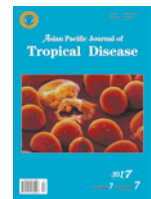


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Morphological and molecular characterization of *Gordius*, a horse hairworm recovered from cricket

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ABSTRACT

Objective: To describe the morphological and molecular characterization of a newly isolated *Gordius* spp. from of the northeastern states of India.

Methods: Identification of the parasite was based on light microscopy and scanning electron microscopy. For molecular identification a partial sequence of ITS2 region was amplified. The product was cloned in cloning vector and subsequently phylogenetic analysis was done.

Results: Based on the morphological and molecular analysis, the nematode infesting cricket was confirmed to be *Gordius* species. Grossly the length of those nematodes ranged between 15.1 and 35.5 cm. The present study also described the light microscopy and fine structural morphological study of the isolated species. Molecular amplification targeting ITS2 region of *Gordius* was found to be approximately 1048 bp. Phylogenetic analysis revealed that the *Gordius* species is a close relative (100%) to Malaysian species.

Conclusions: Occurrence of *Gordius* species in Indian cricket has been confirmed and found 100% similarity with Malaysian species.

1. Introduction

Horsehair worm or sometimes called *Gordius* worms are endoparasites of arthropods under the class Nematomorpha. Juvenile stages are obligatory parasites but the adults of *Gordius* worms are free living found in lakes, ponds, and running water[1-2]. A total of 300 species of different parasites of Nematomorpha have been on record and out of these, 22 species fall under the genera *Gordius*[3]. There are few reports of the occurrence of Nematomorpha in frogs, fishes, bird and even in humans[4-7].

Definite hosts get the infection when they ingest parasitic larvae of *Gordius*. Developing Nematomorphs can grow from microscopic larvae to a large worm whose size exceeds the host

length. At this stage they are ready to emerge from the host. There have been reports that mature Nematomorphs alter the behavior of their terrestrial insects making them find water and jumping into it. They are white in colour when first emerging from the host body, turning to yellowish tan to brownish-black after a while. The worms often squirm and twist themselves into a loose, ball-like shape, resembling the Gordian knot.

To date, there is no record of Gordioidean fauna from cricket in India. The present study describes *Gordius* sp. (Nematomorpha: Gordida) found in the body cavity of crickets in Mizoram India. For morphological identification, the worms were studied under light microscopy. The morphological classification was reinforced on the basis of molecular analysis.

2. Materials and methods

A total of 100 crickets were examined from different districts of Mizoram, India from February 2016 to January 2017. Out of 100 crickets 10 were found positive for the horse hairworms. The

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liberated worms were thoroughly washed in physiological saline and were placed into two groups; One group ($n = 5$) was preserved in 70 % alcohol for light microscopic study and another group ($n = 5$) washed 4–5 times with 0.2 mol/L cacodylate buffer (pH 7.3) and fixed in 2.5% glutaraldehyde at 4 °C for 24 h for scanning electron microscopy (SEM). The fixed samples were washed in phosphate buffer saline (pH 7.2) for three times and then in double distilled water followed by acetone dehydration.

After acetone dehydration, the specimens were dried with liquid carbon dioxide at its critical point (*i.e.* 3.5 °C at 11 P.S.I). The specimens are then immersed in tetramethylsilane for 5–10 min for two changes at 4 °C. Then they were brought to room temperature (25–26 °C) to dry. The samples were mounted on aluminium stubs. The parasitic specimens were then gold coated in a sputter coat and finally examined under SEM [(JSM-6360-JEOL)] at the North Eastern Hill University (NEHU), Shillong, Meghalaya, India in Sophisticated Analytical Instrument Facilities (SAIF) Laboratory.

Genomic DNA was extracted from the Gordian worm by using a DNeasy® blood and tissue kit (Qiagen, Alameda, California USA) as per manufacturer's instructions. Primers were designed using the online tool where several published sequences were utilised from *Gordius robustus* (*G. robustus*), *Gordius albopunctatus*, *Gordius attoni* and *Gordius aquaticus*. 18s rRNA region spanning internal transcribed spacer 2 (ITS2) gene (DNA) was targeted to amplify with the following oligonucleotide sequences: 5'-AGAATTCCTCCAGTAACGGCGAGTG-3' (forward) and 5'-GACGATCGATTGCACGTCAGAACC-3' (reverse).

The primer set was designed to amplify at 1048 bp length. The optimum condition for polymerase chain reaction (PCR) was set for initial temperature at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 10 min. PCR reaction was performed in a BioRad C1000 thermal cycler. PCR product of 5 µL was analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide and visualized on under gel documentation system.

The PCR products were purified from agarose gel using QIA quick PCR purification kit (Qiagen) and subsequently cloned in TA cloning vector (pTZ57R/T, Thermoscientific).

Transformation of the recombinant pTZ57R/T plasmid was done in *Escherichia coli* DH5 cells and sent for custom sequencing. DNA sequences were analyzed by BLAST and multiple sequence alignment by clustal W. Molecular phylogenetic study for Mizoram isolate of *Gordius* was done by using MEGA 6.0, software (<http://www.megasoftware.net>) and compared with isolates from different countries. Bootstarp analysis of 1000 replicates were applied and values were given at relevant nodes of the constructed tree.

All experimental procedures are conducted in according to Institutional Animal Ethic Committee vide grant no. CVSc/CAU/IAEC/no. 6641, dtd, Selesih, the 25th, April, 2016.

3. Results

Grossly, the size of the parasites ranges from 15.1 to 35.5 cm (Figure 1A) and their colour is light brown (Figure 1B). The anterior end of the mouth part is rounded with dark colour and



Figure 1. Length of the parasite (A), light brown coloured parasite (B) and anterior end of the parasite (C).

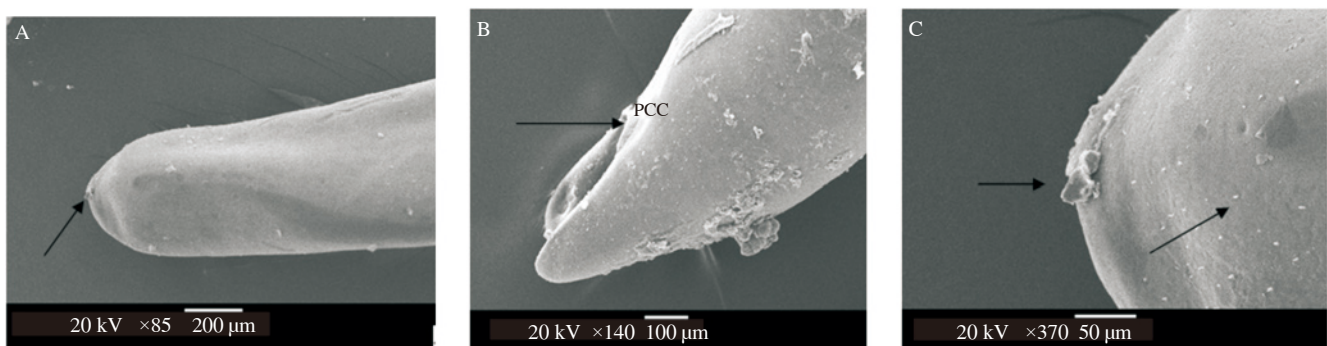


Figure 2. Anterior end (A), posterior part of male (B), and posterior end of female (C). PCC: Post-cloacal crescent.

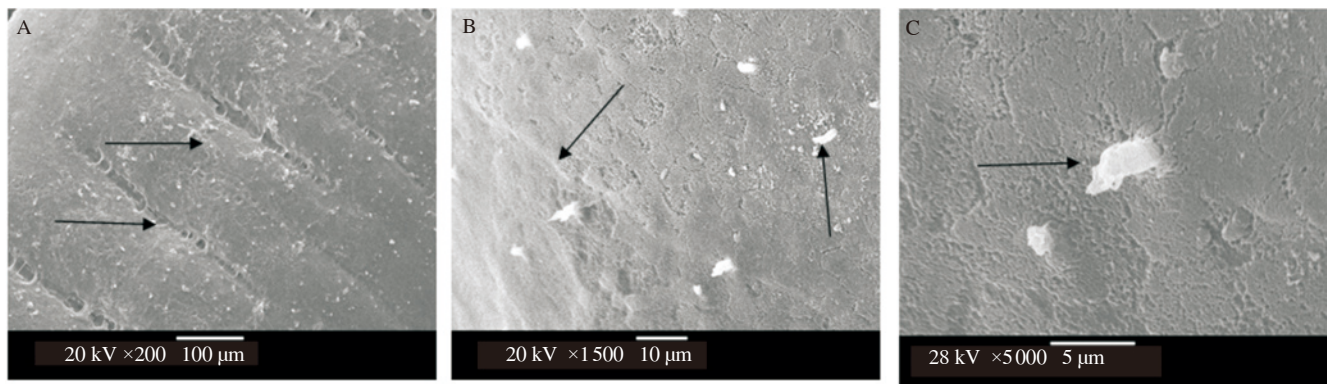


Figure 3. Cuticular elevation and grooves (A), spines with cuticular elevation and grooves (B), and prominent spine on the cuticle (C).

is opened at the centre (Figure 1C). Under SEM, the cuticular structure is found to be divided of cuticular elevation and spines are seen in the anterior part of the body and the posterior part of the male has two lobes with smooth cuticle (Figure 2A–C). Spines are difficult to see in the mid part of the body (Figure 3A–C).

The ITS2 region of 18s rRNA of *Gordius* species was amplified, and the PCR products were cloned in TA cloning vectors and custom sequenced. The sequence compared with *G. robustus*, *Gordius attoni*, *Paragordius tricuspoidatus*, *Paragordius rautheri* and *Chordodes morgani*, respectively. An approximately at 1048 bp of the worm is amplified by conventional PCR (Figure 4) and by colony PCR (Figure 5). The sequence of the present *Gordius* species shows 100% similarity with Malaysian *Gordius* sp. In addition to this, partial ITS2 sequence of the *Gordius* species has low gap difference of nucleotide.

The sequence of the current *Gordius* species identified genetically close to the other sequences available in the data bank for *Gordius* species (KM 382417, KM 382411, KM 382407, KM 382409, KT 202299, AY 210817, AY 863410, AY 863410, KM 382416), *Paragordius* species (AF421771, AF421770) and *Chordodes* species (AT421763, AF036639) (Figure 6).

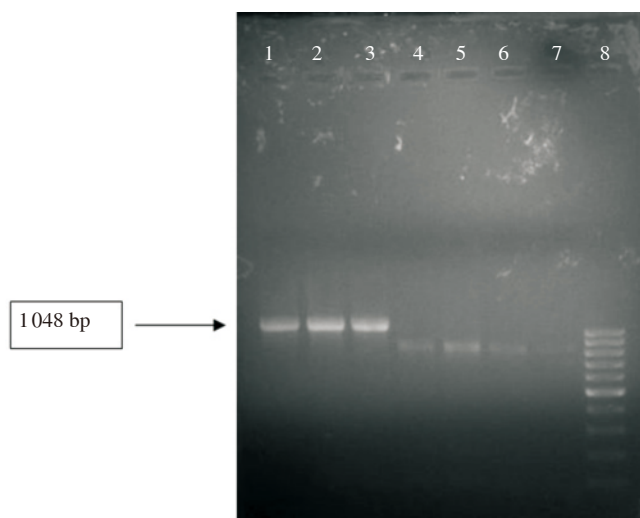


Figure 4. Gel picture of *Gordius* sp. conventional. PCR product in 1% agarose gel. Lanes 1–3: Positive amplification; Lanes 4–7: Negative result; Lane 8: 100 bp marker.

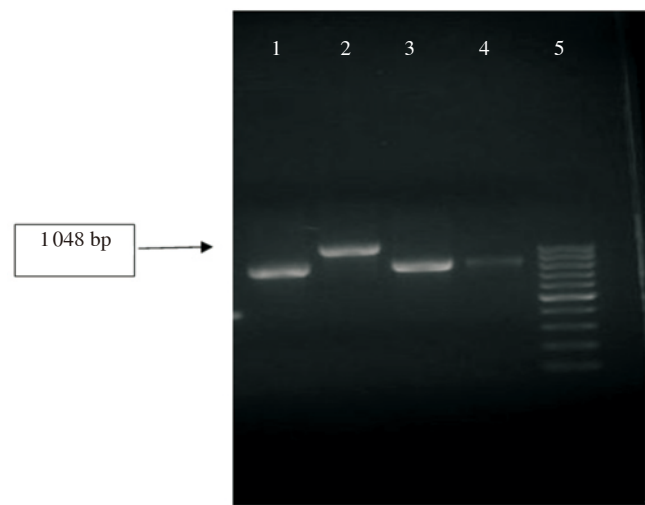


Figure 5. Gel picture of *Gordius* sp. colony. PCR product in 1% agarose gel. Lane 1, 3 & 4: Negative result; Lane 2: Positive amplification; Lane 5: 100 bp marker.

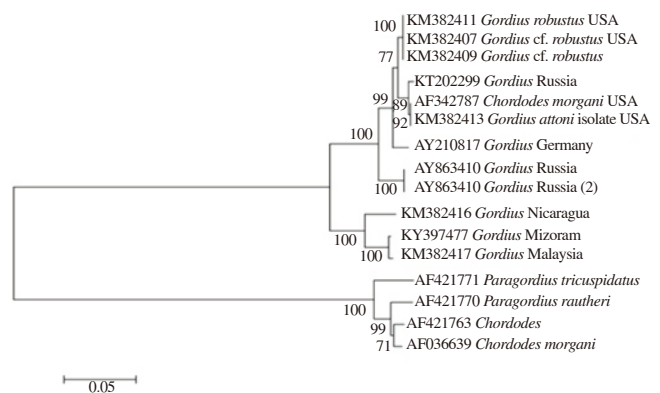


Figure 6. Phylogenetic tree.

4. Discussion

Horse hairworms closely resemble in shape to worm-like body of other worms such as certain nematodes and annelids, but they have some unique physical characteristics that place them in the phylum Nematomorpha. Horsehairworms are mostly enigmatic and because of their large size and sinister in appearance, they draw people's attraction. Hairworms possess some morphological

traits by which specimens are separated. One of the most useful characters is the cuticular structures which vary greatly interspecifically and intraspecifically[8,9].

In the present *Gordius* sp. cuticular ornamentations and cuticular plates are not observed. *G. robustus* of North American species is also devoid of such structures as reported earlier[10,11]. These findings suggest that occurrence or non-occurrence of cuticular structures can be seen within closely related hairworms and this must be taken into account when interpreting taxonomic groupings among Gordioidea.

SEM shows post-cloacal crescent which is semicircular and two distinct tail lobes in the male tail. The same structure is also observed by other researchers[12,13]. One cuticular inflation is seen on the large lobe in the present species which is not recorded by earlier researchers. In contrast to male tail, the female tail appears smooth without lobe or any cuticular inflation.

Sequence of the ITS2 region is used to study inter and intra-specific relationships as it is highly repeated and contains variable nucleotides flanked by more conserved regions. ITS2 regions are also used for diagnosis of different organisms at species level[14].

We compared the Mizoram isolates of *Gordius* with the sequences from the gene bank and found both Malaysian and our isolated species had 100% similarity. This suggests that both species might have similar origin. The divergence value (71%–92%) shown by *Gordius* sp. of USA might be due to diverse evolution as well as the geographical separation.

From the study we can conclude that, genus *Gordius* can be easily identified using light and SEM studies. Amplification of ITS2 region is successful in differentiating horse hairworms. However, sequencing alignment and phylogeny is not sufficient enough to ascertain the correct species. Genetic divergence of *Gordius* species can be ascertained by complete sequencing of full length ITS regions.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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