Taxonomic Identification of *Leidynema appendiculata* using Large Subunit (28S) ribosomal DNA Sequence

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Abstract

Molecular markers have often been used for taxonomic identification and phylogenetic analyses in different species groups. Evolution of rDNA is relatively independent of changes in morphology, and analyses of these genetic data have been shown to provide good phylogenetic resolution. So, it was decided to perform a phylogenetic analysis on species of the genus Leidynema appendiculata, based on ribosomal DNA (rDNA) sequences. In the present study, phylogenetic relationships of species of the genus were investigated using nucleotide sequences of the region of 28S rDNA. Phylogenetic analyses were performed for primary sequence data as well as using neighbour-joining and maximum-parsimony approaches.

Keywords*- Leidynema appendiculata, rDNA, 28srDNA, rRNA, Phylogenetic analysis*

Introduction

Morphological identifications are sometimes based on very small and minute differences. Systematics and taxonomy of nematodes have changed substantially after introduction of DNA sequencing and genomic studies (Aleshin *et. al*., 1998; Blaxter *et. al*., 1998; De Ley and Blaxter, 2002 and 2004; Holterman *et. al*., 2006; Meldal *et. al*., 2007 and Zhao and Buckley, 2009). Evolution of rDNA is relatively independent of changes in morphology and analyses of these genetic data have been shown to provide good phylogenetic resolution (Nadler, 1992; Heise *et.al.*, 1995). In fact, several recent studies of eukaryotes used rDNA sequences in phylogenetic analyses to make strong inferences of ancestor descendant relationships when analyses of morphological data only resulted in more unanswered questions (Carmean *et.al.*, 1992; Sidow and Thomas, 1994). In addition, the analysis of rDNA nucleotide sequences has recently been used to access phylogenetic relationships among taxa of both higher and lower organisms (Hillis and Dixon, 1991; Sidow and Thomas, 1994; Halanych *et.al.*, 1995). Choosing the appropriate segment of DNA within the genome of an organism is a critical step in any phylogenetic study (Hillis and Dixon, 1991; Derr *et.al.*, 1992). The rRNA gene that has been used in molecular systematics is the large subunit rRNA gene (28S) and small subunit rRNA gene (18S). The rRNA gene has been shown to be useful in estimating phylogeny because it contains regions that evolved slowly and other regions evolved more quickly. Thus this gene has been selected to infer divergences used rDNA to examine the evolutionary relationships among animal parasitic nematodes.

During the course of study, it was decided to perform a phylogenetic analysis on species of the genus *Leidynema appendiculata,* based on ribosomal DNA (rDNA) sequences. In the present study, phylogenetic relationships of species of the genus were investigated using nucleotide sequences of the region of 28S rDNA and 18S rDNA.The investigator is convinced that the findings of present work will provide a base line for the study of molecular taxonomy of these species and validating their specific status. Review of literature reveals that some taxonomic studies were carried out using molecular tool by Parasitdogists using either 18S rDNA or 28S rDNA. Nadler *et.al,* (2007) studied 18S rDNA contents of *T. krausi.,* Koubkova *et.al,* (2006) worked out 18S rDNA contents of *Thelastoma gueyei.* Spiridonov (2009) worked out 28S rDNA and 18S rDNA of *Leidynema appendiculata*, *L. portentosae*, *Hammerschmidtiella cristata* and *H.diesingi* and more recently, Spiridonov and Guzeeva (2009) studied 28S rDNA contents of *Thelastoma* sp.

Materials and Methods

Parasite was excised out carefully from alimentary canal of *Periplaneta americana* from Meerut (29801'N, 77845'E), U.P., India. Parasite was identified

up to the level of species morphologically using existing taxonomic keys and descriptions. The parasite found is *Leidynema appendiculata* (Leidy, 1850) Chitwood, 1932. For genomic DNA extraction, one specimen of nematode parasite was fixed in either 95% or 100% Ethanol. DNA was extracted from samples using the Qiagen DNeasy Tissue Kit as per the manufacturer's instructions. Polymerase chain reaction (PCR) for the amplification of 28S ribosomal DNA was undertaken using the specifically designed primers. A total volume of 25 µl was used for the PCR reaction. Each reaction contained 10X PCR buffer, 0.4 mM dNTP, 10 pM of each primer pair, 3 µl template DNA, 1 U Taq polymerase (Biotools) and Milli-Q water. The PCR assay was carried out in an Eppendorf Master Cycler Personal for 35 cycles. The amplification profile consisting of 3 min. at 94 ^{0}C, 30 s at 94 ^{0}C, 45 s at 56 ^{0}C and 1 min at 72 ^{0}C, followed by final extension at 72 ^{0}C for 10 min. The PCR products were visualized using ethidium bromide on a 1.5% agarose TBE gel. The products were then purified by Chromous PCR cleanup kit (# PCR 10), according to manufacturer's instructions. Both DNA strands were sequenced using a Big Dye Terminator ver. 3.1 cycle sequencing kit in an ABI 3130 Genetic Analyzer. Same PCR primers were used for sequencing reaction. Primer sequences designed in the study is (**Forward primer 5'- TTGGCGTCTCA GTGTG AAAG-3' and Reverse primer 5'- TTCACCATCTTTCGGGTCTC-3'**). Phylogenetic analysis of 28S rDNA sequence was used to perform the phylogenetic analysis of the sequences. Sequences were uploaded on NCBI to search for the most similar reference sequences and positions of 28S gene were determined with the help of BLAST (available at www.ncbi.nlm.nih.gov). Subsequently, nucleotide sequences of various species were aligned using the aligning tool Clustal W (Thompson *et.al.,* 1994). The sequences were entered in the MEGA for construction of the phylogenetic tree. Data were analyzed using maximum parsimony (MP) and neighbor- joining (NJ) methods by using MEGA version 4.0 (Tamura *et.al*., 2007). Pairwise comparisons were made by using Kimura-2 parameter model (Kimura, 1980). Nucleotide sequences of related sequences and Electropherogram of sequencing sample is also provided in the thesis. The base pair sequence of large subunit of ribosomal DNA of parasite submitted to NCBI GenBank under the accession number **GQ925910** .

Results and Discussion

Leidynema appendiculata (Leidy, 1850) Chitwood, 1932, (Table 1-2 and Fig.1-4). 28S rDNA sequence of *L appendiculata* (Leidy, 1850) Chitwood, 1932, was aligned using the clustal W to perform the phylogenetic analysis. The reference sequences used in this study are listed in Table 1. Pairwise comparisons were made shown in Table 2 using Kimura-2 parameter model (Kimura, 1980). The phylogenetic reconstructions inferred from analysis of the

28S rDNA sequences showed great resolution for the species of the nematodes. The Electropherogram of sequencing sample is also provided as shown in Fig. 1. Sequence similarity searching for *L. appendiculata* was performed using the NCBI BLAST program. Analyses of multiple sequence alignments were done with the help of program, Clustal W (Thompson *et. al.,* 1994). DNA sequences of closely related species were also download and used in the phylogenetic analysis.

The Phylogenetic analysis was performed using MEGA ver. 4.0 (Tamura *et.al.,* 2007). For distance analyses, the Kimura 2-parameter model was used to construct the distance matrix and the trees were inferred from this using the neighbour-joining (NJ) and maximum parsimony (MP) method with a high degree of confidence (Fig. 2 & 3). Bootstrap resampling (1,000 pseudoreplicates) was done and a bootstrap consensus tree produced. Both the methods gave trees with similar topology and approximate relatively bootstrapped values. These sequences were aligned with the 28S rDNA genes and revealed clear differences in nucleotide sequences among different species (Fig. 4).

Table 1. Reference sequences (28S) used in this study, their geographical origins as well as accession numbers (the asterisk is used due to same species name).

Species	Location/Source	Genbank Accession No.
Leidynema appendiculata	India	GQ925910†
Leidynema appendiculata	Russia	EU365630
Hammerschmidtiella cristata	Russia	EU365629
Leidynema portentosae	USA	GQ401114
Cordonicola sp.	Russia	GQ368464
Cordonicola gibsoni*	Australia	AM232758
Cordonicola gibsoni**	Australia	AM232757
Cordonicola gibsoni***	Australia	AM232759
Hammerschmidtiella diesingi	Russia	EU365628
Aoruroides sp.	Vietnam	FJ936558
Thelastomasp.	India	GU968648cies

†Species sequenced in the present study

Table 2. Kimura 2- parameter distances comparison of sequence differences (in %) in the 28S among species (the asterisk is used due to same species name).

L.appendiculata†	*L.appendiculata*	*H.cristata*	*L portentosae*	*C.*sp.	*C.gibsoni**	*C.gibsoni***	*C.gibsoni****	*H.diesingi*	*A.*sp.	*T.*sp.
L.appendiculata†										
L.appendiculata	0.0546									
H.cristata	0.1252	0.0658								
L.portentosae	0.1308	0.0710	0.0876							
*C.*sp.	0.1249	0.0654	0.0936	0.0394						
*C.gibsoni**	0.1194	0.0762	0.0994	0.0445	0.0145					
*C.gibsoni***	0.1422	0.0976	0.1097	0.0704	0.0393	0.0243				
*C.gibsoni****	0.1252	0.0817	0.1052	0.0497	0.0194	0.0048	0.0293			
H.diesingi	0.1424	0.0817	0.0194	0.0932	0.0823	0.0879	0.0982	0.0936		
*A.*sp.	0.1600	0.0979	0.0979	0.0814	0.0704	0.0758	0.0549	0.0812		0.0923
*T.*sp	0.1600	0.0979	0.0979	0.0814	0.0704	0.0758	0.0549	0.0812	0.0923	0.0000

†Species sequenced in the present study

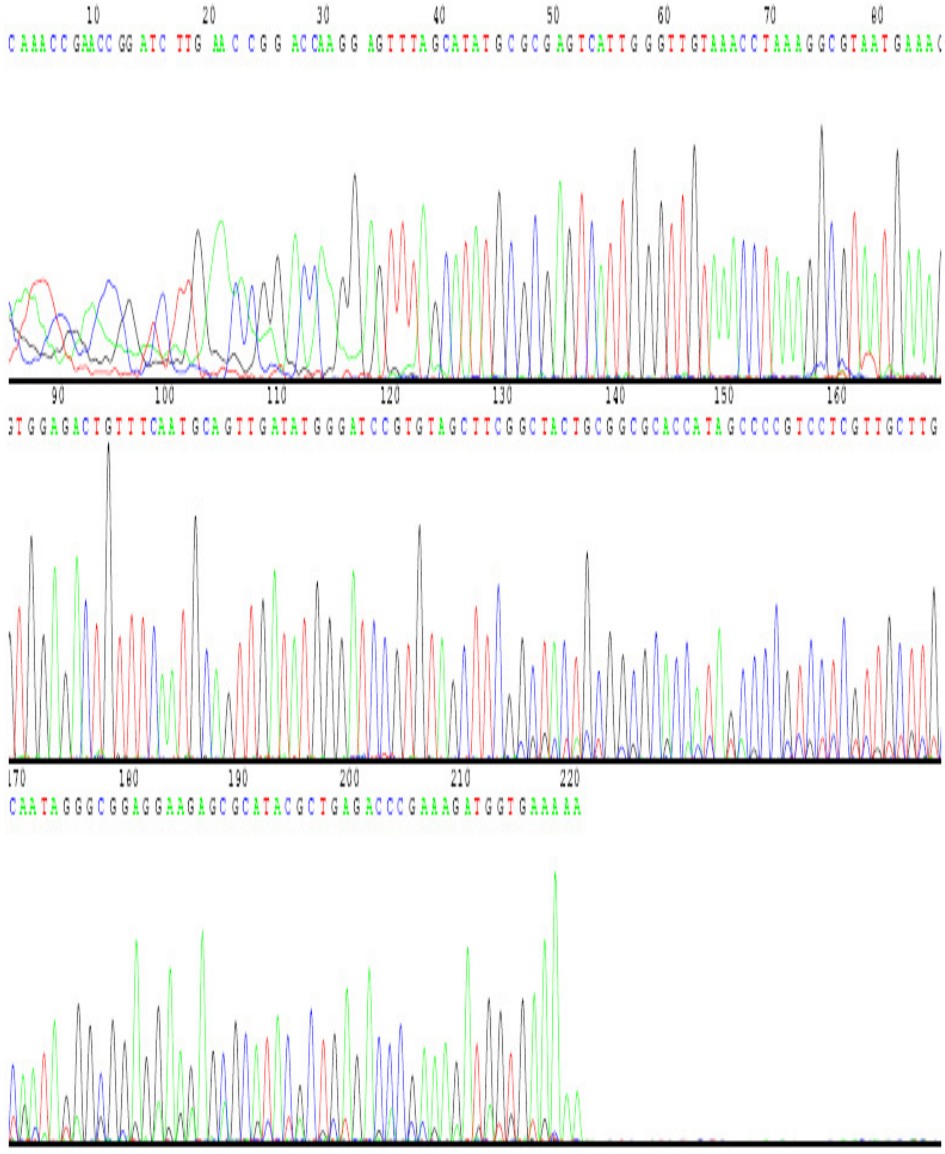

Fig. 1 Electropherogram

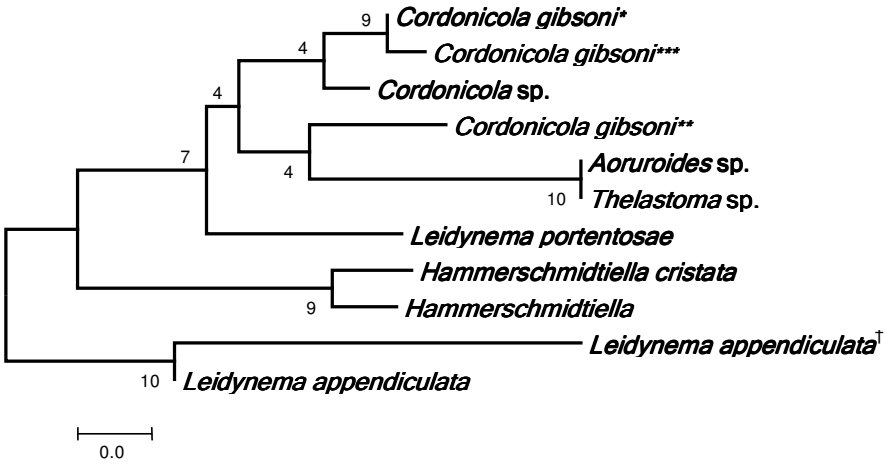

Figure 2. A phylogenetic tree constructed by neighbour-joining method (1,000 bootstraps) for 28S region. Bootstrap values (as percentages) are shown at internal nodes. The scale bar indicates the proportion of sites changing along each branch (the asterisk is used due to same species name). †Species sequenced in the present study.

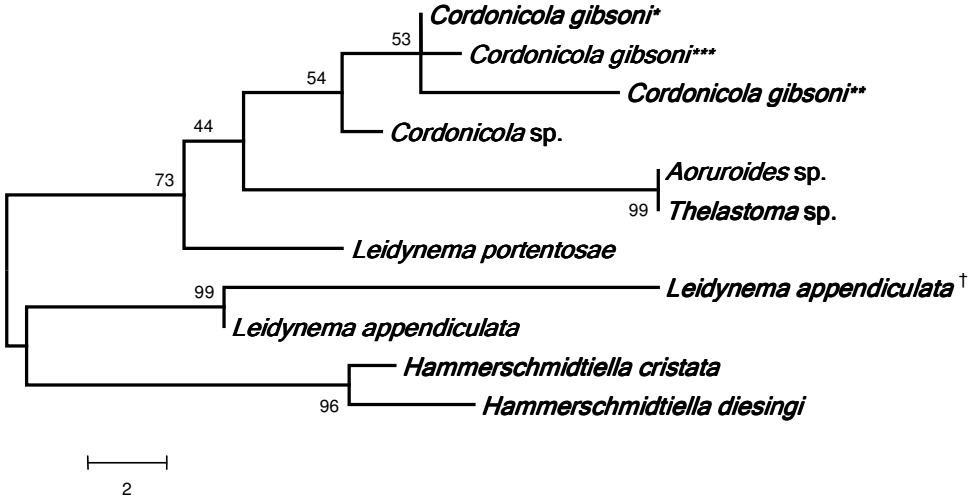

Figure 3. Phylogenetic relationship of the species *L. appendiculata* inferred from the 28S region using the Maximum Parismony (MP) method (1,000 bootstraps). The scale bar indicates the proportion of sites changing along each branch (the asterisk is used due to same species name). †Species sequenced in the present study.

Fig 4. Alignment of 28S sequences for comparative purposes of different species from different geographical locations showed nucleotide identical to *L. appendiculata*. Dots indicate identity with the first sequence and dashes are inferred insertion-deletion. (the asterisk is used due to same species name †Species sequenced in the present study.

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Leidynema appendiculata†     ------CAAA CCGAACCGGA TCTTGAACCG GACCAAGGAG TTTAGCATAT [50]
Leidynema appendiculata      GACCAC.T.T ....C...TC .TGAA.CA.. .......... .......... [50]
Hammerschmidtiella cristata GACCAC.T.T ....C...TC .TGAA.CA.. .......... .......... [50]
Leidynema portentosae        GACCAC.T.T ....C...TC .TGAA.CA.. .......... .......... [50]
Cordonicola sp.              GACCAC.T.T ....C...TC .TGAA.CA.. .......... .......... [50]
Cordonicola gibsoni*         ---------- ---.C...TC .T.GA.CA.. .......... .......... [50]
Cordonicola gibsoni**        ---------- ---.C...TC .T.GA.CA.. .......... .......... [50]
Cordonicola gibsoni***       ---------- ---.C...TC .T.GA.CA.. .......... .......... [50]
Hammerschmidtiella diesingi GACCAC.T.T ....C...TC .TGAA.CA.. .......... .......... [50]
Aoruroides sp.               GACCAC.T.T ....C...TC .TGAA.CA.. .......... ..C....... [50]
Thelastoma sp.               GACCAC.T.T ....C...TC .TGAA.CA.. .......... ..C....... [50]
Leidynema appendiculata†     GCGCGAGTCA TTGGGTTGTA AACCTAAAGG CGTAATGAAA GTGGAGACTG [100]
Leidynema appendiculata      .......... .......... .......... .......... .......... [100]
Hammerschmidtiella cristata .......... ......G... .......... .......... ..AA...T.. [100]
Leidynema portentosae        ....A..... ......G... .......... .......... ..AA...TC. [100]
Cordonicola sp.              .......... ......G... .......... .......... ..AC...TC. [100]
Cordonicola gibsoni*         .......... ......G... .......... .......... ..AA...TC. [100]
Cordonicola gibsoni**        .......... ......G... .......... .......... ..AA...TC. [100]
Cordonicola gibsoni***       .......... ......G... .......... .......... ..AA...TC. [100]
Hammerschmidtiella diesingi .......... ......G... .......... ..A....... ..AA...T.. [100]
Aoruroides sp.               .......... ......G... .......... .......... ..AA...TC. [100]
Thelastoma sp.               .......... ......G... .......... .......... ..AA...TC. [100]
Leidynema appendiculata†     TTTCAA-TGC AGTTGATATG GGATCCGTGT AGCTT-CGGC TACTGCGGCG [150]
Leidynema appendiculata      ......-... .......... .......... .....-.... .......... [150]
Hammerschmidtiella cristata -C.TC.G... .......... .....T.... G...A-.... C.....A... [150]
Leidynema portentosae        C.....-... G......... .......C.. ....G-..... .G........ [150]
Cordonicola sp.              C...T.--.. G......... .......CA. ..T..-..... .G-....... [150]
Cordonicola gibsoni*         C...T.--.. G......... .......CA. ..T..-..... .G-....... [150]
Cordonicola gibsoni**        C...T.--.. G......... .......CA. ..T.--TC.G CTG....... [150]
Cordonicola gibsoni***       C...T.--.. G......... .......CA. ..T..-..... .G-....... [150]
Hammerschmidtiella diesingi GC.TT.G... .......... .....T.... G.T.A-.... C.....A... [150]
Aoruroides sp.               C..T..--.. G......... .......... T.TCGCTT.G CTTC...... [150]
Thelastoma sp.               C..T..--.. G......... .......... T.TCGCTT.G CTTC...... [150]
Leidynema appendiculata†    CACCATAGCC CCGTCCTCGT TGCTTGCAAT AGGGCGGAGG AAGAGCGCAT  [200]
Leidynema appendiculata              .......... .......... .......... .......... ..[200]
Hammerschmidtiella cristata .......... .....T.T.. .......... .......... .......... [200]
Leidynema portentosae        .......... ......CTA. .........A .......... T......... [200]
Cordonicola sp.              .......... ......C... .......... .......... T......... [200]
Cordonicola gibsoni*         .......... ......C... .......... .......... T......... [200]
Cordonicola gibsoni**        .......... ......C... .......... .......... T......... [200]
Cordonicola gibsoni***       .......... ......C... .......... .......... T......... [200]
Hammerschmidtiella diesingi .......... .....T.T.. .......... .......... T......... [200]
Aoruroides sp.               .......... ......C... .......... .......... T......... [200]
Thelastoma sp.               .......... ......C... .......... .......... T......... [200]
Leidynema appendiculata†             ACGCTGAGAC CCGAAAGATG GTGAAAAA       [228]
Leidynema appendiculata               .......... .......... .....CT.      [228]
Hammerschmidtiella cristata          .......... .......... .....CT.       [228]
Leidynema portentosae                .......... .......... .....CT.       [228]
Cordonicola sp.                      .......... .......... .....CT.       [228]
Cordonicola gibsoni*                 .......... .......... .....CT.       [228]
Cordonicola gibsoni**                .......... .......... .....CT.       [228]
Cordonicola gibsoni***               .......... T......... .....CT.       [228]
Hammerschmidtiella diesingi          .......... .......... .....CT.       [228]
Aoruroides sp.                       .......... .......... .....CT.       [228]
Thelastoma sp.                       .......... .......... .....CT.       [228]
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Different studies have demonstrated that the 28S region of nuclear rDNA provide useful genetic marker for the accurate identification of sibling species and morphospecies. Genetic reltaion between the *L. appendiculata* and other species based on molecular data from the 28S rDNA gene sequence indicates closest similarity between *L. appendiculata* from India and *L. appendiculata* from Russia. Both the species are same and shows 99% nucleotide similarity. The reasons for difference of 1% disimilarity between them might be due to different continents and geographical distribution. Molecular phylogenetic analysis of ribosomal 28S rDNA indicates its potential for clarifying species boundaries that are morphologically similar and that occur sympatrically. These findings highlight the utility of the 28S sequence in conjunction with other morphological characters to delineate species boundaries among closely related species. The reasons for difference in genetic similarity between these same species from 2 different continents, remains a challenging question for further investigations. Genetic comparison with other species documented from different parts of the world may alter our taxonomical concept of this group/genus and provide further clues to the understanding of the evolution of the *L. appendiculata*.

Conclusion

This work confirms that for modern identification and understanding of this genus, works should be necessarily accompanied with DNA analyses. Besides this, it is also recommended that the criteria to validate and identify species must be based on morphological characteristics, genetic identification and sequence comparison of genes having taxonomic importance.

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