Molecular identification of *Taenia mustelae* cysts in subterranean rodent plateau zokors (*Eospalax baileyi*)

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Abstract: Cestode larvae spend one phase of their two-phase life cycle in the viscera of rodents, but cases of cestodes infecting subterranean rodents have only been rarely observed. To experimentally gain some insight into this phenomenon, we captured approximately 300 plateau zokors (*Eospalax baileyi*), a typical subterranean rodent inhabiting the Qinghai-Tibet Plateau, and examined their livers for the presence of cysts. Totally, we collected five cysts, and using a mitochondrial gene (*cox1*) and two nuclear genes (*pepck* and *pold*) as genetic markers, we were able to analyze the taxonomy of the cysts. Both the maximum likelihood and Bayesian methods showed that the cysts share a monophyly with *Taenia mustelae*, while Kimura 2-parameter distances and number of different sites between our sequences and *T. mustelae* were far less than those found between the examined sequences and other Taeniidae species. These results, alongside supporting paraffin section histology, imply that the cysts found in plateau zokors can be regarded as larvae of *T. mustelae*, illustrating that zokors are a newly discovered intermediate host record of this parasite.

Keywords: Endoparasites; New host record; Phylogenetic relationships; Subterranean rodent

Larval taeniid cestodes (Taeniidae, Cyclophyllidea, Cestoda) are known to require either human or other herbivorous mammals—generally rodents—to serve as intermediate hosts during their larval stage (Knapp et al, 2011). While there are many parasites within humans and rodents, larval cestodes are a special epidemiological focus, because they can cause serious pathological changes in viscera and tissues, and even death of the host (Eckert et al, 2001; Hoberg, 2002). While humans and other herbivorous mammals are often used as hosts, subterranean rodents are generally not thought to be viable host options. As a widely distributed group of species that live primarily underground and are highly adapted to that environment (Lacey et al, 2000; Nevo, 1999), most major activities—foraging, mating, and breeding, etc. —take place underground. Consequently, these rodents have rare contact with predators (Begall et al, 2007), but more importantly, since subterranean rodents generally forage underground parts of plants, they have a markedly smaller probability of encountering food contaminated by cestode eggs. As a result, it is commonly believed that these animals have a comparatively rare chance of becoming infected by cestodes, but that preconception may be, at best, flawed, or even incorrect, because these animals are typically hidden, the presence of cestodiasis is not well empirically studied.

Boev et al (1971) first identified *E. multilocularis* from cysts isolated from a zokor species (*Myospalax* sp.) in Kazakhstan. Li et al (1985) and Hong & Lin (1987) reported the same identification of cysts from the viscera of Chinese zokors (*M. fontanieri*) in Ningxia, China.

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Because of the paucity of adult cestode phenotypic characteristics and the great plasticity of larvae, identifying the Taeniidae species using traditional histologic examination of paraffin slices is prone to errors (Nakao et al, 2010). Thankfully, modern molecular technologies provide far more accurate methods of identification. In this study, we sought to use both approaches to gain a more complete picture of cestode infections. To that end, we collected several cysts from the livers of captured plateau zokors (*Eospalax baileyi*) (Figure 1A) and used molecular phylogenetic methods to determine the phylogeny of the larval cysts.

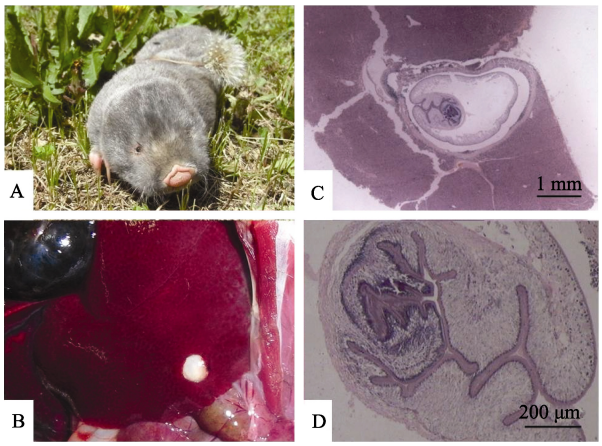

Figure 1 Photographs of Samples

A: Example of plateau zokor *Eospalax baileyi*; B: Cyst in zokor liver tissue; C and D: Histopathological slices of a cyst.

MATERIALS AND METHODS

Totally, 300 plateau zokors were captured for study from Datong County (N37°7.5′, E101°48.7′), in the east of Qinghai Province, China. After euthanizing the specimens and dissecting them, liver tissues were extract and tissues that appeared to be infected (Figure 1B) were fixed in 4% formalin and embedded in paraffin wax, with 3-5 μm sections prepared for histopathological observation. The samples were stained overnight with Delafield's haematoxylin, destained with 70% ethanol containing 1% hydrochloric acid, dehydrated in ethanol, and then cleared with xylene and mounted in Canada balsam. The found cysts that were to be used for molecular analyses were fixed in 95% ethanol before total genomic DNA was extracted using a spin column kit (DNeasy tissue kit, Qiagen, Germany) and then used as a template for PCR. Here, two cysts were randomly selected for molecular analyses. Partial fragments of the mitochondrial gene for cytochrome c oxidase subunit 1 gene (*cox1*) (approximately 880 bp) were amplified using previously published primers cox1/F and cox1/R by Nakao et al (2000). We also selected genes for phosphoenolpyruvate carboxykinase (*pepck*) (approximately 1 650 bp) and DNA polymerase delta (*pold*) (approximately 2 000 bp) to serve as targets for nuclear markers in the cyst DNA (Knapp et al, 2011).

PCR was performed using a 40 μL final reaction volume, with 40 to 60 ng of genomic DNA, 0.6 mmol/L dNTPs, 0.2 μmol/L of each primer, 1U Taq polymerase and the manufacturer-supplied reaction buffer. Thermocycling was conducted in a T-Gradient Thermoblock PCR machine (Biometra, Gottingen, Germany). After initial denaturation at 94 °C for 7 min, the reaction proceeded for 35 cycles as follows: 30 s at 94 °C, 30 s at 54 °C to 56 °C and 90 s at 72 °C and terminated with a final extension step of 72 °C for 5 min. Resulting PCR products were purified using a CASpure PCR Purification Kit following the manufacturer's recommended protocols (Casarray, Shanghai, China), and directly sequenced using the same primers that were previously used for amplifying the sequences mentioned above. Sequencing reactions were conducted in a Biometra thermocycler using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, UK) following the manufacturer's protocols. Sequencing products were later separated and analyzed on an ABI 3730 DNA Analysis System (Applied Biosystems, USA). Putative exon regions for both the *pepck* and *pold* genes were extracted from each of the respective sequence alignments under the guidance of previously published exon–intron maps for *E. multilocularis* (Knapp et al, 2011).

We obtained the sequences of *cox1*, *pepck* and *pold* genes from the available species in the family (9 *Echinococcus* and 15 *Taenia* taxa) from GenBank to serve as a basis for identifying taxonomic status of the cystic larvae using phylogenetic methods. The additional taxa *Hymenolepis diminuta* for *cox1* and *Dipylidium caninum* for the two nuclear genes (*pepck* and *pold*) were included as outgroups for reconstructing maximum likelihood (ML) and Bayesian trees (accession numbers of these sequences are in Table 1). The coding sequences for each gene were aligned using ClustalW in MEGA 5.0 (Tamura et al, 2011), cut to the length of the shortest sequence. Maximum likelihood trees were generated in PAUP 4b10 (Swofford, 2002) and the Bayesian trees in MrBayes 3.2 (Ronquist et al, 2012), each with 1 000

bootstrap replicates. Nucleotide substitution models were selected using the Akaike information criterion (AIC) within Modeltest 3.7 (Posada & Crandall, 1998) running on PAUP 4b10 and in MrModeltest 2.3 (Posada & Crandall, 2001) running on MrBayes3.2. The Kimura 2-parameter pairwise divergence (*K2P*) and the number of different nucleotide substitutions (*N*) among sequences was calculated using MEGA 5.0.

Table 1 GenBank accession numbers for all reference sequences (9 *Echinococcus* and 15 *Taenia* taxa and 2 outgroups *Dipylidium caninum* and *Hymenolepis diminuta*) for each gene used in this study

Species	*cox1*	*pepck*	*Pold*
E. granulosus	AF297617	FN567990	FN568361
E. multilocularis	AB018440.2	FN567985	FN568356
E. shiquicus	AB208064	FN567986	FN568357
E. felidis	AB732958	FN567989	FN568360
E. oligarthrus	AB208545	FN567988[b]	FN568359[b]
E. ortleppi	AB235846	FN567992	FN568363
E. equinus	AF346403	FN567991	FN568362
E. vogeli	AB208546	FN567987	FN568358
E. canadensis (G7)	AB235847	FN567994	FN568365
T. asiatica	AF445798.2	FN567998	FN568369
T. crassiceps	AF216699	FN567999	FN568370
T. hydatigena	FJ518620	FN568000	FN568371
T. multiceps	GQ228818	FN568002	FN568373
T. saginata	AY684274	FN567997	FN568368
T. solium	AB086256	FN567996	FN568367
T. laticollis	JX860624	FR869697	FR869703
T. madoquae	AM503324	FR869699	FR869705
T. martis	AB731758	FR852569	FR869706
T. ovis	JX134121	FN568003	FR869707[b]
T. parva	AB731760	FR869700	FR869708
T. serialis	AB704405	FN568001	FN568372
T. taeniaeformis	AB731761	FR869701	FR869709
T. twitchelli	AB731759	FR852568	FR869710
T. mustelae	EU544570	FR869698	FR869704
Dipylidium caninum	——	FR869702	FR869711
Hymenolepis diminuta	AF314223	——	——

RESULTS

Totally, 300 plateau zokors were captured and then examined for the presence of cestode parasites. Among these five separate cysts were detected in five of the examined zokors, each ranging from 3-5 mm in diameter (Figure 1B). The cysts were found deeply embedded, and partly or fully covered by liver tissue. Physically, they are thin-walled and either transparent to translucent. Slices of the tissues (depicted in Figure 1C, D) showed that only one larva attached to the inner face of the cyst. The larva was about 3.5 mm in major diameter with no sign of scolex formation. Together, these physical characteristics suggest a close relationship of the observed cysts with *Taenia mustelae* Gmelin 1790 (Freeman, 1956).

Three genes each from two cysts were sequenced and the resulting sequences submitted to GenBank (accession numbers: KC898934-KC898939). The *cox1*, *pepck*, and *pold* (partial sequences) were 820 bp, 1 041 bp, and 1 873 bp in length, respectively. The putative exon regions for *pepck* and *pold* genes were likewise 921 bp and 867 bp, respectively. Finally, three alignments in lengths of 384 bp, 921 bp, and 867 bp respectively for the *cox1*, *pepck*, and *pold* genes

were used for molecular analyses. Testing showed that the three genes differed at only one or two sites between the DNA sequences from the larvae from the two cysts.

Further phylogenetic analysis yielded both ML and Bayesian trees, (Figure 2) both of which clearly demonstrated that the sequences of the cysts and *T. mustelae* were closely related. Further analysis between the sequences of the cysts and other sequences yielded *K2P* and *N* values (Table 2), and the values for both measurements between the studied sequences and earlier reported sequences of *T. mustelae* were far less than those between our sequences and other Taeniidae species.

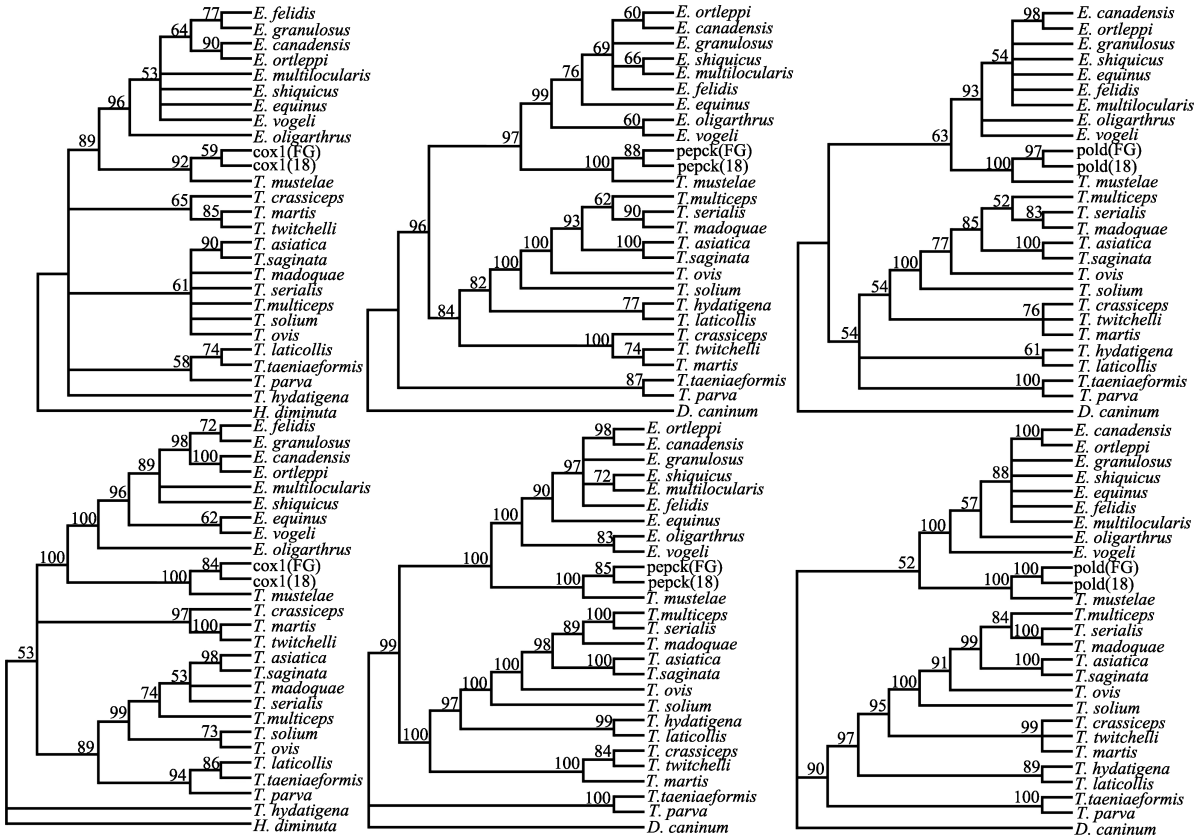

Figure 2 Phylogenetic trees of cyst tissues found in Plateau Zokors using the *cox1* and exon data sets of *pepck* and *pold*

Upper tree is generated by maximum likelihood and the lower by Bayesian analysis. Each tree was rooted with *Hymenolepis diminuta* for cox1 and *Dipylidium caninum* for *pepck* and *pold*. Values at each node are bootstrap proportions (%).

DISCUSSION

Both the histopathological and molecular analyses we conducted indicated that the cysts can be regarded as larvae of *T. mustelae,* meaning that the zokors are a newly identified host for *T. mustelae* infection. A recent DNA barcoding of taeniids using the same *cox1* gene segment as we used in this study found that the optimum threshold for distinguishing a *Taenia* species is 3.6% of K2P distance (Galimberti et al, 2012), which is higher than the value (2.7%) found between our samples and *T. mustelae*. Given this threshold, we can conclude that our speculation on the nature of the cysts as being *T. mustelae* larvae is likely accurate. Unfortunately, the "gold standard" of determining a taeniid species relies on using both molecular data of adult specimens as well as morphological observations. Though our present findings are intriguing, further studies, such as survey of potential hosts for adult taeniids, are still necessary to make a definitive taxonomic review of the cysts.

Combined with former reports, our results make it clear that that zokors can harbor two Taeniidae species with two different genuses. Taeniid parasites require two mammalian hosts to perpetuate their life cycles.

Table 2 Mean values of different sites (*N*) and Kimura 2-parameter distances (*K2P*) between the sampled sequences and earlier published sequences for *cox1*, *pepck*, and *pold* genes

	cox1(384 bp)		*pepck* (921 bp)		*pold* (867 bp)	
	N	*K2P*	*N*	*K2P*	*N*	*K2P*
E. granulosus	52.0	0.1500	83.5	0.0980	92.0	0.1170
E. multilocularis	50.0	0.1430	81.5	0.0955	87.0	0.1100
E. shiquicus	48.0	0.1370	83.5	0.0980	91.0	0.1150
E. felidis	48.0	0.1370	82.5	0.0965	88.0	0.1110
E. oligarthrus	43.0	0.1220	80.5	0.0945	90.0	0.1140
E. ortleppi	48.0	0.1370	83.5	0.0980	90.0	0.1140
E. equinus	46.0	0.1310	82.5	0.0965	85.0	0.1070
E. vogeli	48.0	0.1370	78.5	0.0915	85.0	0.1070
E. canadensis (G7)	51.0	0.1470	82.5	0.0965	90.0	0.1140
T. asiatica	56.0	0.1630	104.5	0.1255	104.0	0.1335
T. crassiceps	50.0	0.1430	99.5	0.1190	122.0	0.1610
T. hydatigena	56.0	0.1630	99.5	0.1185	96.5	0.1230
T. multiceps	55.0	0.1590	110.5	0.1335	103.0	0.1325
T. saginata	54.0	0.1560	104.5	0.1255	103.0	0.1325
T. solium	55.0	0.1590	90.5	0.1070	105.0	0.1355
T. laticollis	54.0	0.1560	111.5	0.1345	129.0	0.1710
T. madoquae	53.0	0.1530	102.5	0.1230	97.0	0.1235
T. martis	55.0	0.1600	99.5	0.1190	110.0	0.1435
T. ovis	56.0	0.1630	101.5	0.1215	100.0	0.1275
T. parva	54.0	0.1560	122.5	0.1505	139.0	0.1855
T. serialis	57.0	0.1660	101.5	0.1215	96.0	0.1225
T. taeniaeformis	59.0	0.1720	117.5	0.1425	143.0	0.1910
T. twitchelli	48.0	0.1370	99.5	0.1195	118.0	0.1555
T. mustelae	10.0	0.0270	3.5	0.0035	4.0	0.0045

Terrestrial carnivorous predators act as hosts for the adult worms, while their prey, such as the zokors and other rodents, act as intermediate hosts for the cystic larvae. Zokors are commonly thought to have only rare contact with predators because of their absolute underground habitat. An earlier study noted that the numbers of plateau zokors in the pellets and food remains of *Buteo hemilasius* and *Bubo bubo* only consisted ~5% of total prey individuals (Cui et al, 2003). Likewise, the average feeding intensity by predators of the zokor, such as the red fox (*Vulpes vulpes*), polecat (*Mustela eversmanni*) and weasel (*M. altaica*) of plateau zokor was much lower than that of the sympatric distributed plateau pika (*Ochotona curzoniae*) (Yang et al, 2007). Moreover, as a typical subterranean rodent, plateau zokors mainly feed on underground roots and shoots of plants (Zhang, 1999), both of which are less likely to be contaminated by cestode eggs contained in the feces of carnivorous hosts. Collectively, these characteristics make the zokors somewhat unlikely infectious targets for taeniid cestodes. The reality that zokors can indeed be infected by both *Taenia* and *Echinococcus* larvae shows that an underground habitat alone cannot prevent infection by such parasites, which may then hold some interesting implications for studies of similar parasitic organisms

One possibility that may explain the infection is that when zokors collect food items from underground, they may also pull down the aboveground parts of plants. In fact, recent studies on winter caches of plateau zokors showed that they collected considerable amount of aboveground plant parts, some of which are even positively selected as food sources (Xie et al, 2013). This novel observation gives some credence to our observed infection of zokors by parasites, because if zokors actually harvest aboveground sources of food, they may have more chances to become infected by cestodes as expected. Another possibility is that while zokors

constitute only a small part of food resources of predators, some carnivores, such as the polecat, frequently invade to their burrows in search of food (Zheng et al, 1983) Even when unsuccessful, such forays into the underground zokor burrows may increase the probability of contamination of cestodes eggs (from feces) in the zokor habitats and consequently increase their infection rate. Since there are many predators and other herbivorous mammals such as plateau pika, root vole (*Microtus oeconomus*), hamster (*Cricetulus longicaudatus*), and marmot (*Marmota himalayana*) that are sympatrically distributed with plateau zokors, the taeniid parasites that infect zokors may complete their life cycles locally, placing many of these wild mammals of the plateau at risk for cestodiasis, though this clearly needs further detailed follow-up. Accordingly, we suggest that the health challenges of endoparasites from zokors (and probably other subterranean rodent species) should not be neglected and indeed warrant greater attention and observation.

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