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Genetic diversity of the potentially therapeutic tapeworm *Hymenolepis diminuta* (Cestoda: Cyclophyllidea)

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ABSTRACT

The cestode *Hymenolepis diminuta* is highly prevalent in wild rat populations and has also been observed rarely in humans, generally causing no apparent harm. The organism has been studied for decades in the laboratory, and its colonization of laboratory rats has recently been shown as protective against some inflammation-associated disorders. Recently, *H. diminuta* has become a leading candidate for helminth therapy, an emerging method of "biota enrichment" used to treat or prevent inflammatory diseases of humans in Western society. While most of the experimental isolates of *H. diminuta* are identified based on typical morphological features, hymenolepidid tapeworms may represent complexes of cryptic species as detected by molecular sequence data. In the present study, we explored the diversity of laboratory-kept strains using partial sequences of two genes (lsrDNA and *cox*1) and determined that *H. diminuta* isolates currently considered for therapeutic purposes in the US and Europe belong to a single, genetically nearly uniform lineage, showing only little genetic deviation from wild-caught isolates.

1. Introduction

Western civilization is plagued by an ever-increasing amount of inflammation-associated diseases, including allergic disorders, autoimmune conditions, digestive diseases, and neuropsychiatric problems (e.g., [1–4]). The rapid rise of inflammatory disorders implicates changes in environmental factors as consequences of industrialized society that includes increased hygiene [5], chronic psychological stress [6], rich diets poor in nutrition [7], and sedentary lifestyles [8,9]. Alteration of the symbiotic biota associated with the human body [10,11] is another factor leading to inflammatory disease in Western civilization [11–13].

Industrialized civilization has induced a number of changes in the human biota, the symbiotic life associated with the human body [5]. One of the most profound changes has been the almost nearly complete loss of helminths [14]. Consistent with the view that normalization of the biome might treat disease, a wide range of studies in animal models have shown that reintroduction of helminths can reduce inflammation and treat disease [15,16]. However, clinical trials are still in early stages, perhaps due to a lack of incentive for development [15,17], and thousands of human individuals are self-treating themselves with helminths, reportedly with beneficial results [18–19].

Helminths most commonly considered for use in helminth therapy are typically nematodes; both hookworms and whipworms are being used [16,18]. The rat tapeworm *Hymenolepis diminuta* (Rudolphi, 1819) (Cestoda: Hymenolepididae) currently stands alone as the only cestode considered for therapeutic use [20–22]. This well-known and common tapeworm with worldwide distribution has been in use as laboratory model for decades (reviewed in [23]) and has become popular for testing host-symbiont interactions (e.g., [20,22,24,25]). The rat tapeworm has an indirect life cycle involving an insect intermediate host – it is typically maintained in the laboratories using tenebrionid beetles ([23,25]) (see Fig. 1). Natural populations of *H. diminuta* colonize mainly Norwegian and black rats [*Rattus norvegicus* (Berkenhout) and *R.*

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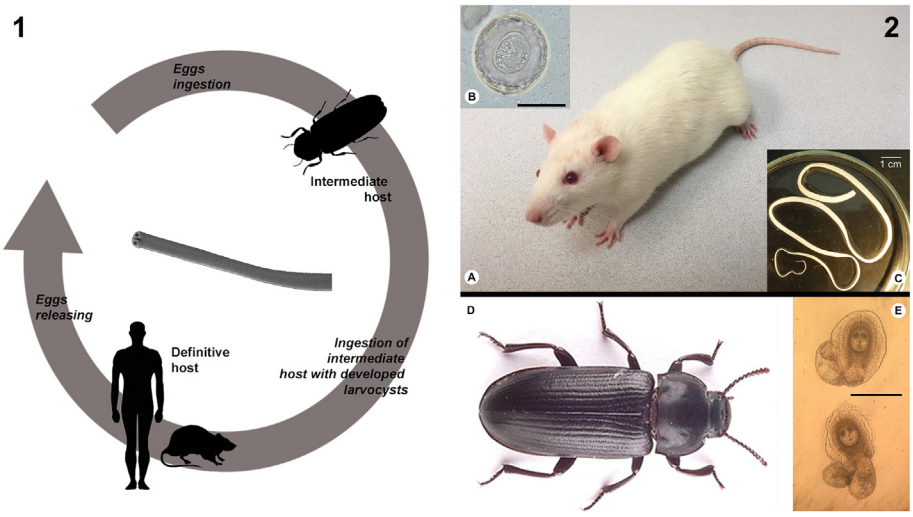

Fig. 1. Life cycle of *Hymenolepis diminuta* under laboratory conditions. [1] graphical visualization of the *H. diminuta* life cycle; [2] Hosts and life stages of *H. diminuta* under laboratory conditions: [2A] SPF outbred rat as a definitive host (DH) under laboratory conditions; [2B] the egg of *H. diminuta* containing typical oncosphere and hexacanth inside, excreted in the feces of DH (scale bar = 40 μm); [2C] the adult of *H. diminuta* isolated from DH's small intestine (adult tapeworm ranging between 20 and 30 cm in length); [2D] *Tenebrio molitor* as an intermediate host (IH); [2E] the cysticercoids of *H. diminuta* developing in body cavity of IH;

rattus (Linnaeus), respectively], but have also been observed in other mammalian hosts, mostly rodents [26]. This tapeworm is able to colonize humans, however, such colonizations are rare and usually asymptomatic [27].

Studies using laboratory animal models have probed the potential utility of *H. diminuta* for helminth therapy. Interestingly, *H. diminuta* ameliorates inflammatory diseases in many, but not all, animal models [20], and proved effective in the majority of self-treating humans surveyed in a recent study [18]. In terms of inflammatory bowel disease, *H. diminuta* protects against chemically induced colitis by dampening the inflammatory response via Th2 type immune response, increased production of regulatory IL-10 with involvement of regulatory T- and B-cells as well as activation of mucosal immunity [20,22,25,28,29]. Colonization of laboratory rats with *H. diminuta* has also been shown to protect neurological function under conditions of stress and inflammation [30]. A broad range of helminths can apparently modulate immune functions [31], however, *H. diminuta* in addition offers several practical advantages over the nematodes currently in use for helminth therapy [21] [32]. Unlike the nematodes currently used for helminth therapy, *H. diminuta* does not breach the gut epithelium and can be raised inexpensively under strictly controlled conditions in the laboratory (see Fig. 2 for illustration) [21].

1. Given its potential medical significance, the origins as well as the genetic diversity of the individual strains of *H. diminuta* currently kept for medical research represent an important piece of information. At present, four isolates of *H. diminuta* have been already tested for the purposes of helminth therapy (e.g., [22,24,33,34]). The main aim of this study was to evaluate the genetic variability between individual laboratory-kept specimens of *H. diminuta*, including three isolates used in helminth therapy research, five isolates obtained from wild rats and one that was obtained from a human patient (for details see Table 1).

2. Material & methods

2.1. Tapeworms sequenced

Nine rat tapeworm isolates morphologically identified as *H. diminuta* were molecularly characterised in this study (Table 1). Four isolates originated from the experimental animal models kept under laboratory conditions (isolates no. 1, 3, 4 & 6; Table 1), three of them being utilized in helminth therapy (isolates no. 1, 3 & 6). Further one isolate originated from a human patient (isolate no. 5, Table 1) and four others were isolated from wild rats (isolates no. 2, 7–9; Table 1). The wild-caught isolates were collected on Tenerife, Canary Islands, (isolates no. 7–9) and the sequence of the last wild isolate no. 2 was obtained from Asahikawa Medical University (Japan) [35].

2.2. Molecular-phylogenetic analyses

Total DNA was extracted from a snip of worm strobila using High Pure PCR Template Preparation Kit (Roche s.r.o., Praha, Czech Republic). Partial sequences of nuclear large subunit ribosomal RNA gene (lsrDNA) and cytochrome *c* oxidase subunit 1 (*cox*1) were amplified according to the previously published protocols of Brabec et al. [36] and Nkouawa et al. [35], respectively. Both genes were amplified with Phusion High-Fidelity DNA Polymerase (New England BioLabs Inc., MA, USA), the amplicons were gel-checked and enzymatically purified with Exonuclease I and FastAP alkaline phosphatase (Thermofisher Scientific, Waltham, USA) and directly Sanger sequenced at GATC Biotech (Konstanz, Germany). Sequence assembly, manual check

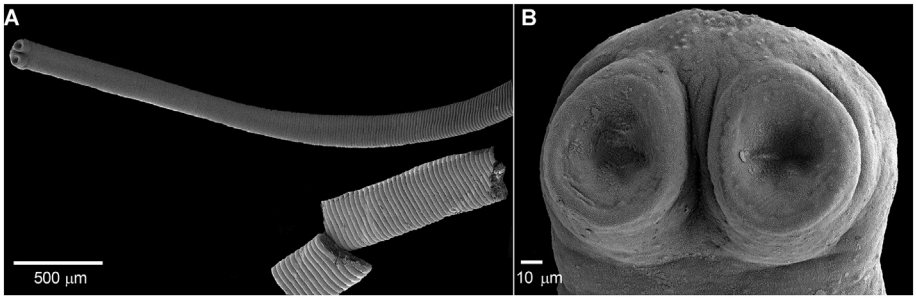

Fig. 2. Scanning electron micrographs of the *Hymenolepis diminuta* adult from experimental infection of SPF Wistar rats. [A] part of the strobila, [B] detail of scolex, dorsoventral view. For this SEM, the *H. diminuta* strain 1 according the Table 1 was used.

Table 1
List of used isolates of *Hymenolepis diminuta* and their origin. RN-*Rattus norvergicus*, RR-*Rattus rattus*, Ho-human.

[b]	Isolates` origin	Host	lsrDNA	*cox*1
1	Institute of Parasitology, BC CAS, Czech Rep. (therapeutic model)	RN/lab	MH472992	MH472983
2	Asahikawa Medical University, Japan	RN/lab[a]	MH472993	MH472984
3	Biome Restoration, Lancaster, UK (therapeutic model)	RN/lab	MH472989	MH472980
4	W. Stefanski Institute of Parasitology, PAS, Poland	RN/lab[a]	MH472994	MH472985
5	W. Stefanski Institute of Parasitology, PAS, Poland	Ho	MH472991	MH472982
6	Department of Surgery, Duke University, NC, USA (therapeutic model)	RN/lab	MH472988	MH472979
7–9	El Batan locality, Tenerife, Canarian Islands	RR/wild[b]	MH472990, −95, −96	MH472981, −86, −87

[a] *H. diminuta* isolate obtained from wild rats, but now kept under laboratory conditions.
[b] Only rat feces with *H. diminuta* eggs were collected and preserved in 96% ethanol for molecular analyses.

and further analyses were undertaken in Geneious ver. 7 (http://www.geneious.com/; [37]). The lsrDNA and *cox*1 alignments were constructed using the *E*- and G-INS-i algorithms of the program MAFFT [38], respectively. End gaps were encoded as missing data and datasets were analysed individually under the maximum likelihood criterion in PHYML [39]. GTR + I + Γ model of nucleotide evolution and sub-tree pruning and regrafting branch-swapping algorithm were employed when searching for the best tree. Nodal supports were estimated over 100 bootstrap replicates.

3. Results

The lsrDNA- and *cox*1-sequences of nine isolates of *H. diminuta* were characterised and compared with available sequences of *H. diminuta* and closely related species deposited in GenBank (Fig. 3). The lsrDNA and *cox*1 gene alignments consisted of 1439 and 996 nucleotide sites, respectively, both clearly delineating *H. diminuta* as a distinct, monophyletic lineage. Eight of the nine new lsrDNA sequences (MH472988–96; Table 1) were identical; one of the Tenerife isolates (MH472990) differed by a single nucleotide. Divergence between the nine *cox*1 sequences (MH472979–87; Table 1) ranged from 0 to 0.4% (i.e. 0–4 nucleotides). Evaluating the nucleotide distances within all available representatives of *H. diminuta*, the level of divergence along the 996 nt-long gene fragment ranged from 0 to 7.9% (total nucleotide differences 0–35), yet containing little phylogenetic signal to reveal phylogenetic structure within this lineage (Fig. 3). There are four *cox*1 sequences (AB494470, AF096244, KY079337, KY079338) forming a distinct branch within the lineage of *H. diminuta*; all originating from rats collected in Asia (Japan, Korea and China) [40–42].

4. Discussion

Hymenolepidid tapeworms [*Hymenolepis diminuta*, *Rodentolepis* (*Hymenolepis*) *nana* (Siebold, 1852) or *Rodentolepis* (*Hymenolepis*) *microstoma* (Dujardin, 1845)] are commonly maintained under laboratory conditions and used as helminth models for a range of studies because of their easy cultivation [23,43]. The life cycle includes insect intermediate hosts and rodents or rarely humans as definitive host (see Fig. 1), with exception of *R.* (*H.*) *nana* that is able to multiply in the definitive host without any need for an intermediate host. Recent research addressed *H. diminuta* in particular as a suitable candidate for helminth therapy given its potential for asymptomatic colonizations of the human host (e.g., [18]) and other criteria for use in helminth therapy as defined by Lukeš et al. [32] and later (re)formulated by Sobotková et al. [44].

So far, several isolates of *H. diminuta* have been tested for their impact on the host health [20–22,30] but without any detailed morphological description or molecular identification. Intriguingly, recent observations demonstrated that the accurate identification of the hymenolepidid tapeworms in general might be problematic since this

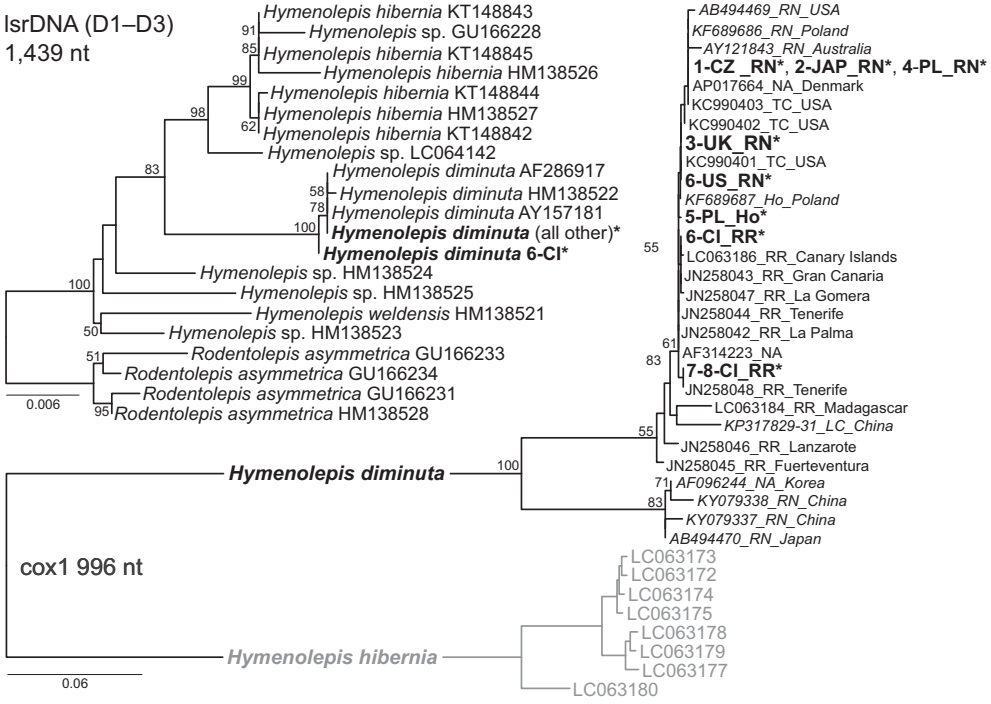

Fig. 3. Phylogenetic relationships of various isolates of *Hymenolepis diminuta* based on partial large subunit ribosomal RNA (left) and partial cytochrome *c* oxidase subunit 1 (right) sequences. Maximum likelihood phylogenetic estimates using GTR + I + Γ substitution model, unrooted phylogenies. Bootstrap nodal support values (100 repetitions) are stated only when > 50. Terminals might represent multiple specimens with identical sequences. Sequences of *cox1* in italics reach only 50% of the total alignment length (996 nt). Host and origins stated when available: Ho, Human; R, rat; RN, *Rattus norvegicus*; RR, *Rattus rattus*; TC, *Tribolium castaneum*; LC, *Lemur catta*; NA, not available; isolates characterised in the present study are marked with asterisks.

cestode group includes several cryptic species complexes (e.g., [26,45,46]) that colonize a broad spectrum of hosts (e.g., [46–48]). For example, one recent report demonstrated the human colonization with *Hymenolepis* sp. closely related to *Hymenolepis hibernia* [49], a sister species of *H. diminuta*, and previously assumed to colonize solely Euroasian *Apodemus* mice [35,50]. This group of hymenolepidid species has highly similar egg structure more or less indistinguishable from each other, and the adults are essential for proper diagnostics based on the tapeworm morphology [49]. However, in such cases of human colonizations, only the eggs or pieces of strobila are available from the stool sample, which is insufficient for proper diagnostics. Analogous cases involve two other hymenolepidid species from rodents, *R.* (*H.*) *nana* commonly reported from man and *R.* (*H.*) *microstoma* reported recently for the first time also from man [51]. In these cases, adult morphology rather than egg morphology is needed for identification of the species [23,51]. Since there are two groups of hymenolepidid tapeworms that are indistinguishable based on the egg structure, the analyses of adult morphology or, better, molecular approaches are critical for exact identification. Along with all these facts above, even evolutionary relationships within both genera, *Hymenolepis* and *Rodentolepis*, are not satisfactorily resolved yet [46,48].

In our study, molecular phylogenetic comparisons of *H. diminuta* isolates originating from different laboratories across the world and wild-caught isolates showed virtually no variability within the fragment of lsrDNA, a commonly utilized (although relatively conserved) genetic locus in phylogenetic studies. Very low numbers of nucleotide differences were revealed even within the relatively more variable genetic locus, *cox*1, ranging between 0.0 and 0.3%. Intraspecific diversity within *H. diminuta* has not been studied intensively so far, and the nucleotide difference of partial *cox*1 of 6.4% between two isolates from Japan and USA reported by Okamoto et al. [40] might actually correspond to variation between cryptic species. In contrast, pairwise differences of *cox*1 sequence data obtained by Foronda et al. [47] from strains isolated in the Canary Islands range between 0 and 1.3%. In comparison, two isolates of *R. (H.) nana* from mouse and human showed a 5% difference in the *cox*1 gene fragment [45]. Comparing the intraspecific molecular diversity of *H. diminuta* in our *cox*1-based phylogenetic estimates, we detected a very divergent branch of four Asian isolates forming a sister lineage to all remaining representatives of *H. diminuta*. Given the fact that the sources of those sequences were identified solely based on sequence comparisons according to the original articles [40–42], we cannot rule out the possibility that those sequences actually represent a cryptic species. Without any further data to address this issue, however, we refrain from making any further comments on the presence of a separate species. Systematic collection of *H. diminuta* across its geographical distribution along with reliable species identification (based on the adult morphology) needs to be carried out first to allow for an accurate assessment of the actual diversity of the parasite and its closest relatives.

Very low levels of genetic diversity observed within characterised specimens of *H. diminuta* (notably within the laboratory-kept strains) suggest that medical research may be using tapeworms historically originating from a limited number of sources. The tapeworm strain maintained at Duke University Medical Center (isolate no. 6; Table 1) [19] and those currently sold for therapeutic purposes by WormThreapy® and Biome Restoration® (isolate no. 3; Table 1) were derived from stocks at Carolina Biological Supply (North Carolina, USA). Those stocks, in turn, were obtained either from Shelly Michalski (University of Wisconsin Oshcosh) or from John Oaks (University of Wisconsin Madison, School of Veterinary Medicine). Michalski had obtained the tapeworms originally from Oaks, so the organisms have the same origin, regardless of the source. The Oaks´ strain was originally obtained from Tulane University and brought to the University of Iowa by Richard Lumsten. It is probable the Lumsten obtained the organism from his mentor, who in turn probably obtained the organism from a wild rat at the docks in New Orleans, USA (John Oaks, personal communication). Oaks as well as Lumsten widely shared their specimens for research purposes, which could explain in part the limited diversity of the laboratory strains studied. Similarly, the *H. diminuta* isolate used for research of helminth therapy at the Biology Centre of the Czech Academy of Sciences (isolate no. 1, Table 1) [22] was passed through several laboratories. Jirků-Pomajbíková obtained it from the laboratory of Faculty of Pharmacy at Charles University, but originally it was derived from the W. Stefanski Institute of Parasitology, Poland (isolate no. 4 from rat; see Table 1). This *H. diminuta* strain was isolated from a wild rat in the 1950s [52]. These two cases highlight the fact that laboratory-kept helminth strains from different laboratories can have complex and shared histories.

The observation that multiple suppliers of helminths for helminth therapy and multiple laboratories are using genetically uniform isolates of *H. diminuta* has several practical implications. First, results obtained in the laboratory are more likely to be applicable to results seen in individuals using the organisms for therapy. Perhaps more importantly, any potential variation in the results obtained by different investigators is less likely to be due to the biological properties unique to specific strains. At the same time, this observation begs the question as to whether particular strains of *H. diminuta* may be more effective therapeutically than are other strains. Given that clinical trials with helminthic therapy are in their infancy in general, and that clinical trials with *H. diminuta* have yet to begin, it seems prudent to focus on the single common strain for the moment, postponing additional testing of other variants until a “baseline” with the most commonly used strain is established.

5. Conclusion

Molecular characterisation of helminths used for helminth therapy is rare, and the sequences of this common symbiont are scattered among specimens of *H. diminuta* isolated from rodents and other mammals. This study demonstrates very low genetic diversity in *H. diminuta* isolates used for the helminth therapy, a finding that could have important implications for this field. Finally, the eggs obtained from a human case reported by Nkouawa et al. [35] morphologically similar to *H. diminuta* but yet genetically distinct from *H. diminuta* demonstrates that there are more genetically distinct lineages of *Hymenolepis*, related to the *H. diminuta/hibernia* or other species, providing potential alternatives for future clinical trials with the helminthic therapy.

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