGGTTTA-3'; pr-b: 5'-AGAAAGAACGTAATGAAAATGAGC-AAC-3'; 3S: 5'-CGGTGGATCACTCGGCTCGTG-3'; A28: 5'-CCTGGTTAGTTTCTTTTCCCTCCGC3'

markers for revealing phylogenetic relationships among related groups, because mtDNA is maternally inherited, it evolves fairly rapidly, and most of the nucleotide substitutions occur at neutral sites. In the present study, we examined partial sequences from the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene to infer phylogenetic relationships of 3 hymenolepidid species. In addition, we also obtained nuclear ribosomal internal transcribed spacer 2 (ITS2) sequences which are inherited in a Mendelian fashion, and inferred the phylogeny of the 3 hymenolepidid species from phylogenetic relationships of both sequences.

MATERIALS AND METHODS

Parasite materials

Two isolates of *H. diminuta* from Hokkaido, Japan (HdHok1 and HdHok2) were isolated from wild Norway rats (*Rattus norvegicus*) captured at a farm in Hokkaido University in a separate year. An isolate of *H. nana* from Montevideo, Uruguay (HnMon) was isolated from a laboratory golden hamster (*Mesocricetus auratus*). A strain of *H. nana* from Tokyo, Japan (HnTok) was isolated from a laboratory mouse (*Mus musculus*) (Okamoto, 1968). The strains of both *H. diminuta* from USA (HdUSA) and *H. microstoma* (Hm), originally isolated at Rice University, Texas, were supplied by Dr J. Andreassen, Copenhagen, to

A. Ito (Ito *et al.* 1988). Three strains, HnTok, HdUSA and Hm, are maintained in beetles, *Tribolium confusum*, and rats (HdUSA) and mice (HnTok and Hm) at Gifu University (Ito *et al.* 1988).

Preparation of DNA

Total DNA was prepared from adult hymenolepidid tapeworms using Easy-DNA™ Kit (Invitrogen, USA). A single adult worm for each strain or isolate was used for DNA extraction, and examined.

Amplification and sequencing of DNA

Total DNA was used as a template for amplification of DNA fragments by the polymerase chain reaction (PCR). PCR amplifications were performed using AmpliTaq DNA Polymerase (Perkin Elmer, USA) according to the manufacturer's instructions. Schematic representation of amplified region, primer locations and their sequences are shown in Fig. 1. The PCR primers for the CO1 gene were designed for amplification of the planarian CO1 gene (Bessho, Ohama & Osawa, 1992*a*) and primers for ITS2 were designed from ribosomal DNA sequences of *Schistosoma* spp. (Blair *et al.* 1997). The amplification conditions were: denaturation at 94 °C for 50 sec (except for the first cycle for 1 min 50 sec), annealing at 42 °C for 1 min 30 sec and extension at 72 °C for 1 min 30 sec (30 cycles) for CO1 gene and denaturation at 94 °C for 30 sec (except for the first cycle for 3 min 30 sec), annealing at 50 °C for 30 sec and extension at 72 °C for 1 min (30 cycles) for ITS2 region. After these cycles, a final extension step of 7 min at 72 °C was performed. PCR products were purified with QIAquick-spin PCR purification Kit (Qiagen, Germany). Purified double-stranded PCR products were directly sequenced from both ends using Dye Terminator Cycle Sequencing FS Ready Reaction Kit and a Model 373A DNA sequencer (Perkin Elmer). At least 2 independent PCR products have been used to sequence.

Phylogenetic analysis

DNA sequence data were aligned using the CLUSTAL W computer program (Thompson, Higgins & Gibson, 1994). Amino acid sequences of the CO1 were predicted using known mitochondrial modifications to the universal genetic code (Bessho, Ohama & Osawa, 1992*b*). The evolutionary distances were computed by Kimura's two-parameter method (Kimura, 1980), and the phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the CLUSTAL W computer program. The positions with gaps were excluded. The tree was evaluated using the bootstrap test (Felsenstein, 1985) based on 1000 resamplings. Another tree was also

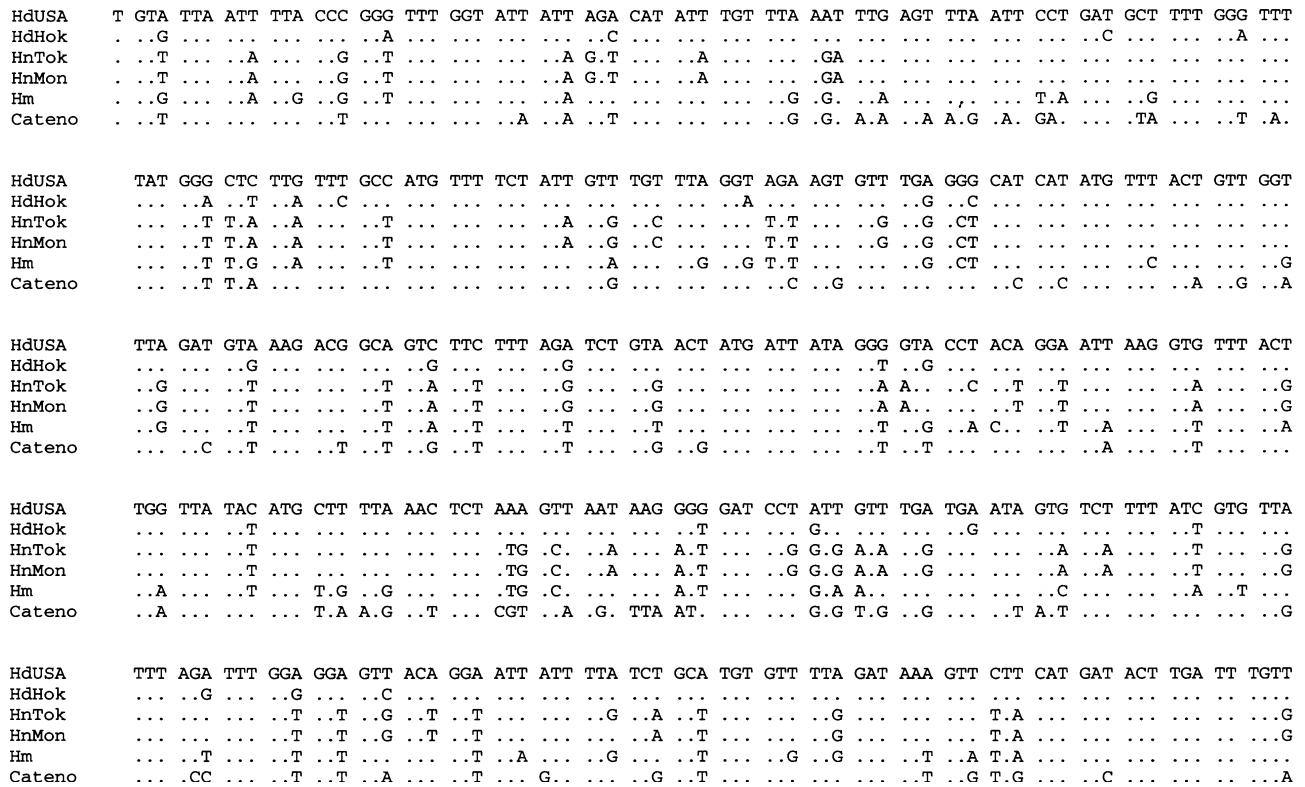


Fig. 2. Nucleotide sequences of 391 bp fragment of the mitochondrial CO1 gene. Dots denote homology with the HdUSA sequence. For explanations of abbreviations of species names, see text.

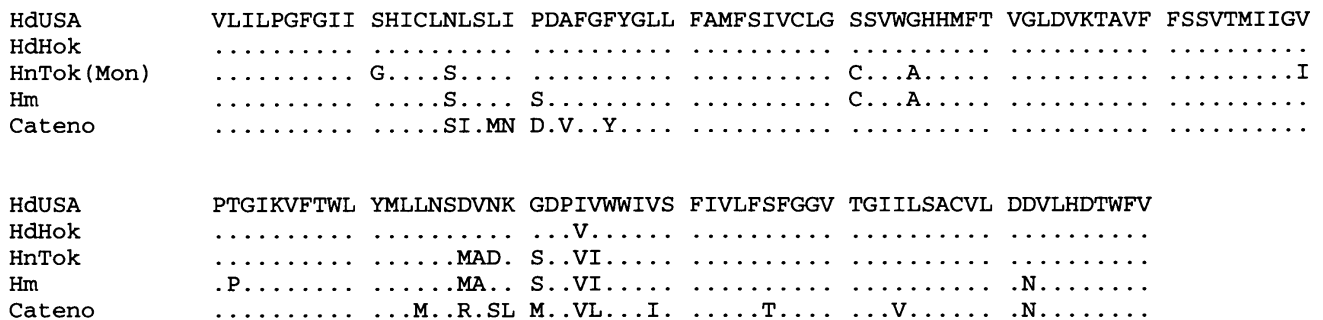


Fig. 3. Predicted partial amino acid sequences of the mitochondrial CO1. Dots denote homology with the HdUSA sequence. For explanations of abbreviations of species names, see text.

inferred using the Dnaml in the PHYLIP 3.5 phylogeny package (Felsenstein, 1993), which implements the maximum likelihood method (Felsenstein, 1981) for DNA sequences. *Catenotaenia* sp. from Uruguay was used as an outgroup species to root the tree.

RESULTS

HdHok1 and HdHok2 shared the same sequences in both the CO1 gene and the ITS2 region. Hence, HdHok represents HdHok1 and HdHok2 in the following. The CO1 gene sequence obtained was the same in length (391 bp) among all specimens (Fig. 2). Predicted partial amino acid sequences of mitochondrial CO1 are shown in Fig. 3. HnTok and HnMon shared the same amino acid sequences. HdUSA showed a sequence different from that of

HdHok at only 1 amino acid. Fifteen or more amino acids differed among the hymenolepidid species. In the case of ITS2, several insertions and deletions were detected (671–741 bp) not only among species but also between the American isolate (HdUSA) and the Japanese isolate (HdHok) of *H. diminuta* (Fig. 4). ITS2 sequences of HnTok and HnMon shared the same sequence.

The levels of nucleotide variation detected between pairs of hymenolepidid samples for the CO1 gene and for the ITS2 region are presented in Table 1. Percentage nucleotide differences between *H. diminuta* and *H. microstoma*, or *H. diminuta* and *H. nana* were 16.6–18.2 % for the CO1 gene and 21.3–22.9 % for ITS2. The differences between *H. microstoma* and *H. nana* were about 14 % for both sequences.

The nucleotide sequence data of the CO1 gene and ITS2 region were analysed by the neighbour-joining

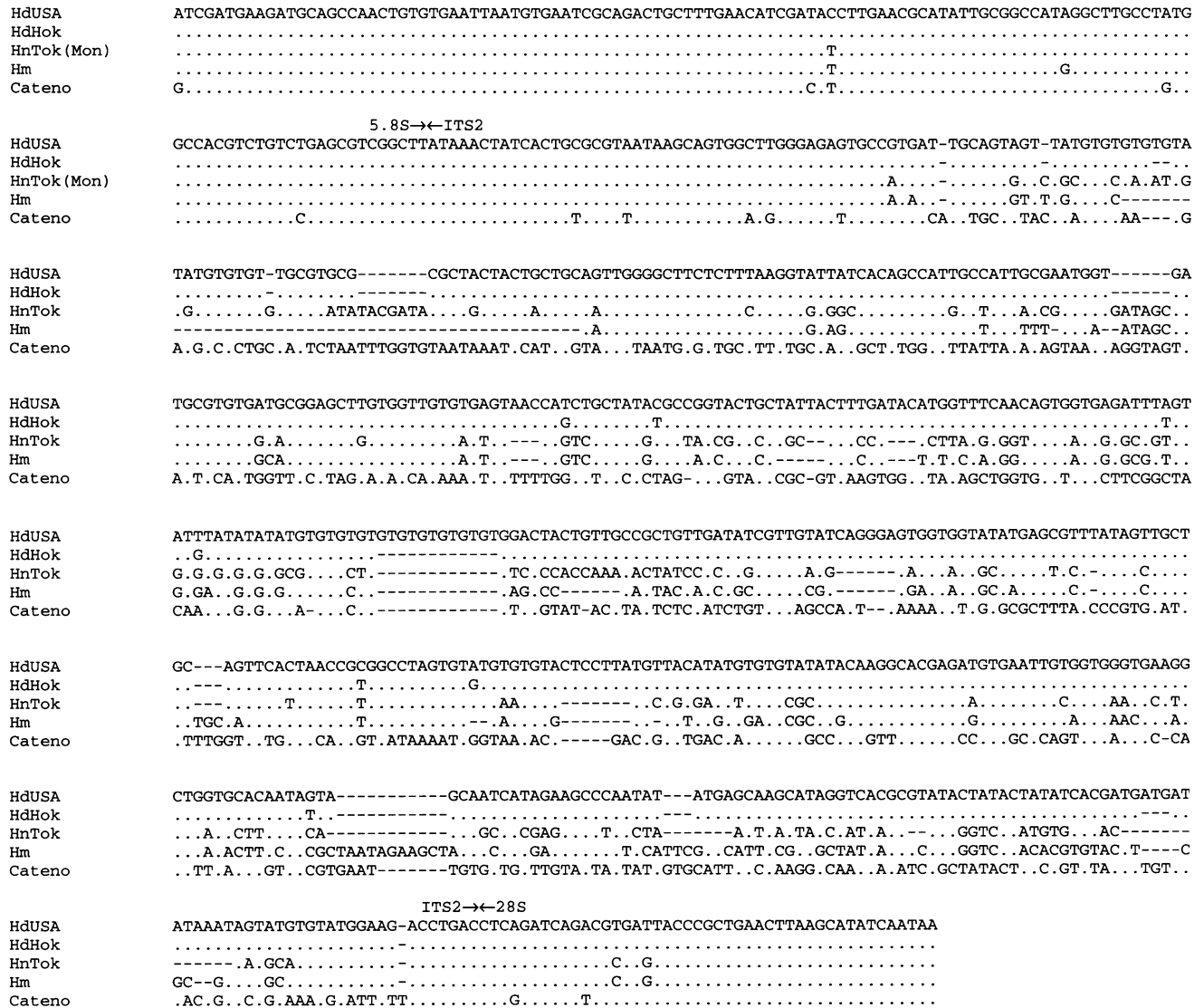


Fig. 4. Nucleotide sequences of nuclear ribosomal ITS2. Dots denote homology with the HdUSA sequence. For explanations of abbreviations of species names, see text.

Table 1. Levels of nucleotide variation between pairs of hymenolepidid species for the CO1 gene (below the diagonal) and ITS2 (above the diagonal), expressed as a percentage

(Nucleotide positions with gaps were excluded. For explanations of abbreviations of species names, see text.)

	HdUSA	HdHok	HnTok	HnMon	Hm
HdUSA	—	1.0	22.9	22.9	21.8
HdHOK	6.4	—	22.3	22.3	21.3
HnTOK	17.1	17.1	—	0	14.1
HnMon	16.6	16.6	0.5	—	14.1
Hm	18.2	17.7	13.8	14.1	—

method and maximum-likelihood method to obtain the phylogenetic relationships. Phylogenetic trees of *Hymenolepis* spp. inferred from each sequence are shown in Figs 5 and 6, respectively. In both figures, 2 trees inferred by different algorithms showed similar topology. Phylogenetic trees inferred from

different sequences were also very similar, except for genetic distances. Furthermore, bootstrap values of the node in neighbour-joining trees were large. The reliability of the phylogeny inferred in this study seems to be very high.

DISCUSSION

Nuclear and mitochondrial genomes exhibit different patterns of inheritance. In general, when phylogenetic trees inferred from these separate genomes show the same topology, it is highly probable that these trees indicate the true phylogeny of the organisms. In the present study, 2 phylogenetic trees inferred from the mitochondrial CO1 gene and from nuclear ribosomal ITS2 showed similar topology. These trees demonstrate that *H. diminuta* may have diverged from the common ancestral line the earliest and that *H. nana* is closer to *H. microstoma* than to *H. diminuta*. These results are considered to support the classification proposed by Schmidt (1986).

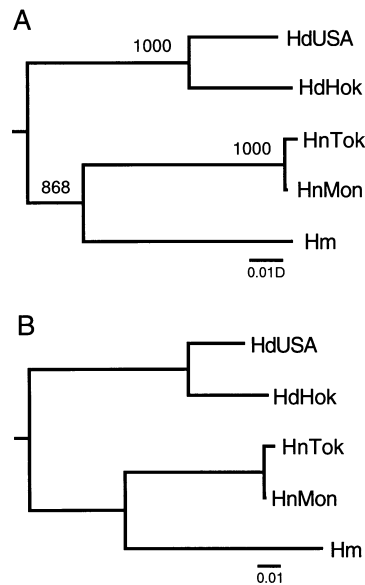


Fig. 5. Phylogenetic trees of *Hymenolepis* spp. inferred from the mitochondrial CO1 gene nucleotide sequences shown in Fig. 2. The phylogenetic trees were outgroup-rooted using the CO1 gene nucleotide sequence of *Catenotaenia* sp. For explanations of abbreviations of species names, see text. (A) Neighbour-joining tree. D indicates Kimura's 2-parameter evolutionary distance. The values at the nodes represent bootstrap confidence level (based on 1000 resampling). (B) Maximum-likelihood tree. The parameter of transition/transversion ratio was set at 2.0. The log likelihood of the tree was -1307.86 . Scale indicates the expected number of substitutions per site.

Comparing the variations of the CO1 gene and those of ITS2, intraspecific variations of the CO1 gene were larger than those for ITS2, whereas interspecific variations of the CO1 gene were smaller than those for ITS2. In the case of the CO1 gene, sequences obtained from all samples were the same in length, so that alignment of sequences was easy and probably accurate. However, the length of ITS2 regions amplified were varied among specimens because several deletions and insertions occurred. ITS2 is a non-coding region, so that accurate positions of deletions or insertions are difficult to determine. Alignment of ITS2 by use of the CLUSTAL W computer program is not always correct, especially at the regions adjacent to the 3' end of ITS2. Genetic distances among hymenolepidid species inferred from ITS2 regions may be overestimated as compared with real genetic distances. When phylogenetic relationships are inferred from nucleotide sequences of a non-coding region such as ITS2, the reliability of their alignment should be noted.

The level of nucleotide variation in the CO1 gene between HdHok and HdUSA was 6.4%. This level is almost the same as that between *Taenia saginata* and *T. multiceps* (McManus & Bowles, 1994). Okamoto *et al.* (1995) reported that the CO1 gene sequence of some isolates of *T. taeniaeformis* from

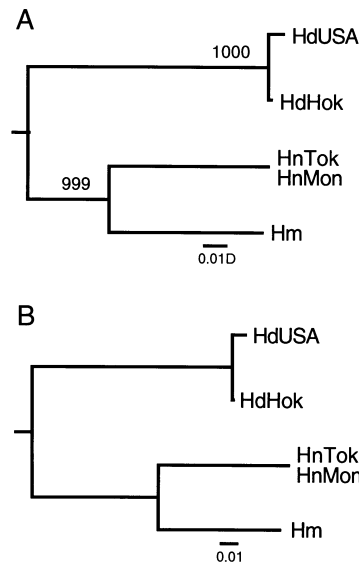


Fig. 6. Phylogenetic trees of *Hymenolepis* spp. inferred from nuclear ribosomal ITS2 nucleotide sequences shown in Fig. 4. The phylogenetic trees were outgroup-rooted using the ITS2 nucleotide sequence of *Catenotaenia* sp. For explanations of abbreviations of species names, see text. (A) Neighbour-joining tree. D indicates Kimura's 2-parameter evolutionary distance. The values at the nodes represent bootstrap confidence level (based on 1000 resampling). (B) Maximum-likelihood tree. The parameter of transition/transversion ratio was set at 2.0. The log likelihood of the tree was -2769.49 . Scale indicates the expected number of substitutions per site.

grey red-backed vole (ACR isolate) was different from those of other isolates with levels being 9.0–9.5%. The ACR isolate differs from the others in morphology, infectivity and protein composition of the metacestode (Azuma *et al.* 1995). *H. diminuta* is one of the most popular tapeworms that has been used as an experimental model of tapeworm infection, and many reports on studies with this tapeworm have been published. It should be emphasized that biological features of *H. diminuta* in respective experiments are not always identical.

On the contrary, although 2 samples of *H. nana* have been isolated from distinct geographical areas, these isolates were very close genetically. This fact does not necessarily indicate that there is little variation within *H. nana* species. It was reported that in the case of cestodes parasitizing domesticated animals, genetic distances were not always related to geographical distances between the locations where each cestode had been collected (Bowles & McManus, 1993; Okamoto *et al.* 1995). It seems that *H. nana* in this study has been introduced to each country with laboratory rodents. Actually it has been reported that there might be at least 3 different strains, mouse-, rat- and human-adapted, in *H. nana* (Ito & Smyth, 1987). Although *H. nana* is an important parasite of humans, most studies seem to have been performed using the mouse-adapted

strain. Further investigation should be carried on including the isolates derived from human, wild rodents and those reared in each laboratory.

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