

## New molecular data for parasites *Hammerschmidtella indicus* and *Thelandros scleratus* (Nematoda: Oxyurida) to infer phylogenetic position

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**Abstract:** A phylogenetic study of nematode species belonging to Nematoda: Oxyurida from India has been conducted using molecular characters. Molecular marker 18S rRNA (18S) from the nuclear gene was tested and analyses were conducted using the minimum evolution, maximum parsimony, and maximum likelihood methods. Phylogenetic analysis revealed that both of the species *Hammerschmidtella indicus* and *Thelandros scleratus* clustered as sister species with species of *Thelastoma* and *Leidynema*, and *Parapharyngodon*, respectively. Interestingly, the results confirm the taxonomic status of *H. indicus* and *T. scleratus* from India.

**Key words:** *Hammerschmidtella indicus*, *Thelandros scleratus*, rDNA, 18S, Meerut, India

### 1. Introduction

Nematodes of the order Oxyurida parasitize both invertebrate and vertebrate hosts. This order consists of 2 superfamilies: those parasitizing vertebrate hosts belong to the superfamily Oxyuroidea, and those parasitizing invertebrate hosts belong to the superfamily Thelastomatoidea. During a survey of nematode parasites in Meerut, Uttar Pradesh, India, we found 2 species of oxyurids belonging to *Hammerschmidtella* and *Thelandros* from 2 different hosts.

The genus *Hammerschmidtella* was established by Chitwood (1932) for the worms described by Hammerschmidt (1838) as *Oxyuris diesingi* and contains more than 10 species. Several studies on *Hammerschmidtella* species associated with host *Periplaneta americana* have been conducted in India on the basis of morphology (Biswas and Chakravarty, 1963; Singh and Kaur, 1988; Singh and Malti, 2003; Shah, 2007).

On the other hand, *Thelandros scleratus* Travassos, 1923 is an intestinal parasite of the Brook's house gecko, *Hemidactylus brooki* Gray, 1845. *Thelandros* parasites are commonly found in both carnivorous and herbivorous reptiles from India (Gupta and Kumari, 1968; Singh et al., 2003). In India, parasite identification based on molecular criteria is a less common practice in comparison to other regions, and only a few studies using molecular tools have been carried out on parasitic nematodes (Chaudhary et al., 2011; Kumari et al., 2011). PCR methods are widely used to identify parasites because of their accuracy and specificity.

Nematode identification could greatly benefit from the use of molecular tools, as these may provide more reliable estimates of nematode diversity and their validation. Small subunit (SSU) rDNA has been widely used for studies of nematode phylogenetic analysis, including studies within the main nematode clades (Fitch, 1997; Van Megen et al., 2009; Bhadury et al., 2010; Blanco et al., 2012; Callejón et al., 2013; Singh et al., 2013; Zhang et al., 2014). The present study's goal was to evaluate the molecular phylogeny of the nematodes by using sequences of the 18S ribosomal gene. These sequences were compared with publicly available sequences of nematodes to contribute to the knowledge of the species, and to use these sequences to put them in a phylogenetic framework.

### 2. Materials and methods

#### 2.1. Parasites

*P. americana* and *H. brooki* were euthanized and their digestive tracts were examined for the presence of parasites. Nematodes *H. indicus* and *T. scleratus* were collected from the intestines of hosts *P. americana* and *H. brooki*, respectively (04 ♀, 10 ♂ from *P. americana* and 14 ♀, 01 ♂ from *H. brooki*), from Meerut (29°01'N, 77°45'E), Uttar Pradesh, India. Upon dissection, nematodes were removed from the intestine and placed in 0.6% saline solution. They were then collected and fixed in hot 70% alcohol for slide preparation. For the morphological examination, the nematodes were cleared gradually in glycerin. A light microscope (Motic Digital Microscope) was used

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for morphological analysis. The slides were deposited in the museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut, Uttar Pradesh, India, under the voucher numbers Nem/2010/03 for *H. indicus* and Nem/2010/1 for *T. scleratus*.

## 2.2. DNA extraction, amplification, and sequencing

Collected materials were also preserved in 95% ethanol and then processed for DNA isolation. Extraction of genomic DNA for both species was performed on individual female nematodes using the DNeasy Tissue Kit from QIAGEN according to the manufacturer's protocol. The primer set Nem 18S F (5'-CGCGAATRGCTCATTACAACAGC-3') and Nem 18S R (5'-GGGCGGTATCTGATCGCC-3') designed by Floyd et al. (2005) was used for amplification of the region of 18S ribosomal DNA. PCR was performed in a total volume of 25 µL, comprising 1X PCR buffer with MgCl<sub>2</sub>, a mixture of dNTPs (at a concentration of 0.4 mM), primers at 0.8 µL each, 1 U Taq DNA polymerase (Biotoools, Madrid, Spain), and 3 µL of extracted nematode DNA template. The PCR reaction consisted of 1 cycle of initial denaturation at 94 °C for 3 min; 35 cycles at 94 °C for 30 s, 54 °C for 45 s, 72 °C for 1 min; and final extension at 72 °C for 10 min. Amplified products were sent for sequencing to a national facility (Chromous Biotech Ltd.), and sequences were obtained for both directions with the same primers.

## 2.3. Phylogenetic analysis

DNA sequences were subjected to further analysis with the help of bioinformatics tools, including similarity search using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST>). Alignment between nucleotide sequences obtained from this study and similar 18S sequences of nematodes was performed with the ClustalW multiple alignment program. Pairwise distances between sequences were calculated; the distance matrix was elaborated using the Kimura-2 parameter model (Kimura, 1980). Phylogenetic trees were reconstructed using MEGA version 5.0 (Tamura et al., 2011), based on the methods of minimum evolution (ME), maximum parsimony (MP), and maximum likelihood (ML). The MP tree was obtained using the close-neighbor-interchange algorithm. Robustness of the inferred phylogeny was assessed using a bootstrap procedure with 1000 replications.

## 2.4. Sequence data

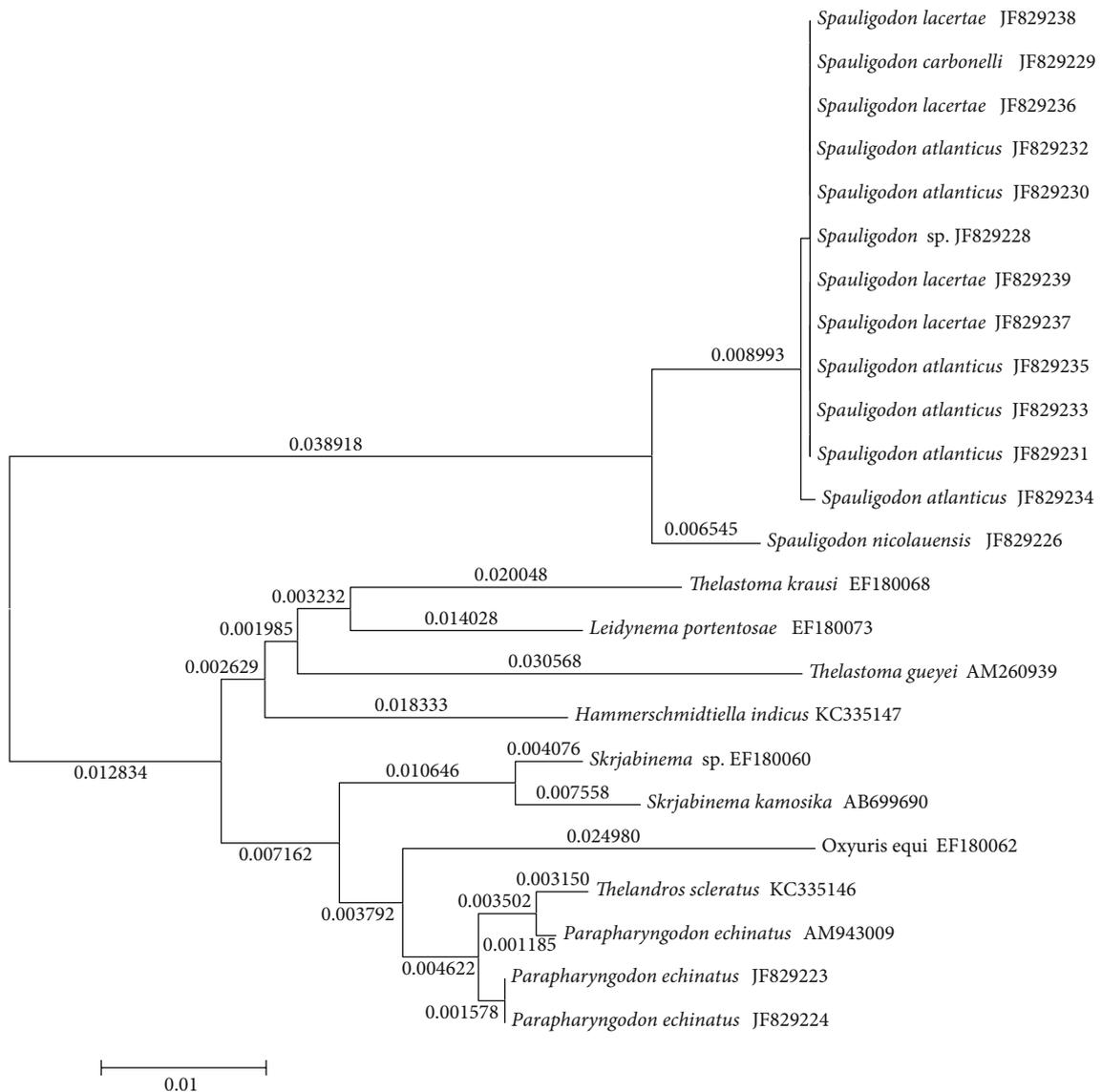
The SSU rRNA sequences of *H. indicus* and *T. scleratus* contained 770 and 750 bp, respectively (GenBank accession numbers KC335147 and KC335146).

## 3. Results and discussion

Nucleotide sequences of 18S of *H. indicus* and *T. scleratus* were queried in the National Center for Biotechnology Information (NCBI) database (<http://blast.ncbi.nlm.nih.gov/>). On the basis of the BLAST search, we chose

sequences closely related to the queried sequences for phylogenetic study. Regarding the similarity to 18S rRNA sequences of *H. indicus*, the closest score match was 95% with other members of thelastomatoids. The closest score match for *T. scleratus* was 99% with *Parapharyngodon* species *P. echinatus* (AM943009, JF829223, JF829224), 97% with *Oxyuris equi* (EF180062), 96% with *Skrjabinema* species (EF180060, AB699690), and 92% with the species of *Spauligodon* (*S. lacertae*, *S. carboneli*, *S. atlanticus*, *Spauligodon* sp., and *S. nicolauensis*). Phylogenetic trees of 18S sequences showed similar groupings using the different methods employed. The ME tree showed a well-resolved distinct clade for the species *H. indicus* and *T. scleratus* with other members of the nematode species belonging to the families Thelastomatidae and Parapharyngodonidae, respectively (Figure 1). Both MP and ML analyses inferred similar topology; thus, only the ML tree is shown (Figure 2).

The present investigation thus provides strong molecular evidence of the validation of *H. indicus* and *T. scleratus* distributed in India. *H. indicus* grouped with the other nematode species of genera *Leidynema* and *Thelastoma* (Figures 1 and 2). The SSU rRNA gene of *H. indicus* shares significant sequence identity with species of the same family, Thelastomatidae, i.e. *L. portentosae* (95%), *T. gueyei* (95%), and *T. krausi* (95%). The presence of these parasites has previously been documented based on morphological and morphometric analyses in India (Singh and Malti, 2003; Singh et al., 2003). However, morphological characteristics can cause misidentification, as when Chitwood (1932) erected the genus, he indicated that the female reproductive system was amphidelphic. Later, Adamson and Van Waerebeke (1992) diagnosed the genus and indicated that it was prodelphic. The 18S rDNA sequence of *H. indicus* shows 95% identity with related species such as *L. portentosae*, *T. gueyei*, and *T. krausi* and provides strong molecular evidence. Additionally, this study provides sufficient information for the validation of thelastomatoids at the genus level. *H. indicus* was found to be closely related to other species of thelastomatoids harbored by the same host, *P. americana*. Only 2 (including the sequence generated from this study) sequences are available in GenBank from the genus *Hammerschmidtella*. For *Leidynema* and *Thelastoma*, only 1 and 3 sequences respectively are available on NCBI. Hence, further molecular studies with more molecular data from this group of parasites would be important to evaluate their validity for being in the family Thelastomatidae. The close relationship between the genus *Hammerschmidtella* and *P. americana* suggests that this genus of nematode has been spread worldwide via the dispersal of its host, including to Germany, Brazil, India, Malaysia, and Poland (Leibersperger, 1960; Kloss, 1966; Khairal and Paran, 1977;



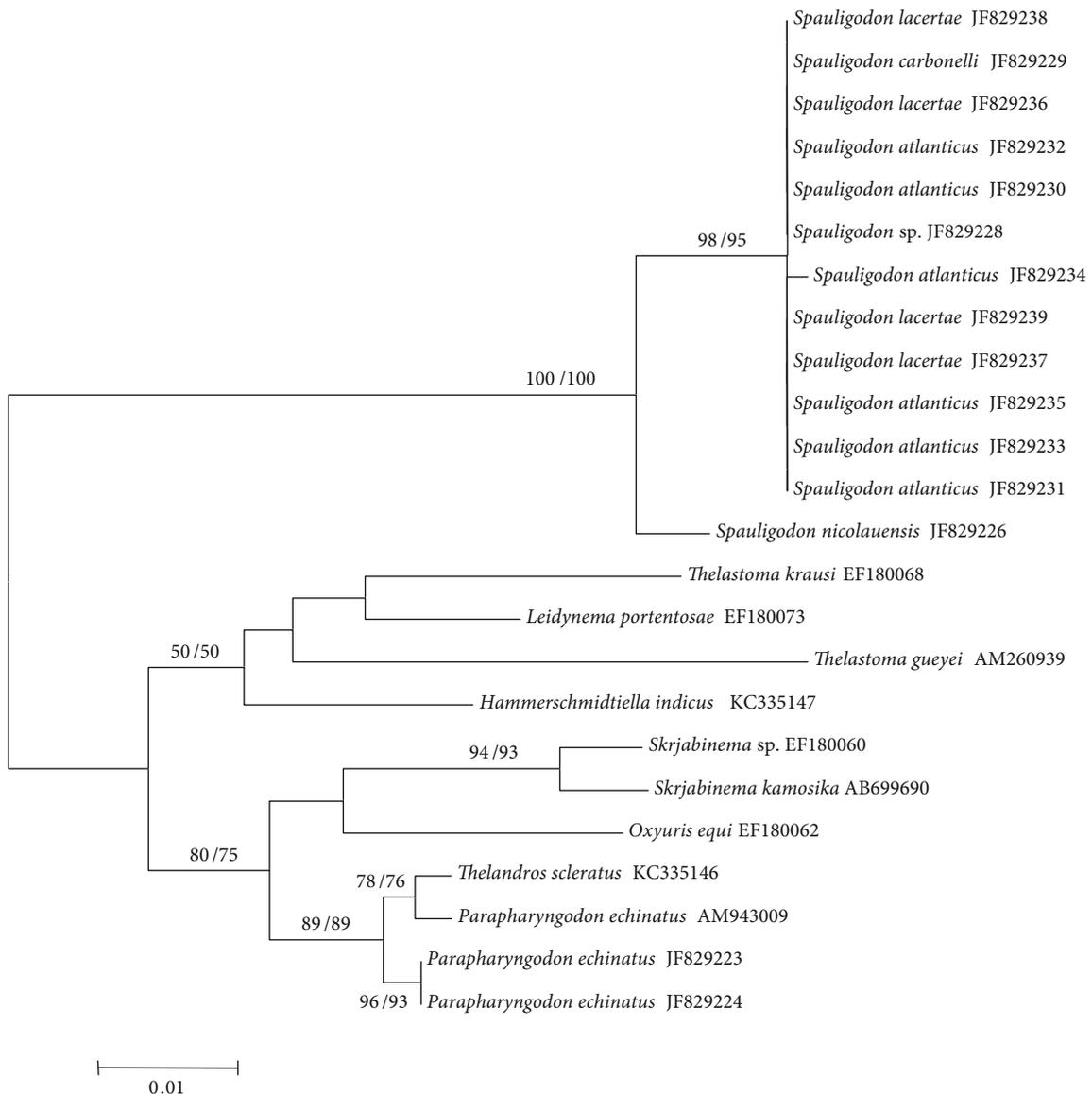
**Figure 1.** A phylogenetic tree based on the 18S rDNA sequences was constructed by using the ME method. The evolutionary distance values are indicated at the nodes. The GenBank accession number for each sequence is given adjacent to the name of the corresponding species.

Gabryelow and Lonc, 1986; Adamson and Van Waerebeke, 1992; Jain and Kaur, 2001; Shah, 2007).

From the phylogenetic tree inferred from the SSU rRNA gene sequences, *T. scleratus* species grouped in the same clade of *Parapharyngodon* with *P. echinatus* isolates (Figures 1 and 2). The SSU rRNA gene of *T. scleratus* also shares high sequence identity with isolates of *P. echinatus* (99%). Previously, *Parapharyngodon* was considered a synonym of *Thelandros* and the percentage of similarity has proven to show a reliable relationship between them. *T. scleratus* was described by Travassos (1923) from Brazil from the host iguanid *Tropidurus torquatus*. Due to specimens exhibiting close morphological resemblance with *Parapharyngodon*,

Baylis (1936) considered the genus *Parapharyngodon* to be a synonym of the genus *Thelandros* Wedl (1861/62) after its proposal by Chatterji (1933). However, Adamson (1981), Castaño-Fernandez et al. (1987), and Hering-Hagenbeck et al. (2002) considered *Parapharyngodon* to be distinct from *Thelandros*. Identification of *Thelandros* within the family Parapharyngodonidae has previously been based on morphological and morphometric studies.

Molecular methods provided in our earlier study (Kumari et al., 2011) analyzed information from the LSU rRNA gene sequences of *T. scleratus*, but due to lack of data it was hard to validate the status of this species. In order to know more about this nematode, we provide



**Figure 2.** Phylogenetic tree constructed on the basis of maximum likelihood analysis of the 18S rDNA sequences of selected nematodes. Number at nodes indicates the bootstrap values for ML and MP (ML/MP), respectively.

data on its phylogenetics. The percentage of genetic similarity of the sequence of *T. scleratus* with species of *Parapharyngodon* (0.004–0.007) has proven to be a reliable criterion for scoring taxonomic relationships. No sequences of species of *Thelandros* are available in GenBank, with the taxonomically closest being those of *Parapharyngodon*; both are members of the same family (Parapharyngodonidae). This study produced results that by the addition of other taxa related to *Thelandros*, *Spauligodon*, and *Parapharyngodon* could offer a topology that better explains the relations among these genera. To assess deeper or more intensive relationships between the members of the same family, future studies should be further supplemented with more markers.

Therefore, the present approach of molecular methods could serve as a precise tool in parasitological studies. SSU sequences have good potential as informative molecular markers and thus can be used for identification and systematics of parasites. This basic approach should be supplemented further by additional molecular studies for identification of nematodes.

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## References

- Adamson ML (1981). *Parapharyngodon osteopili* n. sp. (Pharyngodonidae: Oxyuroidea) and a revision of *Parapharyngodon* and *Thelandros*. *Syst Parasitol* 3: 105–117.
- Adamson ML, Van Waerebeke D (1992). Revision of the Thelastomatoidea, Oxyurida of invertebrate hosts. I. Thelastomatidae. *Syst Parasitol* 21: 21–63.
- Baylis HA (1936). Nematoda. I. Ascaridoidea and Strongyloidea. The Fauna of British India. London, UK: Taylor and Francis.
- Bhadury P, Austen MC (2010). Barcoding marine nematodes: an improved set of nematode 18S rRNA primers to overcome eukaryotic co-interference. *Hydrobiologia* 641: 245–251.
- Biswas PK, Chakravarty GK (1963). The systematic studies of the zoo-parasitic oxyuroid nematodes. *Z Parasitenkd* 23: 411–428.
- Blanco MV, Lax P, Dueñas JCR, Gardenal CN, Doucet ME (2012). Morphological and molecular characterization of the entomoparasitic nematode *Hammerschmidtella diesingi* (Nematoda, Oxyurida, Thelastomatidae). *Acta Parasitol* 57: 302–310.
- Callejón R, Nadler S, De Rojas M, Zurita A, Petrášová J, Cutillas C (2013). Molecular characterization and phylogeny of whipworm nematodes inferred from DNA sequences of *cox1* mtDNA and 18S rDNA. *Parasitol Res* 112: 3933–3949.
- Castaño-Fernández C, Zapatero-Ramos LM, Solera-Puertas MA, González-Santiago PM. (1987). Descripción de *Parapharyngodon lilfordi* n. sp. (Oxyuroidea, Pharyngodonidae) en *Podarcis lilfordi* (Reptilia, Lacertidae) de las Islas Baleares. *Rev Ibér Parasitol* 47: 275–281 (in Spanish).
- Chaudhary A, Pal S, Malti, Singh HS (2011). Genomic DNA Sequence of *Leidynema appendiculata* from Meerut, U.P., India. *Asian Journal of Animal Sciences* 5: 243–255.
- Chitwood BG (1932). A synopsis of the nematodes parasitic in insects of the family Blattidae. *Z Parasitenkd* 5: 14–50.
- Fitch DHA (1997). Evolution of male tail development in rhabditid nematodes related to *Caenorhabditis elegans*. *Syst Biol* 46: 145–179.
- Floyd RM, Rogers AD, Lambshead PJD, Smith CR (2005). Nematode-specific PCR primers for the 18S small subunit rRNA gene. *Mol Ecol Notes* 5: 611–612.
- Gabryelow K, Lonc E (1986). Parasites of *Periplaneta americana* from laboratory rearing. *Wiad Parazytol* 32: 75–78.
- Gupta NK, Kumari U (1968). Observation on four nematode parasites of lizards. *Res Bull Punjab Univ* 22: 77–88.
- Hammerschmidt KE (1838). Helminthologische Beiträge. *Isis von Oken* 5: 351–358 (in German).
- Hering-Hagenbeck SFBN, Petter AJ, Boomker J (2002). Redescription of some *Spauligodon* spp., and *Parapharyngodon* spp., and of *Skrjabinodon mabyae* (Sandground, 1936) Inglis, 1968 (Pharyngodonidae: Oxyuroidea) from insectivorous South African lizards. *Onderstepoort J Vet* 69: 7–29.
- Jain M, Kaur L (2001). Qualitative and quantitative analysis of nematode parasites of cockroaches from Chandigarh. *J Parasitol Appl Anim Biol* 10: 55–58.
- Khairal AA, Paran TP (1977). Parasites of *Periplaneta Americana* Linn., in Penang, Malaysia. I. *Thelastoma malaysiense*, new species, with notes on *Hammerschmidtella diesingi* and *Leidynema appendiculata*. *Malay Nat J* 30: 69–77.
- Kimura M (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111–120.
- Kloss GR (1966). Revisao dos nematoides de Blattaria do Brasil. *Pap Avulsos de Zool (São Paulo)* 18: 147–188 (in Portuguese).
- Kumari S, Chaudhary A, Malti, Singh HS (2011). Further observation of *Thelandros scleratus* Travassos, 1923 based on 28S rDNA. *J Nematode Morphol System* 14: 63–66.
- Leibersperger E (1960). Die Oxyuroidea der europäischen Arthropoden. *Parasitologische Schriftenreihe* 2: 1–150 (in German).
- Shah MM (2007). Some studies on insect parasitic nematodes (Oxyurida, Thelastomatoidea, Thelastomatidae) from Manipur, North-East India. *Acta Parasitol* 52: 346–362.
- Singh HS, Arya P, Kumar N, Kumar Y (2003). Helminth fauna of wall lizards at Meerut. *J Exp Zool* 6: 195–204.
- Singh HS, Kaur H (1988). On a new nematode, *Hammerschmidtella bisiri* n. sp. from *Periplaneta americana* Linn. *Indian J Parasitol* 12: 187–189.
- Singh HS, Malti (2003). Studies of some nematode parasites of *Periplaneta americana* Linn. at Meerut, Uttar Pradesh, India. *Flora and Fauna* 2: 59–66.
- Singh N, Chaudhary A, Singh HS (2013). Morphological redescription and molecular characterization of three species of Travassosinematidae (Nematoda: Oxyurida: Thelastomatoidea) from *Gryllotalpa africana* Beauv (Orthoptera: Gryllotalpidae). *Bioinformation* 9: 818–823.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739.
- Travassos L (1923). Informações sobre a fauna helminthologica de Mato Grosso. *Folha Medica (Rio de Janeiro)* 4: 58–60 (in Portuguese).
- Van Megen H, Vandenelsen S, Holterman M, Karssen G, Mooyman P, Bongers T, Holovachov O, Bakker J, Helder J (2009). A phylogenetic tree of nematodes based on about 1200 full-length small subunit ribosomal DNA sequences. *Nematology* 11: 927–950.
- Wedl K (1861/62). Zur Helminthenfauna Egyptens. *Sitzungsberichte Mathematisch-Naturwissenschaftliche Akademie der Wissenschaften* 44: 463–482 (in German).
- Zhang SL, Liu GK, Janssen T, Zhang SS, Xiao S, Li ST, Couvreur M, Bert W (2014). A new stem nematode associated with peanut pod rot in China: morphological and molecular characterization of *Ditylenchus arachis* n. sp. (Nematoda: Anguinidae). *Plant Pathol* 63: 1193–1206.