Parasitology International (2016.4) 65(2):83-86.

Cryptic diversity in hymenolepidid tapeworms infecting humans.

Agathe Nkouawa, Voitto Haukisalmi, Tiaoying Li, Minoru Nakaoa, Antti Lavikainen, Xingwang Chen, Heikki Henttonen, Akira Ito

PARINT-D-15-00219-R2

Short communication

Cryptic diversity in hymenolepidid tapeworms infecting humans

Agathe Nkouawa [a,1], Voitto Haukisalmi [b,1], Tiaoying Li [c,1], Minoru Nakao [a,1,*], Antti Lavikainen [d], Xingwang Chen [c], Heikki Henttonen [e], Akira Ito [a]

[a] Department of Parasitology, Asahikawa Medical University, Asahikawa, Japan

[b] Finnish Museum of Natural History, University of Helsinki, Helsinki, Finland

[c] Institute of Parasitic Diseases, Sichuan Center for Disease Control and Prevention, Chengdu, China

[d] Department of Bacteriology and Immunology/Immunobiology Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland

[e] Natural Resources Institute Finland (Luke), Vantaa, Finland

[1] These authors contributed equally to this article.

* Corresponding author.

E-mail address: nakao@asahikawa-med.ac.jp (M.Nakao)

ABSTRACT

An adult hymenolepidid tapeworm was recovered from a 52-year-old Tibetan woman during a routine epidemiological survey for human taeniasis/cysticercosis in Sichuan, China. Phylogenetic analyses based on sequences of nuclear 28S ribosomal DNA and mitochondrial cytochrome *c* oxidase subunit 1 showed that the human isolate is distinct from *Hymenolepis diminuta* and *Hymenolepis nana*, the common parasites causing human hymenolepiasis. Proglottids of the human isolate were unfortunately unsuitable for morphological identification. However, the resultant phylogeny demonstrated the human isolate to be a sister species to *Hymenolepis hibernia* from *Apodemus* mice in Eurasia. The present data clearly indicate that hymenolepidid tapeworms causing human infections are not restricted to only *H. diminuta* and *H. nana*.

Keywords:

hymenolepiasis

Hymenolepis diminuta

cryptic species complex

The family Hymenolepididae is a diverse group of tapeworms consisting of approximately 620 species in birds and 230 species in mammals, and has been assigned to many genera based on their morphological traits [1]. However, molecular phylogenetic studies on interspecific and intergeneric relationships within the family are still in their infancy [2]. Although a few members of the genus *Hymenolepis* sensu lato are of medical importance as pathogenic organisms, their taxonomy is still controversial, particularly that of *Hymenolepis nana* [2]. Rodent tapeworms of this genus generally require arthropod intermediate hosts in their life cycles. The adult tapeworms parasitize in rodent intestines, and the eggs develop into cysticercoid larvae in the hemocoel of insects, mainly beetles (Coleoptera).

Human infections with adult hymenolepidid tapeworms (hymenolepiasis) occur worldwide, particularly in tropical and subtropical countries under poor hygiene conditions. Most patients remain asymptomatic. The human hymenolepiasis has been generally believed to be caused only by the mouse tapeworm *H. nana* and the rat tapeworm *Hymenolepis diminuta*, of which *H. nana* is by far the most common because human-to-human infections occur frequently in children by directly ingesting the parasite eggs as a result of contamination of house dust, food and water with human feces [3]. Human infections with *H. diminuta* via beetle intermediate hosts have been found less frequently [3]. Humans seem to become infected with *H. diminuta* due to the accidental ingestion of small beetles in stored cereal crops. Diagnosis of hymenolepiasis in human patients and differentiation of causative species are usually based on the morphology of eggs recovered from feces.

The taxonomy and identification of *H. diminuta* are problematic issues since the taxon includes a complex of cryptic species [2], indicating a possibility that clinical samples (i.e. proglottids and eggs) from human patients might be often misdiagnosed as *H. diminuta*. Originally, *H. diminuta* was discovered in the brown rat, *Rattus norvegicus*, from Europe. Several species of Eurasian field mice

(*Apodemus* spp.) were subsequently listed as definitive hosts for *H. diminuta* [4]. However, additional descriptions of *Hymenolepis apodemi* [4], *Hymenolepis pseudodiminuta* [5] and *Hymenolepis hibernia* [6] from *Apodemus* spp. suggested that true *H. diminuta* is a specific parasite of *Rattus* spp. The infectivities of these newly defined *Hymenolepis* spp. to humans are completely unknown. We report here an unexpected and novel finding about a causative agent of hymenolepiasis in humans.

During a routine epidemiological survey for human taeniasis/cysticercosis in remote communities of Ruoergai region of Sichuan, China (located at the eastern margin of the Tibetan Plateau), hymenolepidid eggs were detected in a fecal sample from a 52-year-old Tibetan woman. She showed no clinical signs. Under approval of the local informed consent form, a deworming treatment was done for her using pumpkin seeds and areca nut extract [7]. An adult tapeworm expelled was washed with tap water and then kept in 70% ethanol for subsequent morphological observation and molecular identification. Mature eggs were obtained from the terminal gravid proglottids. Measuring the diameter of eggs, the thickness of outer coat (egg-shell), the size of oncospheres, and the length of embryonic hooks was done after mounting the eggs in Berlese's medium.

The human-derived hymenolepidid tapeworm was subjected to a molecular phylogenetic analysis, together with 13 reference samples (*H. diminuta* and *H. hibernia*) from collections of the Finnish Museum of Natural History and 3 laboratory strains (*H. diminuta*, *H. nana* and *Hymenolepis microstoma*) kept in Asahikawa Medical University, Japan. Parasite genomic DNA was purified from a small part of proglottids using DNeasy tissue kit (QIAGEN) and then used as a template for PCR. Nuclear 28S ribosomal DNA (rDNA) and mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) were selected as target genes. The 28S rDNA primers XZ-1 and 1500R [2] and the original *cox1* primers Hym-cox1F (5'-GTT ACT AAT CAT GGT ATT ATT ATG-3') and Hym-cox1R (5'-CCA AAA TAA TGC ATA GGA

AAA-3') were used for PCR amplification and subsequent DNA sequencing. Procedures of the PCR and sequencing were the same as those reported previously [8]. The resultant sequences were submitted to BLAST homology search [http://blast.ncbi.nlm.nih.gov] to check sequence identity. All of the sequences determined in this study have been deposited into DDBJ/EMBL/GenBank databases (Supplementary Table 1). In the case of 28S rDNA, sequences retrieved from the databases were also added to the present analysis. Nucleotide data sets of nuclear 28S rDNA and mitochondrial *cox1* were prepared using the multiple aligner MAFFT [9]. Gaps were completely removed from the alignments. The genetic software MEGA 6 [10] was used to find nucleotide substitution models and to estimate phylogenetic trees by maximum likelihood (ML) method. Midpoint-rooted ML trees were generated from the data sets by 500 bootstrap repetitions under the model HKY+G for 28S rDNA and the model TN93+G for *cox1*. Pairwise divergence values were also computed at interspecific and intraspecific levels using the MEGA6.

The adult tapeworm from a Tibetan woman was approximately 10 cm in length and 3 mm in maximum width. The scolex was lost, and furthermore the contracted body in ethanol was unsuitable for morphological observation of reproductive organs in mature proglottids. As shown in Fig. 1, eggs obtained from the gravid proglottids had a spherical shape similar to those of *H. hibernia, H. pseudodiminuta* and *H. apodemi*. The egg size of the human tapeworm was 63 μm in mean diameter (n=12), overlapping with those of the above-mentioned three species [4]. The egg outer coat was relatively thick; 4.0 μm in mean thickness (n=7). The oncosphere was oval; 28.4 × 34.6 μm in mean size (n=10). The embryonic hook was relatively long; 16.5 μm in mean length (n=7). These egg features appear to be similar to those of *H. apodemi* [4]. However, the lack of information about morphological features of reproductive organs prevented us to definitively identify the human tapeworm in China.

The BLAST homology search using nuclear 28S rDNA and mitochondrial *cox1* sequences demonstrated the unidentified tapeworm not to be identical to any of the hymenolepidid tapeworms recorded in DNA databases. To clarify its taxonomic position, a preliminary molecular phylogeny of human-infecting hymenolepidid tapeworms was made based on DNA sequences of 28S rDNA and *cox1* (Fig. 2). The data sets 28S rDNA and *cox1* consisted of 1,243 and 1,000 nucleotide sites, respectively. Both the gene data sets resulted in a very similar phylogeny, showing that the unidentified tapeworm is distinct from the human-infecting tapeworms, *H. diminuta* and *H. nana*. The unidentified tapeworm occupied a sister position relative to *H. hibernia*. Intraspecific divergence values of variable *cox1* ranged from 0.054 to 0.000 in *H. hibernia* isolates (n=11) and from 0.021 to 0.004 in *H. diminuta* isolates (n=3). Whereas, divergence values of *cox1* between the unidentified tapeworm and each isolate of *H. hibernia* ranged from 0.141 to 0.131, suggesting that the unidentified tapeworm differs from *H. hibernia* at species level.

This report clearly demonstrates that hymenolepidid tapeworms causing human infections are not restricted to only *H. diminuta* and *H. nana*. Although the human-derived hymenolepidid tapeworm in China remained unidentified, the present molecular phylogeny showed that the human isolate is the most related to *H. hibernia* from Eurasian *Apodemus* mice. As indicated in Fig. 2, *H. hibernia* is widely distributed in the Palaearctic region. Recently, a new species of *Hymenolepis* from *Apodemus peninsulae*, *Apodemus uralensis* and *Apodemus agrarius* in the south of Russian Far East, western Siberia and Kazakhstan has been described as *H. apodemi* [4]. In the highlands of the eastern margin of the Tibetan Plateau where the unidentified tapeworm was found, the Sichuan field mouse (*Apodemus latronum*) and the South China field mouse (*Apodemus draco*) are endemic [11], together with *A. peninsulae* and *A. agrarius* from which *H. apodemi* has been found. The shared rodent fauna and the morphological similarity of parasite eggs suggest that *H. apodemi* is a potential candidate for the

unidentified human tapeworm, although a possibility of a new species also should be considered. Further taxonomic studies are needed to integrate molecular and morphological data of *H. diminuta* species complex.

The Eurasian *Apodemus* spp. generally inhabit forests, forest edges and grasslands, and perpetuate the sylvatic life cycles of *Hymenolepis* spp. with arthropod intermediate hosts. As compared with *Apodemus* mice, house rats and house mice are more directly linked with human living environments. An early experimental study of *H. hibernia* [6] indicated that the *Apodemus*-derived parasite can infect rats (*Rattus norvegicus*) more easily than mice (*Mus musculus*). Another *Apodemus*-derived parasite, *H. pseudodiminuta*, also has a loose host-specificity at the adult stage [12]. The host-switching of *Hymenolepis* spp. from *Apodemus* to *Rattus* has an important implication because the resultant synanthropic life cycles could be associated with human infections.

Moreover, in the cases of human infections with *H. nana*, researchers and health workers should pay attention to the possible involvement of cryptic species originating from wild rodents [13]. In Australia, *H. nana*-like eggs in human feces were identified as *H. microstoma* using a mitochondrial DNA analysis, although the adult tapeworms were not confirmed from the patients [14]. Even at the present time, the generic assignment of *H. nana* and *H. microstoma* is a problematic issue, and these species cannot be unambiguously assigned to any existing genus [2]. Based on the morphological distinctiveness of the scolex, they are sometimes classified into the genus *Rodentolepis* [1, 2] or *Vampirolepis* [15, 16]. However, the species of *Rodentolepis*, *Vampirolepis* and other hymenolepidids with rostellar hooks do not truly belong to *Hymenolepis*, because the members of latter genus have a rudimentary rostellum without hooks [1, 2]. Therefore the generic assignment “*Hymenolepis* sensu lato” is preferred for *H. nana* and *H. microstoma*, and “*Hymenolepis* sensu stricto” should be used only for *H. diminuta* species complex. *Rodentolepis*-like species are morphologically similar to each other, and

utilize many species of rodents as definitive hosts, including the house mice *Mus musculus* and *Mus domesticus*. A PCR-based molecular identification using clinical samples of fecal eggs and ploglottids is necessary to clarify whether other hymenolepidid tapeworms are involved in human infections with so-called "*H. nana*". A molecular phylogenetic survey using *H. nana* isolates from humans and rodents suggests a possibility that *H. nana* is a cryptic species complex containing at least two morphologically indistinguishable species [17], one of them possibly representing *Hymenolepis fraterna* [18]. However, the occurrence of the two cryptic species was not related to the host origins (humans and rodents). A mitochondrial DNA barcoding system should be prepared for hymenolepidid cestodes parasitizing humans and rodents in collaboration with tapeworm taxonomists to better understand causative species of hymenolepiasis.

Acknowledgments

Jukka T. Lehtonen, Carlos Feliu and Pilar Foronda are acknowledged for collecting some of the cestode specimens used in the phylogenetic analysis. Thanks are also due to Munehiro Okamoto for his early suggestions on PCR identification. This work was supported, in part, by the Japan Society for the Promotion of Science (JSPS) Postdoctoral Fellowship for Foreign Researchers (no. 26-04103) to Agathe Nkouawa under the assistance of Yasuhito Sako and by a Grant-in-Aid for Scientific Research (no. 26460503) from JSPS to Minoru Nakao.

References

1. Czaplinski B, Vaucher C. Family Hymenolepididae Ariola, 1899, in: L. F. Khalil, A. Jones, R.A. Bray (Eds.), Keys to the Cestoda Parasites of Vertebrates, CAB International, Wallingford, Oxfordshire, 1994, pp. 595-663.

207 2. Haukisalmi V, Hardman LM, Foronda P, Feliu C, Laakkonen J, Niemimaa J, et
208 al. Systematic relationships of hymenolepidid cestodes of rodents and shrews
209 inferred from sequences of 28S ribosomal RNA. Zool Scr 2010; 39: 631-41.
210 3. Garcia LS. Intestinal Cestodes, in: Diagnostic Medical Parasitology, fifth ed.,
211 ASM Press, Washington D.C., 2006, pp. 357-380.
212 4. Makarikov AA, Tkach VV. Two new species of *Hymenolepis* (Cestoda:
213 Hymenolepididae) from Spalacidae and Muridae (Rodentia) from eastern
214 Palearctic. Acta Parasitol 2013; 58: 37-49.
215 5. Tenora F, Asakawa M, Kamiya M. *Hymenolepis pseudodiminuta* sp. n.
216 (Cestoda: Hymenolepididae) from *Apodemus* spp. (Rodentia: Muridae) in
217 Japan. Helminthologia 1994; 31: 185-9.
218 6. Montgomery SSJ, Montgomery WI, Dunn TS. Biochemical, physiological and
219 morphological variation in unarmed hymenolepids (Eucestoda: Cyclophyllidae).
220 Zool J Linn Soc 1987; 91: 293-324.
221 7. Li T, Ito A, Chen X, Long C, Okamoto M, Raoul F, et al. Usefulness of pumpkin
222 seeds combined with areca nut extract in community-based treatment of human
223 taeniasis in northwest Sichuan Province, China. Acta Trop 2012; 124:152-7.
224 8. Hailemariam Z, Nakao M, Menkir S, Lavikainen A, Yanagida T, Okamoto M, et
225 al. Molecular identification of unilocular hydatid cysts from domestic ungulates
226 in Ethiopia: implications for human infections. Parasitol Int 2012; 61: 375-7.
227 9. Katoh K, Standley DM. MAFFT multiple sequence alignment software version
228 7: im- provements in performance and usability. Mol Biol Evol 2013; 30:
229 772–80.
230 10. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular
231 evolutionary genetics analysis version 6.0. Mol Biol Evol 2013; 30: 2725–9.
232 11. Sakka H, Quéré JP, Kartavtseva I, Pavlenko M, Chelomina G, Atopkin D, et al.
233 Comparative phylogeography of four *Apodemus* species (Mammalia: Rodentia)

in the Asian Far East: evidence of Quaternary climatic changes in their genetic structure. Biol J Linn Soc 2010; 100: 797-821.

12. Ishih A, Sekijima T, Asakawa M, Tenora F, Uchikawa R. *Hymenolepis pseudodiminuta* Tenora et al. 1994 from *Apodemus speciosus* and *H. diminuta*: a comparison of experimental infections in rats. Parasitol Res 2003; 89: 297-301.
13. Ito A. Basic and applied problems in developmental biology and immunobiology of cestode infections: *Hymenolepis*, *Taenia* and *Echinococcus*. Parasite Immunol 2015; 37: 53-69.
14. Macnish MG, Ryan UM, Behnke JM, Thompson RCA. Detection of the rodent tapeworm *Rodentolepis* (=*Hymenolepis*) *microstoma* in humans. A new zoonosis? Int J Parasitol 2003; 33: 1079-85.
15. Schmidt GD. CRC handbook of tapeworm identification, CRC Press Inc., Boca Raton, 1986, 675 pp.
16. Spasskii AA. Klassifikaciya gimenolepidid mlekopitayuschih. Trudy Gel'mintologicheskoi Laboratorii, Akademiya Nauk SSSR 1954; 7: 120-167.
17. Macnish MG, Morgan-Ryan UM, Monis PT, Behnke JM, Thompson RCA. A molecular phylogeny of nuclear and mitochondrial sequences in *Hymenolepis nana* (Cestoda) supports the existence of a cryptic species. Parasitology 2002; 125: 567-75.
18. Baer JG, Tenora F. Some species of *Hymenolepis* (Cestoidea) from rodents and from primates. Acta Sc Nat Brno 1970; 4: 1-32.

Figure legends

Fig. 1. Spherical eggs of a hymenolepidid tapeworm derived from a Tibetan woman in China. Scale bar represents 50 μm. Resolution of the microscopic photograph was enhanced using Nomarski prism.

Fig. 2. Midpoint-rooted phylogenetic trees of *Hymenolepis* spp. including a human isolate from China. Code names of the isolates and their localities are shown in parentheses. The trees were made by maximum likelihood method using data sets of nuclear 28S rDNA (1,243 nucleotide sites) and mitochondrial *cox1* (1,000 sites). Database accession numbers of the original sequences are shown in Supplementary Table 1. Values of the main nodes are bootstrap percentages after 500 replicates. Scale bars represent the estimated number of substitutions per nucleotide site. A) The tree of 28S rDNA. Sequences published in a previous report by Haukisalmi *et al*. [2] are shown by asterisks, and those published by them only in databases are indicated with hash symbols. B) The tree of *cox1*.

Fig. 1

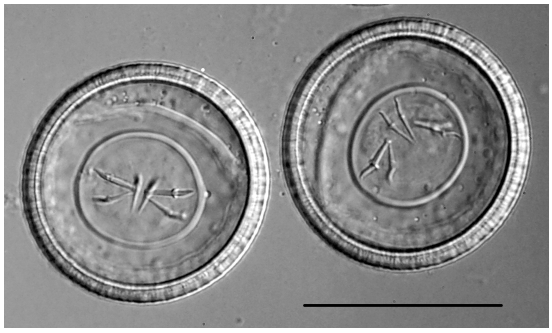
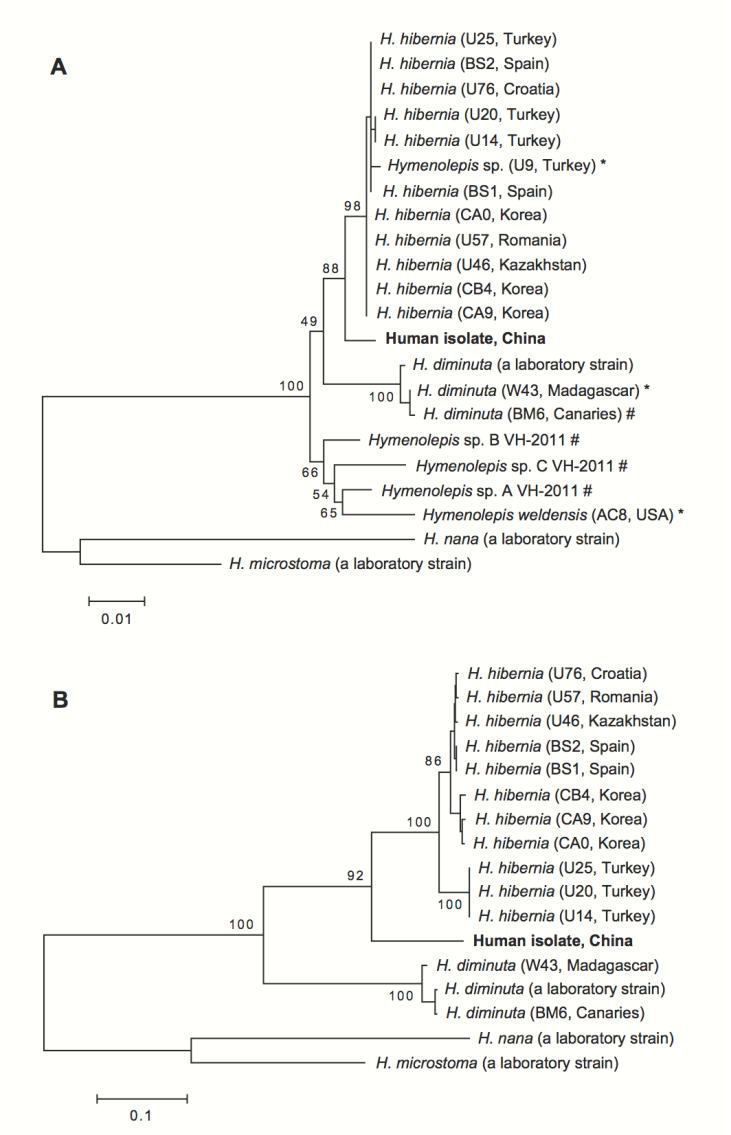


Fig. 2


A
H. hibernia (U25, Turkey)
H. hibernia (BS2, Spain)
H. hibernia (U76, Croatia)
H. hibernia (U20, Turkey)
H. hibernia (U14, Turkey)
Hymenolepis sp. (U9, Turkey) *
H. hibernia (BS1, Spain)
H. hibernia (CA0, Korea)
H. hibernia (U57, Romania)
H. hibernia (U46, Kazakhstan)
H. hibernia (CB4, Korea)
H. hibernia (CA9, Korea)
Human isolate, China
H. diminuta (a laboratory strain)
H. diminuta (W43, Madagascar) *
H. diminuta (BM6, Canaries) #
Hymenolepis sp. B VH-2011 #
Hymenolepis sp. C VH-2011 #
Hymenolepis sp. A VH-2011 #
Hymenolepis weldensis (AC8, USA) *
H. nana (a laboratory strain)
H. microstoma (a laboratory strain)
98
88
49
100
100
66
54
65
0.01
B
H. hibernia (U76, Croatia)
H. hibernia (U57, Romania)
H. hibernia (U46, Kazakhstan)
H. hibernia (BS2, Spain)
H. hibernia (BS1, Spain)
H. hibernia (CB4, Korea)
H. hibernia (CA9, Korea)
H. hibernia (CA0, Korea)
H. hibernia (U25, Turkey)
H. hibernia (U20, Turkey)
H. hibernia (U14, Turkey)
Human isolate, China
H. diminuta (W43, Madagascar)
H. diminuta (a laboratory strain)
H. diminuta (BM6, Canaries)
H. nana (a laboratory strain)
H. microstoma (a laboratory strain)
86
100
92
100
100
100
0.1

Supplementary Table 1

Database accession numbers of nucleotide sequences used in this study.

Species	Codes	Accession nos.	
		28S rDNA	*cox1*
Hymenolepis hibernia	U25, Turkey	KT148842	LC063180
Hymenolepis hibernia	BS2, Spain	KT148842	LC063175
Hymenolepis hibernia	U76, Croatia	KT148842	LC063172
Hymenolepis hibernia	BS1, Spain	KT148842	LC063176
Hymenolepis hibernia	U20, Turkey	KT148844	LC063181
Hymenolepis hibernia	U14, Turkey	KT148844	LC063182
Hymenolepis hibernia	U57, Romania	KT148843	LC063173
Hymenolepis hibernia	U46, Kazakhstan	KT148843	LC063174
Hymenolepis hibernia	CB4, Korea	KT148843	LC063177
Hymenolepis hibernia	CA9, Korea	KT148843	LC063178
Hymenolepis hibernia	CA0, Korea	KT148843	LC063179
Hymenolepis diminuta	Laboratory strain	LC064143	LC063185
Hymenolepis diminuta	W43, Madagascar	GU166229 [a]	LC063184
Hymenolepis diminuta	BM6, Canaries	HM138522 [b]	LC063186
Hymenolepis nana	Laboratory strain	LC064145	LC063187
Hymenolepis microstoma	Laboratory strain	LC064144	LC063188
Hymenolepis sp.	Human isolate	LC064142	LC063183
Hymenolepis sp.	U9, Turkey	GU166227 [a]	n.d. [c]
Hymenolepis weldensis	AC8, USA	GU166230 [a]	n.d.
Hymenolepis sp. A VH-2011	BP4, Thailand	HM138523 [b]	n.d.
Hymenolepis sp. B VH-2011	C31, Madagascar	HM138524 [b]	n.d.
Hymenolepis sp. C VH-2011	U45, Kazakhstan	HM138525 [b]	n.d.

[a] Sequences published in a previous report [2].

[b] Sequences published by Haukisalmi *et al*. only in databases.

[c] not determined.