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Molecular Phylogeny of Nematodes (Oxyurida: Travassosinematidae) from Orthoptera (Gryllotalpidae) Inferred by Mitochondrial Cytochrome C Oxidase Subunit 1 Gene

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Abstract:
In this study, we sequenced mt Cox 1 gene sequences of five nematode spp. that were infective to arthropod, *Gryllotalpa africana*. The nematode belongs to Thelastomatoidea, a group of pinworms that parasitizes only invertebrates. Currently, in India spp. of this group are distinguished mainly on the basis of morphological characters that present possible confusions. Therefore, we identified the species through morphological and genetic analysis. We selected mt Cox 1 gene region to show their phylogenetic position with closely related spp. and confirmed their molecular validation. The present findings are important to confirm the phylogenetic position and relationship among five nematode spp. and avoid misidentification regarding their validation, as it is more necessary in that case when many species harbours the same host.

Keywords: Nematoda, Thelastomatoidea, Cox 1, *Gryllotalpa africana*, India

Background:
Thelastomatoidea is one of the two superfamilies belonging to order Oxyurida which include nematodes that parasitizes invertebrate animals, mainly arthropod and live within the hindgut of the host **[1].** It includes family Travassosinematidae, which have been reported worldwide **[2-10].** The Travassosinematids are mainly found in mole crickets **[11-13].** So far, in India, *Gryllotalpa africana* prevalent **[14, 15]** including other countries and morphological adaptations enable it to live in a warm climate like in India. The mole cricket is characterized by its brownish colour, shape of adult male pronotum, trochanter, fore wing, transverse sclerite and epiphallus. Although, *G. africana* is more closely related to *G. orientalis* but can be easily separated from it by body color, characters of posterior tibiae, forewing and base of transverse sclerite. During a survey of entomopathogenic nematodes of *G. africana* in Meerut (U.P.), India, five thelastomatoid nematodes belonging to four genera were found. The five species belong to genera, *Binema* **[16]**, *Chitwoodiella* **[17]**, *Isobinema* **[18]**, and *Mirzaiella* **[17].** Morphological diagnosis of the species, collected from the same host is very difficult because many of the characters that serve to distinguish them, sometimes overlap. In that case, where different species harbours same host, morphological identification requires most reliable characters. Identification of species using DNA sequences is the base for molecular taxonomy. Therefore, the identification of these five species needs to be favoured by molecular tools as these are very useful for validation of nematode species **[19-21].** Regarding the spp. of Travassosinematidae, little attention has been paid to use of mitochondrial genes despite the fact, that mtDNA evolves very quickly in closely related species of nematodes **[22, 23].** It has been proved useful in phylogenetics

because of its maternal inheritance and rapid rate of divergence **[24].** For phylogenetic relationships of nematodes, mitochondrial genes have been frequently used **[25, 26]** specifically Cox1 has been effective to resolve the phylogenies **[27, 28].** The mtDNA Cox1 gene sequence enables the discrimination of closely allied species including the nematodes **[29].** This study is needed as it will help in the further phylogenetic analysis of these different nematodes species and also in resolving the existing taxonomic confusion at genus and species levels. The finding of this study will also form a base line for the subsequent workers on this group of nematode systematics.

In this study, we obtained mt Cox 1 sequence data for five nematode spp. collected from India to analyse the phylogenetic position and relationships among nematode species.

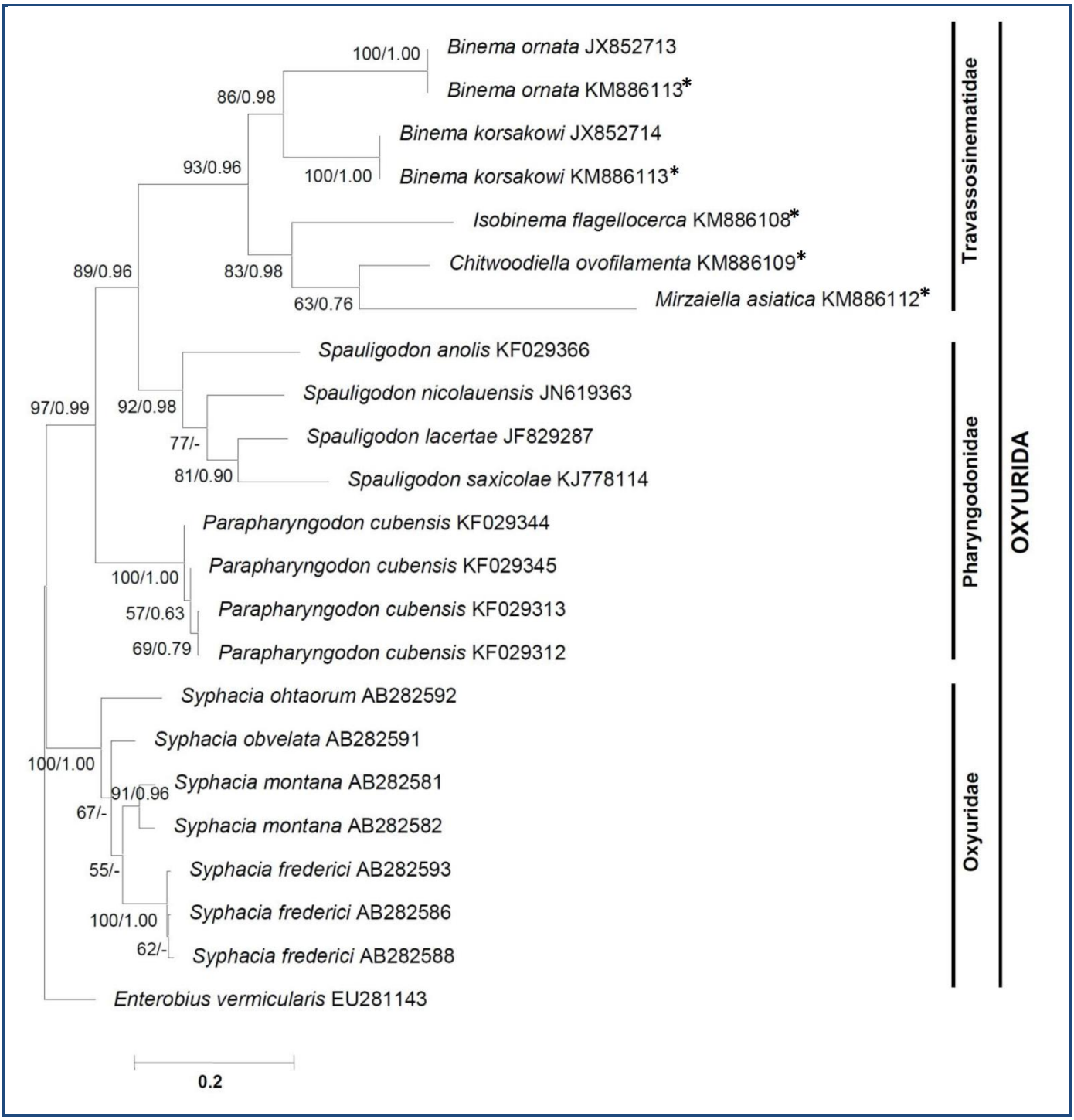

Figure 1: Maximum likelihood tree for representatives of the Travassosinematidae in relation to other closely related nematode species available in GenBank. Scale bar shown number of substitutions per site. Posterior probabilities for Bayesian inference are given behind the bootstrap values for ML. -: unsupported node by BI. GenBank accession numbers are indicated following the species name. *E. vermicularis* was used as outgroup. The newly generated sequences are indicated by an asterisk.

Methodology:

Sample collection and morphological study

G. africana found in Meerut (U.P.), India (29° 01′ N, 77° 45′E) were placed in individual vials in the field and dissections were performed in laboratory under stereoscopic microscope with the insects submerged in saline water in petridish. Nematodes were collected from intestine of the host and were fixed for morphology (in hot 70% alcohol) and molecular studies (in 95% alcohol). For morphology, the nematodes were first gradually cleared in glycerin and identified using a light microscope equipped with differential interference contrast and digital image analysis system (Motic digital microscope for Windows) according to existing keys and descriptions. Voucher specimens are deposited in Museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut (U.P.), India.

Molecular methods

Genomic DNA was isolated using the DNeasy Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. PCR was used to amplify the mt Cox 1 region using primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HC02198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) **[30].** The PCR reactions (in 25 µl) were performed under standard conditions **[31].** Subsequently, PCR products were purified using Purelink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) following the manufacturer's instruction. Amplicons were sequenced (in both directions) using the Big Dye Terminator version 3.1 cycle sequencing kit in ABI 3130 Genetic Analyser, Applied Biosystems with the same primers. For nucleotide sequence analysis was done by using the BLAST available in the NCBI database (http:/ / www.ncbi.nlm.nih. gov). All sequences of nematodes generated in this study were aligned and compared with one another and with those sequences of Cox1 of other nematodes available in the GenBank. The Cox 1 sequences of closely related species were downloaded and used for further analysis. Multiple sequence alignment were made with the the Clustal W **[32]** implemented in MEGA 6 **[33]** with default parameters. DNA pairwise distances were calculated using p-distance model.

Maximum likelihood (ML) and Bayesian inference (BI) analysis were performed to determine the phylogenetic position of analysed five species. The dataset was tested using MEGA 6.06 for the best fit nucleotide substitution model, shown Akaike Information Criterion (AIC) as one that best fits was chosen. ML analyses were performed in MEGA 6 with GTR+G+I model. To support topology, it was tested by bootstrapping over 1,000 replications. A Bayesian inference (BI) analysis was computed by Topali 2.5 **[34].** The substitution models were tested by the Bayesian Information Criterion and GTR+G+I was chosen. Posterior probabilities searches for 1 000 000 generations via two independent runs of four simultaneous MCMCMC chains with every 100th tree saved. The first 25% of the sampled trees for each dataset were discarded as 'burn in'. The ML tree was visualised using the tree explorer of MEGA 6. *Enterobius vermicularis* (EU281143) was used as outgroup in the final alignment. The sequences of the Cox 1 mt DNA fragment obtained were ranged from 589 to 700 bp submitted to GenBank under the following accession numbers: *Isobinema flagellocerca* (KM886108), *Chitwoodiella ovofilamenta* (KM886109), *Mirzaiella asiatica* (KM886112), *Binema ornata* (KM886113) and *Binema korsakowi* (KM886114).

Results & Discussion:

Travassosinematidae specimens analyzed in this study were morphologically identified as *I. flagellocerca, C. ovofilamenta, M. asiatica, B. ornata* and *B. korsakowi*. Measurements were taken and compared with those described originally the species **Table 1 (see supplementary material) .**In BLAST search, five spp. studied showed all closest match with nematodes of the family Pharyngodonidae, but none had closer than 85% similarity. This low similarity observed, might be due to lack of mitochondrial sequence data from the family Travassosinematidae on database. The phylogenetic analyses resulted with bootstrap values for ML and BI in tree. The generated ML and BI represented similar topologies, therefore, to depict the topology of tree, only ML tree is presented **(Figure 1)** with the posterior probabilities for BI. As shown in figure **(Figure 1)**, the present five species were differ phylogenetically from each other. The sequences obtained from two species of *Binema* species (KM886113, KM886114) corresponded 100% with sequences of *B. ornata* (JX852713), *B. korsakowi* (JX852714) and formed clades with high bootstrap support **(Figure 1)**. The phylogenetic position of *I. flagellocerca* was sister to *C. ovofilamenta* and *M. asiatica* with maximum similarity of 75% to 73% respectively **(Figure 1)**. The mt Cox 1 sequence of *C. ovofilamenta* (KM 886109) also showed the closest similarity with *I. flagellocerca* (75%) and *M. asiatica* (81%) **(Figure 1)**. The DNA sequence of *M. asiatica* is also most closely related to *Chitwoodiella* (81%) and *Isobinema* (73%) spp. respectively **(Figure 1)**. All the above species belonging to family Travassosinematidae, formed a separate clade and show that they are closely related to each other.

Several parasitological examinations from India have been conducted on the basis of morphology of travassosinematids. Majority of nematode species infecting *G. africana* have been described only by the morphology except few studies **[35, 36].** The descriptions available were based on the morphology of mouth parts, oesophagus structures, tail, male and female reproductive organs and sometimes were not sufficiently informative. Therefore, for precise identification, support of molecular analysis is necessitated. Attempts are now being made to implement molecular analyses as approaches which should provide an effective means to characterize the nematode species **[37].** The BLAST search revelaed that all five species sequences reported in this study showed most similar with *Spauligodon, Parapharyngodon* and *Syphacia* species (85-75%) as all of these belong to order oxyurida. On the basis of molecular data, all the above species of Travassosinematidae are distinguished from each other by their mt Cox 1 sequences. The molecular result and phylogenetic analysis **(Figure 1)** also supported the identification of these five species of travassosinematids, *I. flagellocerca, C. ovofilamenta, M. asiatica, B. ornata* and *B. korsakowi*. All were placed within the same clade along with high bootstrap values. Mitochondrial Cox 1 sequences have potential for DNA based identification and diagnosis of nematode species. The genetic examination in the presented study using Cox 1 mt gene, reveals a close relationship with the members of other Pharyngodonidae.

Phylogenetic analysis study by Singh *et al.* **[31]** for *Binema* species provided phylogenetic trees with the same topology as observed during the present study. Except spp. of *Binema*, all other species were sequenced first time in this study. The precise relationship of travassosinematids is still not very clear and it might be assessed in the future with more sequences from other genera of this group.

Conclusion:

The data presented here will facilitate future research on these important nematode parasites of India. For the species of family Travassosinematidae, further work is necessitates to fully understand the affinities of these species with detail morphological features using SEM and should be supplemented by molecular data from more genes. Our study demonstrated that mt Cox 1 gene was useful for determining the relationships among nematode species. We suggest that further phylogenetic analysis of a range of species of Travassonematidae will elucidate more compherensive relationship that will provide robust taxonomic data.

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Conflict of Interest:

Authors declare that they have no conflict of interests.

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Supplementary material:

Table 1: Comparison of the measurements of present species collected in this study with the original descriptions.

Body features	***B. korsakowi* (Sergiev, 1923) Basir, 1956**	***B. korsakowi* present observation (n=6)**	***B. ornata* (Travassos, 1925) Basir, 1956**	***B. ornata* present observation (n= 8)**	***C. ovofilamenta* (Basir, 1948) Basir, 1956**	***C. ovofilamenta* present observation (n=7)**	***I. flagellocerca* Rao, 1958**	***I. flagellocerca* present observation (n=4)**	***M. asiatica* (Basir, 1942) Basir, 1956**	***M. asiatica* present observation (n=10)**
Body Length	1.35-4.10	2.01-2.16	2.5 to 3.5mm	3.09-3.20	1.11-2.15	2.0-2.08	2.82-4.09	2.80-3.59	2.25-2.98	2.40-2.81
Body Width	230µ	0.29-0.30	225µ	0.29-0.31	200 µ	0.20-0.27	0.34-0.36	0.32-0.34	400µ	0.240-0.515
Buccal cavity	-	11 µm - 14 µm x 9 µm - 12 µm	20 µ x 10µ	0.015-0.03 x 0.017-0.02	10 µ wide	0.055-0.06 x 0.008-0.01	-	0.009-0.010 x 0.037-0.040	85µ x 25-30 µ	0.034-0.04 x 0.027-0.033
Oesophagus	420 µ	0.38-0.40	345µ	0.35-0.36	300 µ-475 µ	0.41-0.44	0.35-0.45	0.35-0.45	650 µ	0.64-0.71
Corpus	310 µ x 30 µ	27-29	240 µ x 300µ	0.24-0.25	225-370 µ x 30 µ	0.29-0.31	0.273-0.315	0.281-0.340	530 µ x 55 µ	0.53-0.57
Isthmus	10 µ x 20 µ	0.01	15 µ x 24 µ	0.01-0.02	22-25 µ x 20 µ	0.012-0.015	0.015-0.021	0.009-0.010	10 µ x 30 µ	0.006-0.010
Oesophageal bulb	100 µ x 105 µ	0.10-0.11 x 0.12-0.13	90 µ x 90 µ	0.09-0.10 x 0.09-0.11	60-80 µ x 60-85 µ	0.09-0.11 x 0.09-0.11	0.078-0.086 x 0.084-0.098	0.079-0.100 x 0.080-0.110	110 µ x 120 µ	0.10-0.121 x 0.13-0.125
Nerve ring from the anterior end	150 µ	0.13-0.16	130 µ	0.16-0.17	105-180 µ	0.13-0.16 (0.14 ± 0.01)	-	0.10-0.19	290 µ	-
Excretory pore from the anterior end	-	0.52-0.56	520 µ	-	-	-	-	-	560 µ	-
Vulva	1.61	1.23-1.33	1.8 mm	1.95-1.97	1.25 mm	1.21-1.24	1.8-2.2	1.92-2.10	1.67	1.51-1.74
Anus	-	0.22-0.24	15 µ	0.11-0.13	150-290 µ	0.43-0.47	-	0.21-0.27	200 µ	0.15-0.19
Tail	-	0.19-0.21	-	0.10-0.12	-	0.42-0.45	0.21-0.256	0.20-0.26	-	0.16-0.18
Eggs	59 µ x 64 µ- 34 µ x 40 µ	0.05-0.06 x 0.03-0.04	56 µ -62 µ x 32 µ -36 µ	0.05-0.06 x 0.03-0.04	80 µ x 40 µ	0.07-0.08 x 0.045-0.05	0.054-0.060 x 0.030-0.036	0.062-0.080 x 0.034-0.050	66-70 µ x 42-45 µ	0.060-0.062 x 0.04-0.05