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Morphological and molecular identification of the metacestode parasitizing the liver of rodent hosts in bamboo growing areas of mizoram, northeast India

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Abstract:
In Mizoram (Northeast India), rodent outbreaks are known to occur periodically with the onset of bamboo flowering causing a tremendous destruction to food grains and as per the folk belief, often resulting in famine. In an exploratory survey of rodent pests in bamboo growing atreas for their helminth parasite spectrum, metacestodes of tapeworms were frequently encountered infecting the liver lobes and body cavity of the host. The morphological criteria were found to be closely consistent with the metacestode of *Taenia* species. In molecular characterization of the parasite, the ribosomal DNA (ITS1, ITS2) and mitochondrial COI were amplified and sequenced. Based upon both morphological data and molecular analysis using bioinformatic tools, the metacestode is identified as confirmed to be representing *Cysticercus fasciolaris*. The adult form of which (*Taenia taeniaeformis*) commonly occurs in felid and canid mammalian hosts.

Keywords: metacestode; rodent; internal transcribed spacer; ribosomal DNA; polymerase chain reaction

Background:
In the state of Mizoram (Northeast India) the rodent outbreaks are intertwined with the onset of bamboo flowering, causing a tremendous destruction to food crops. This gregarious bamboo flowering, which is known locally as 'Mautam', occurs periodically after every 48±1 years and since the last confrontation with the flowering was in 1959, it was again expected during the period 2007-2009. About 31% of the total forest area of Mizoram is covered by bamboo forests, of which about 90% is covered by *M. baccifera* alone, which has been flowering since the year 2003. Although various measures have been taken to control outbreak of rodents in Mizoram, exhaustive studies on their parasites have never been undertaken. In view of their underlying threat as serious pests of crop plants and also as reservoir of zoonoses, a study on the parasite of rodent hosts in Mizoram was undertaken. Studies on the phylogeny of tapeworms (Eucestoda) have achieved considerable progress and the ribosomal DNA (rDNA) clusters have been used for genetic studies **[1].** PCR techniques that utilize the second internal transcribed spacer (ITS2) sequences and mitochondrial coding region COI,have been used to be a reliable tool in identifying the platyhelminth parasite species and their phylogenetic relationships **[2-11].** Mitochondrial DNA has minimal non-coding DNA and no introns and it has been used in taxonomic studies as it is a rapidly evolving genome as compared to nuclear DNA **[12].** For the design of oligonucleotide primers which are used for amplification of variable region of the genome conserved coding regions are

ideal and the cytochrome oxidase subunit1 (COI) gene quiet useful for studying closely related species.

Cestodes of the family Taeniidae are parasites of carnivore animal and human hosts which use mammals as their intermediate hosts where the larval stage develops in the tissues causing significant harm to the host. As they are of great medical and veterinary significance several studies have been focused at the species level **[13-15].** Approximately 40 species of the genus *Taenia* have been recognized based on morphological studies of the adult specimen **[16].** Phylogenetic relationships of taeniid cestodes have been constructed using morphology, host specificity and biological traits. However it is still difficult to come up with a reliable conclusion on the phylogenetic relationship between the members of Taeniidae **[17].** However, recent studies have shown high level of interspecific variations for molecular characters, which are useful for characterization of species of *Taenia* and many new taeniid species have also been reported using more recent molecular biological techniques **[18-20].** *Taenia taeniaeformis* is a taeniid cestode parasite, the adult form of which is found in cats and other carnivores and uses rodents as the intermediate hosts where the larval form or the cysticercus develops as a fluid filled larva in different organs **[21-23].** Taxonomic revisions of Taenia spp have been done based on morphological criteria **[24].** During an exploratory survey of rodent pests (burgeoning coincident with bamboo-flowering times in forests of Mizoram Northeast India) for their helminth parasite spectrum, metacestodes of cyclophyllidean tapeworms were frequently encountered infecting the liver lobes and body cavity of the host. The present study aimed at identifying this larval form based on morphological studies supplemented with molecular characterization.

Methodology:

Rattus rattus the commonly prevalent rodent species in the region were collected and examined. The parasites were found encysted in the liver tissues. The capsules were opened by making a small slit to release the parasite. The recovered parasites were processed for morphological studies following standard procedures of fixation and stained whole mount preparation. Measurements of the specimens were taken using stage and ocular micrometers and/or morphometric software in the image analyser (Leica DM 1000).

Note: The Nucleotide sequence data reported in this paper have been submitted to the GenBank with the accession numbers FJ939133-FJ939135.

Scanning electron microscopy (SEM)

The specimens were fixed in 10% neutral buffered formalin (NBF) and processed as per the protocol described elsewhere **[25].** The gold coated specimens were observed using LEO 435 VP scanning electron microscope at electron accelerating voltages between 10 and 20 kV.

Molecular characterization

The genomic DNA of the parasite was isolated and amplified following standard procedure as described earlier **[26]** using the universal trematode primers of *Schistosoma* species **[27].** These include ITS1: BD1 (forward) and 4S (reverse); ITS2: 3S (forward) and A28 (reverse); CO1: JB3 (forward) and JB4 (reverse). The PCR amplification was performed following the standard protocol with minor modifications as described elsewhere **[28-29].** The PCR product was purified and sequenced in both directions on an automated sequencer. The sequences were submitted to GenBank for obtaining their accession number. The Sequences obtained were analysed using bioinformatic tools such as Basic Local Alignment Search Tools (BLAST), ClustalW and the extent of variation was compared by doing pairwise alignment of the nucleotides. Initially the sequencesc were aligned using ClustalW multiple alignment with the default gap and extention penalties used by this program. The phylogenetic trees of the metcestodes were constructed using distance and character based method in MEGA 4.0 **[30].** Branch support was given using 1000 bootstrap replicates.

```
  1 aaatttttgg tttttgtatt gtgtggatgc ctgggtgtgt gtgagtcaga gggcagcaaa
 61 ggagtgaatt aatttaatac agattgattt gtacattacc ctcttgactt ccattgcgtc
121 cataggctcg cgtctggcca tgtctgtgct agtgtcgtat aataaaatat cacttagcgt
181 aacgtgcagt ggctcgggcg attcccttcc ctgcccgcct ctggtcgtgt gcggtgctgt
241 cctgtgcggt gcgtttcagt gcatgtcatg tagtgtaagg ttgacggtgc tgccgaggac
301 agggcagtac aggtcagctg tgctgtcctg tgctgtgctg tgcagtgctg tgctgcgttg
361 cgttgcactc ctggtcgtcc tatccacaag acggtggata ggggctgtat ggagtggcgc
421 attgtgtgtc gcgcttgtgc ggtagtggtg cttgcagttc acggatcgtg gcccagtgag
481 tggtggtgtg gtgctggcgc gattggcttt gtcagttatt cgcgtctgta gtgcacgcgc
541 acgtgaagtg gtctgcgcgg gtgcgcccac ctaggcggtt gggcagcggc agcggcagcg
601 gcattaatag cagcggcgga ggcgcaccgt gtttgggtta gggtgtgctg atgcagcatc
661 tacaacagca acagcaacaa acagcagtaa caactgacct cggattaggc gagagtaccc
721 gctgaacata agcatatcaa taat
```

ITS2

```
  1 agagatatgt tttttttttg ggtggggggg gggggtgtg tgagcagggg gggggtgttt
 61 tgtcttcctt cttcgcgcgc gtgtccgtct ctctctcttt ctgtgtgtgt gtgtgtgtgc
121 gcgggggagt gggagcccgg tccatcccgg gggcggaggg gtttatacac atgcgcgcgc
181 gattttgtgt gtgtgtgtgc gtgtgtgcga ggcgtaagat agtgatggat gcgacggttg
241 tgcagttgcg tccgtccgcg ccccatcatg tgtcatgtgt ttggtgtagt ggtgatggtt
301 gtgcgtgagt gtaaggcgat gtgaagtgga gaagtggaat ggcatggcaa taactgtgcg
361 cggtggatca ctcggctcgt gtgtcgatga agggtgcagc caactgtgag aattagtgtg
421 tagtagccag aggtgctctc gta
```

ITS1

```
  1 gtatcatgta acactttatc taaaacacaa gccgataata ctataccagt aaccccacca
 61 aacgtaaaca gaataataaa agaaacaatt catcataaaa caggatcact ctttttgact
121 cgagcattca acaacatata caatcatgta aaaaccttta tacctgtagg tactccaata
181 atcatagtta tagagctaaa aaacacagcc gtctttacat ctaacccaac agtaaacata
241 tgatgacctc acacccttct tcctaaacaa actatagaaa acatagcaaa caataaacca
301 taaaaaccaa acacatccga agacatacta attcttaaac atatatgtct aataatacca
361 aatccaggaa gaat
```

CO1

Figure 1: ITS1, ITS2 and COI sequence of Taenia taeniaeformis

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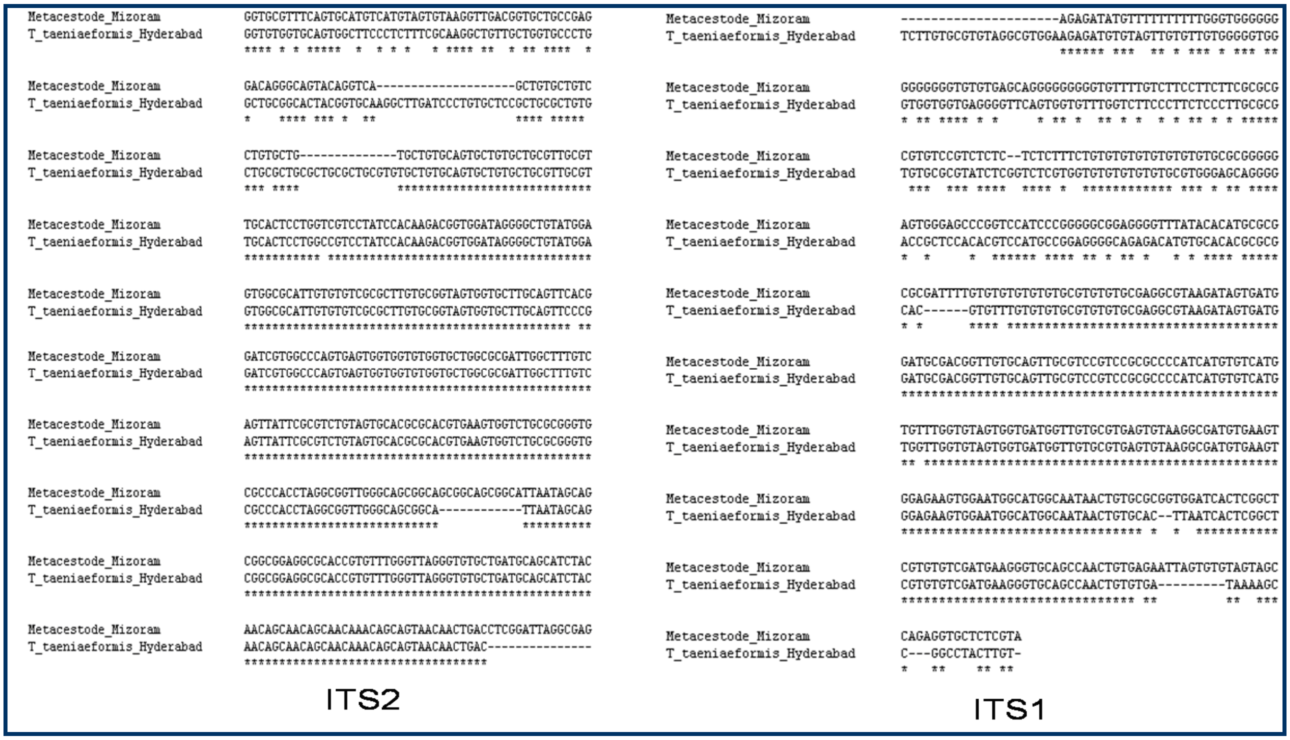

Figure 2: Pairwise Alignment of ITS2 and ITS1 sequences of Taenia taeniaeformis

Figure 3: Multiple alignment of CO1 sequences of Taenia taeniaeformis geographical isolates

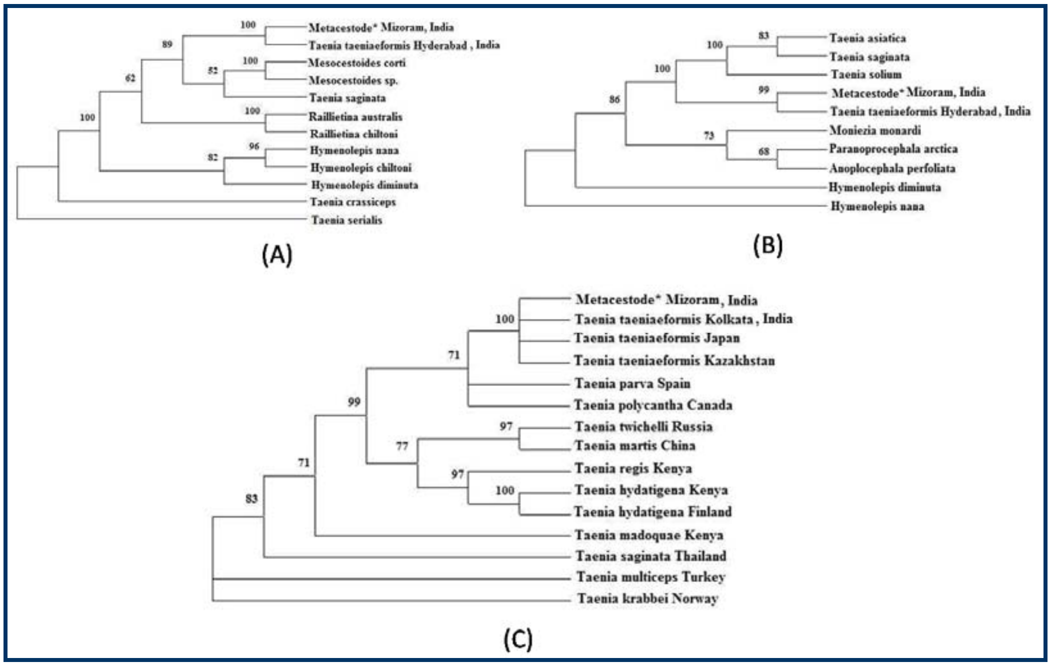

Figure 4: MP phylogenetic trees of the metacestode for- **(A)** ITS2 **(B)** ITS1 **(C)** COI. The numbers on the branches refer to bootstrap values.

Results:

Morphological Analysis

The morphological examination of the parasite revealed typical taeniid features: body measuring 0.9-9.6 cm in length; large scolex 0.8-1.6 mm long and 1.1-2.09 mm wide, with four prominent lateral suckers; rostellum armed with double rows of 34 - 42 hooks, the outer larger hooks 0.36-0.42 mm in length, the smaller inner hooks 0.23-0.27 mm, all hooks typically taenoid type having long blunt handle with sharp pointed blade; and short or elongated and segmented strobila terminating with a bladder, thus resembling a small tapeworm but without reproductive organs **(Figure. 5).** All these morphological characters are in consistence with those of *Cysticercus fasciolaris*, the adult form of which is *Taenia taeniaeformis*.

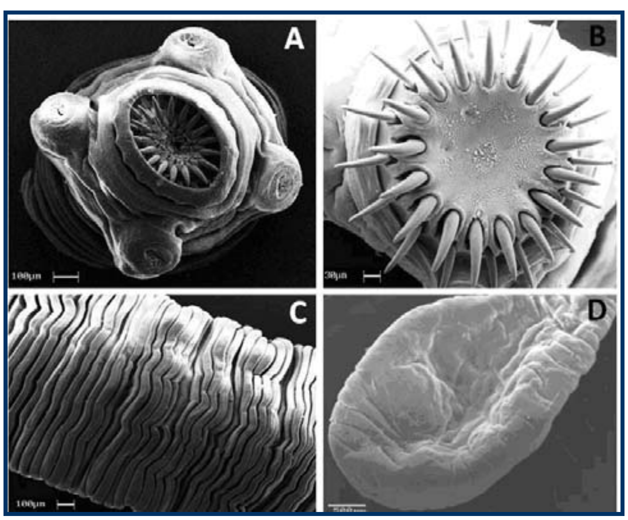

Figure 5: SEM **(A-D)** view of the metacestode. **(A)** Scolex with hooks and four lateral suckers; **(B)** Double rows of hooks on the scolex; **(C)** A portion of the Strobila showing segmentation, suggestive of proglottidization; **(D)** The terminal bladder like end

Molecular Analysis

The rDNA ITS and mtCOI regions of the metacestode were successfully amplified using primers as mentioned above (**Figure. 1).** PCR amplification of ITS regions and the mtCOI showed a single band of size 744bp, 443bp and 374bp, respectively **(Figure. 6).** The nucleotide sequences obtained from the PCR products were put to BLAST and compared with other available cyclophyllidean cestode sequences from GenBank **(Table. 1 see supplementary material).** The BLAST hits result shows that the sequences of the metacestode are closer to those of species of *Taenia,* with maximum similarity to *Taenia taeniaeformis*. In pairwise alignment of the ITS2 and flanking regions of the query sequences with the sequences of *Taenia taeniaeformis* from Hyderabad India isolate shows the presence of 6.4% mismatches. Similarly, with regard to ITS1 pairwise alignment of query sequences with the sequences of *T. taeniaeformis* from Hyderabad India shows 14.3% mismatches **(Figure. 2),** multiple alignment of COI of query sequences with three different isolates shows the presence of 2.1% mismatches with no gap **(Figure. 3).** Phylogenetic trees were obtained by comparing the ITS and mtCOI sequences of the metacestode with other cyclophyllidean cestode species using the NJ and MP methods **(Figure. 4).** The topology of the trees obtained through both the methods emerged to be quiet similar placing both the *Taenia taeniaeformis* and the query sequences in the same clade; giving high bootstraps values of 90% and above. Bootstsrap value of 99% in the tree constructed for ITS1 and 100% for ITS2 with *T. taeniaeformis* obtained from Wistar rats indicates perfect phylogenetic accuracy and reliable grouping. In the trees constructed for COI the query sequences are placed in the same clade with *T. taeniaeformis* isolates from Hokkaido (Japan),

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Kazakhstan and Kolkata (India) showing high bootstrap values of 100%.

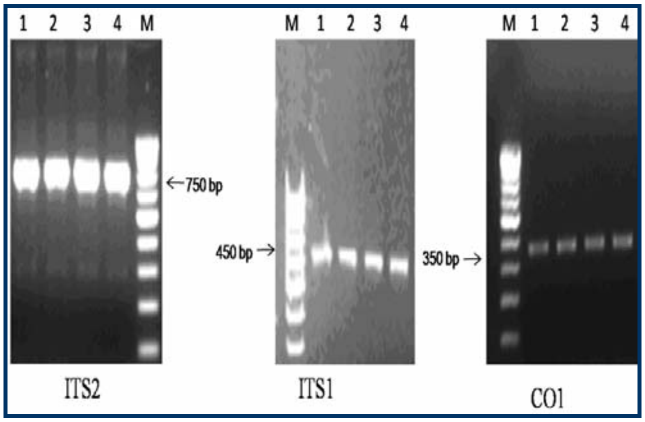

Figure 6: PCR products of metacestode DNA using different primer sets (M) = 100bp DNA ladder

Discussion:

The LM and SEM observations clearly shows the presence of double rows of hooks and four lateral suckers which are the distinguishing characters for *T. taeniaeformis.* The size and the number of hooks are important features for identification of Taeniidae species. The numbers of hooks reported in the metacestode under study were also found to be in confirmity with those of *T. taeniaeformis* **[31].** Ribosomal DNA or the rDNA clusters has also been widely used for taxonomic studies. Mitochondrial genes have also been used to study phylogenetic relationships as they have fast evolutionary rate **[32-33].** In analysis of the sequences of the rDNA ITS2 and ITS1 and the mtCO1 and comparing with the so far known sequences of other cyclophyllidean cestodes, the sequence of ITS and CO1 showed close similarity with the sequence of *T. taeniaeformis* showing high bootstrap value of 90% and above. If the bootstrap value is 70% or higher than the topology at that branch is considered reliable or correct **[34].** Intraspecific variations of four different isolates of *T. taeniaeformis* by using several criteria such as morphology of the parasite, infectivity, protein component of the parasites and restriction fragment length polymorphism of the parasite DNA have also been reported earlier by **[35].** In the present study when pairwise and multiple alignment of the query sequences was done with different isolate only a few mismatches and gaps were seen. On the basis of the morphological similarities with earlier studies supplemented by close matching of the ITS and the mitochondrial CO1 sequences of the metacestode under study with *T. taeniaeformis* it can be concluded that the parasite found in the liver cysts of rodents in the study area is indeed the metacestode of *T. taeniaeformis* the adult of which occurs in the various carnivorous animals.

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Supplementary materials:

Table 1: Taennid species and their various geographical isolates used in the study with respective GenBank accession numbers.

Name of parasites	Host	Geographical Isolate	Accession No. ITS2	ITS1	CO1
Metacestode*	*Rattus rattus*	Mizoram, India	FJ 939133	FJ939134	FJ939135
Taenia taeniaeformis	Wistar rat , *Apodemus sylvaticus* , *Rattus norvegicus* , *Rattus rattus*	India , Kazakhstan, Japan	EU 051352	EU051351	EU544597, AB221484, EF090612
Taenia asiatica	PMD-3 Clone	Taiwan		AY606272	-
Taenia saginata	*Bos indicus*	China , Thailand	AY825542	AY392045	AB465239
Taenia solium	*Sus scrofa domestica*	Kolkata, India		EF090614	
Taenia crassiceps	*Mus* sp.	USA	DQ099564	-	-
Taenia serialis	*Canis latrans*	USA	DQ099574		-
Taenia hydatigena	*Canis familiaris* , *Rangifer tarandus*	Kenya, Finland	-	-	AM503316 , EU544552
T. Regis	*Panthera leo*	Kenya	-	-	AM503330
T. Twichelli	*Gulo gulo*	Russia	-	-	EU544598
T. martis	*Myodes rufocanus*	China	-	--	EU544558
T. madoquae	*Canis mesomelas*	Kenya	-	-	AM503325
T. multiceps	*Ovis aries*	Turkey	-	-	EF393620
T. krabbei	*Vulpes lagopus*	Norway	-	-	EU544579
T. polyacantha	*Lemmus trimucronatus*	Canada	-	-	EU544595
T. parva	*Apodemus sylvaticus*	Spain	-	-	EU544580
Hymenolepis diminuta	Rodents , *R. rattus*	Australia , India	FJ939132	AF461125	-
Hymenolepis nana	Rodents , *Mesocricetus auratus*	Australia , Uruguay	AB494477	AF461124	-
Hymenolepis microstoma	Laboratory rodents	Japan	AB494478	-	-
Raillietina australis	*Dromaius novahollandiae*	Australia	AY382317	-	-
Raillietina chiltoni	*Dromaius novahollandiae*	Australia	AY382319	-	-
Mesocestoides corti	*Canis familaris*	USA	AF119696	-	-
Mesocestoides sp.	*Canis familaris*	USA	AF119697	-	-
Moniezia monardi	*Capricornis crispus*	Japan	-	AB367791	-
Paranoplocephala arctica	*Dicrostomyx spp.*	Finland	-	AY299558	-
Anoplocephala perfoliata	*Equus ferus caballus*	Germany	-	AJ578151	-

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