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Phylogenetic Analysis of the Hoplolaiminae Inferred from Combined D2 and D3 Expansion Segments of 28S rDNA[1]

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Abstract: DNA sequences of the D2-D3 expansion segments of the 28S gene of ribosomal DNA from 23 taxa of the subfamily Hoplolaiminae were obtained and aligned to infer phylogenetic relationships. The D2 and D3 expansion regions are G-C rich (59.2%), with up to 20.7% genetic divergence between *Scutellonema brachyurum* and *Hoplolaimus concaudajuvencus*. Molecular phylogenetic analysis using maximum likelihood and maximum parsimony was conducted using the D2-D3 sequence data. Of 558 characters, 254 characters (45.5%) were variable and 198 characters (35.4%) were parsimony informative. All phylogenetic methods produced a similar topology with two distinct clades: One clade consists of all *Hoplolaimus* species while the other clade consists of the rest of the studied Hoplolaiminae genera. This result suggests that *Hoplolaimus* is monophyletic. Another clade consisted of *Aorolaimus*, *Helicotylenchus*, *Rotylenchus*, and *Scutellonema* species. Phylogenetic analysis using the outgroup species *Globodera rostocheinsis* suggests that Hoplolaiminae is paraphyletic. In this study, the D2-D3 region had levels of DNA sequence divergence sufficient for phylogenetic analysis and delimiting species of Hoplolaiminae.

Key words: 28S, analysis, *Aorolaimus*, clade, D2-D3, *Helicotylenchus*, Hoplolaiminae, *Hoplolaimus*, lance, nematode, phylogenetic, *Rotylenchus*, species, spiral, *Scutellonema*,, taxonomy.

The subfamily, Hoplolaiminae Filip'ev, 1934, belongs to the family Hoplolaimidae Filip'ev, 1934 and is of economical importance because members have a wide host range, a wide geographic distribution, and occur on and damage cultivated crops. Hoplolaiminae is systemically related with the family Heteroderidae Filip'ev and Schuurmans Stekhoven, 1941 (Fortuner, 1991; Siddiqi, 2000; Subbotin et al., 2006). In the Hoplolaiminae subfamily, *Helicotylenchus* Steiner, 1945 and *Scutellonema* Andrássy, 1958 are cosmopolitan whereas *Aphasmatylenchus* sher, 1965 is distributed in few sites of Africa and *Antarctylus* is distributed in limited areas of the Antarctic (Germani and Luc, 1984; Sher, 1973). The genera belonging to Hoplolaiminae are disinguished by several phenotypic traits such as the location, presence, or absence of enlarged phasmids (scutella), whether or not esophageal glands overlap the intestine, and whether the esophageal glands overlap dorsal or ventral. Fortuner (1987) included eight genera in the Hoplolaiminae: *Aorolaimus* Sher, 1963, *Aphasmatylenchus* Sher, 1965, *Antarctylus* Sher, 1973, *Helicotylenchus* Steiner, 1945, *Hoplolaimus* von Daday, 1905, *Pararotylenchus* Baldwin and Bell, 1981, *Rotylenchus* Filipjev, 1936, and *Scutellonema* Andrássy, 1958. Among them, *Aorolaimus*, *Hoplolaimus*, and S*cutellonema* are characterized by presence of enlarged phasmids whereas *Helicotylenchus* and *Rotylenchus* are characterized by normal phasmids.

The D expansion segments of 28S ribosomal DNA (rDNA) have been widely used for resolving phylogenetic relationships at lower and higher taxonomic levels and are also useful diagnostic markers for species identification (Al-Banna et al., 1997; Al-Banna et al., 2004; Duncan et al., 1999; Subbotin et al., 2000). The large subunit (LSU) ribosomal DNA or 28S gene is composed of core segments that are highly conserved across broad taxonomic lineages and variable regions described as divergent D domains or expansion segments (Hillis and Dixon 1991). The coexistence of variability and conservation within the 28S gene makes this region more suitable for estimation of phylogenetic relationships because sequence variation provides phylogenetically informative characters while the conserved region makes it easy to identify homology positions and thus facilitate multiple sequence alignment with confidence (Hillis and Dixon. 1991; Gillespie et al., 2004). When it is considered that the extent of sequence variation is an important criterion to delimit species, the genetic information of D expansion segments is useful for inferring evolutionary relationships and species discrimination of nematodes (De Luca et al., 2004; Handoo et al., 2001).

Molecular phylogenies of parasite nematodes have recently been studied by several authors based on D expansion segments of the 28S rDNA (AL-Banna et al., 1997; de Bellocq et al., 2001; He et al., 2005; Subbtoin et al., 2005). In previous studies, D1-D2 expansion domain sequences of the 28S gene from the order Strongylida were analyzed to evaluate phylogenetic relationships (de Bellocq et al., 2001). They found some species have high AT nucleotide content (67.3-70.4%) in the D2 region and this sequence composition made sequence alignment and construction of phylogenetic analysis ambiguous. Based on the D2-D3 sequences, Subbotin et al. (2005) constructed phylogenetic relationships of Criconematina Siddiqi, 1980 and found that several species have sibling species determined by comparative sequence analysis. He et al. (2005) studied evolutionary relationship with family Longidoridae Thorne, 1935. Subbotin et al. (2006) studied phylogeneic relationships of Tylenchida. In their study, the

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phylogenetic analysis proposed that Hoplolaimidae is clustered with Heteroderidae.

Fortuner (1987) proposed two Hoplolaimidae subfamilies, Hoplolaiminae and Rotylenchulinae Husain and Khan, 1967. According to Siddiqi (2000), family Hoplolaimidae consists of the subfamilies Hoplolaiminae, Aphasmatylenchinae Sher, 1965, and Rotylenchoidinae Whitehead, 1958 as well as 11 genera based on variation of morphological and morphometric characters such as the presence and size of phasmids. In our study, we followed the systemic view of Fortuner (1987) with *Aphasmatylenchus*, *Antarctylus*, *Helicotylenchus, Rotylenchus*, and *Pararotylenchus* included in the subfamily Hoplolaiminae. Though each genus under the Hoplolaiminae is separated by several phenotypic traits, their phylogenetic status is still questioned (Germani et al., 1985).

The objective of this study was to investigate phylogenetic relationships of Hoplolaiminae species using D2 and D3 expansion segments of the 28S gene.

Materials and Methods

The species name and geographical origin of nematode populations used in this study are presented in Table 1. Nematode samples were acquired between

Table 1. The nematode species and populations of Hoplolaiminae used in this study.

Sample code	Species	Host	Location	GenBank Accession number
LA 67	*Hoplolaimus columbus*	Corn	Pointe Coupee County, LA	EU554665
LA92	*H. columbus*	Cotton	Franklin County, LA	EU554666
LA94	*H. columbus*	Cotton	Pointe Coupee County, LA	EU554667
SC103	*H. columbus*	Cotton	Lee County, SC	EU554668
GA105	*H. columbus*	Cotton	UGA research station Midville, GA	EU554669
SC144	*H. columbus*	Corn	Dorchester County , SC	EU554670
SC147	*H. columbus*	Soybean	Dorchester County , SC	EU554671
SC195	*H. columbus*	Cotton	Blackville, SC	EU443780
SC196	*H. columbus*	Cotton	Floence, SC	EU554673
SC198	*H. columbus*	Soybean	Blackville, SC	EU554674
NC242	*H. columbus*	Cotton	Johnston , NC	EU554676
TX115	*H. galeatus*	Corn	Texas city, TX	EU626788
SC109	*H. galeatus*	Cotton	Colleton County, SC	EU626785
FL60	*H. galeatus*	Cotton	B.P.I, FL	EU626784
FL184	*H. galeatus*	Bermuda grass	Fort Lauderdale Research and Education center, FL	EU626786
FL185	*H. galeatus*	Floratam St. Augustinegrass	Fort Lauderdale Research and Education center, FL	EU626787
AR221	*H. magnistylus*	Cotton	Ashley County. AR	EU626789
AR248	*H. magnistylus*	Willow tree	Hope County, AR	EU626790
FL181	*H. seinhorsti*	Peanut	IFAS Experiment Station, Jay, FL	EU626791
AR135	*H. concaudajuvenchus*	Hackberry	Perry County, AR	EU626792
TN241	*Hoplolaimus* sp. 1	?	Smoky Mountains, TN	EU626793
IL172	*Hoplolaimus* sp. 2	Turfgrass	University of Illinois, IL	EU626794
KS237	*Hoplolaimus* sp. 2	Turfgrass	Manhattan, KS	EU626795
SC110	*Hoplolaimus* sp.3	Birch tree	Clemson Univ., SC	EU586798
AL108	*Hoplolaimus* sp.3	Cotton	Belle Mina, Limestone County, AL	EU586797
AR160	*Aorolaimus logistylus*	Black walnut	Devil's Den State Park, AR	FJ485640
AL108	*Scutellonema brachyurum*	Cotton	Belle Mina, Limestone County, AL	FJ485641
AR201	*Scutellonema brachyurum*	Corn	University of Arkansas, AR	FJ485642
KR192	*Scutellonema brachyurum*	Forsythia	Daegu, Korea	FJ485643
AR116	*Scutellonema brachyurum*	Soybean	St Francis County, AR	FJ485644
SC199	*Scutellonema brachyurum*	Cotton	Floence, SC	FJ485645
AR194	*S. bradys*	Tomato	University of Arkansas, AR	FJ485652
VA191	*Rotylenchus buxophilus*	Cotton	University of Virginia, VA	FJ485646
AR189	*Rotylenchus buxophilus*	Cotton	Chicot County, AR	FJ485647
FL180	*Helicotylenchus microlobus*	Floratam St. Augustinegrass	Fort Lauderdale Research and Education Center, Ft. Lauderdale, FL	FJ485648
GA177	*H. dihystera*	Cotton	UGA research station Midville, GA	FJ485651
IL171	*H. pseudorobustus*	Turfgrass	University of Illinois, IL	FJ485649
KR210	*H. vulgaris*	Apple	University of Arkansas, AR	FJ485650
	Rotylenchus laurentinus			DQ328757
	Rotylenchus goodeyi			DQ328758
	Rotylenchus eximinus			DQ328741
	Rotylenchus uniformis			DQ328755
	Helicotylenchus digonicus			DQ328758
	Helicotylenchus multicinctus			DQ328745

2002 and 2006 from soil field samples or living specimens in water. Adult females were selected for extraction of total genomic DNA. Forty-five populations representing 22 species of the subfamily Hoplolaiminae were obtained from a wide range of geographical locations and various hosts. Previously published GenBank sequences of *Aorolaimus perscitus* (DQ328744), *Helicotylenchus multicinctus* (DQ328745), *Helicotylenchus digonicus* (DQ328758), *Rotylenchus goodeyi* (DQ328758), *Rotylenchus laurentinus* (DQ328757), *Rotylenchus eximius* (DQ328741), *and Rotylenchus uniformis* (DQ328755) were included in the analysis.

DNA Extraction: One or two individuals from each population were hand-picked and transferred to a microcentrifuge tube with 0.5 µl RNA free water. DNA was extracted with REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich Co., St. Louis, MO).

Amplification and sequencing of D1-D3 expansion segments of 28S gene: The PCR primers used to amplify the D1 to D3 expansion segments of 28S gene were primers LSUD-1f (5'- ACCCGCTGAACTTAAGCATTA-3') which was designed using comparative sequence alignment of *Globodera tabacum* sequence found in GenBank (DQ097515) and LSUD-2r (5'-TTTCGCCCCTATACCC AAGTC-3') which was designed using comparative sequence alignment of *Globodera rostochiensis* sequence from GenBank (AY592993). Amplification was carried out in a thermal cycler with the following protocol: After initial denaturation at 95°C for 3 min, there were 35 cycles of 95°C for 45 s, 57°C for 1 min 30 s, 72°C for 2 min, and a final extension step of 72°C for 10 min. Each reaction included a negative control without DNA. After amplification, 8 µl of each reaction was loaded into a 1.5% agarose gel (120 V, 50 min) and photographed under UV light. This amplified fragment was purified using the Quantum Prep PCR Kleen Spin Columns (BIO-RAD) and samples were sent to the University of Arkansas DNA sequencing and Synthesis Facility (Little Rock, AR) for direct sequencing in both directions.

Alignment and Phylogenetic analysis: Consensus sequences were obtained using BioEdit 5.89 (Hall 1999) to align sequence data. The distance matrix option of PAUP* 4.0b10 (Swofford 2001) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura 1980) of sequence evolution. *Globodera tabacum* (Genebank AF339502) was used as the outgroup taxon for the ITS1 dataset and *G. rostocheiensis* (AY592993) for the 28S dataset. DNA sequences were aligned using Clustal W (Thompson et al. 1994). The best-fitting nucleotide substitution model was chosen according to the GTR+G model among 64 different models using ModelTest v 3.7 (Posada and Crandall 1998) and PAUP* 4.0b10 (Swofford 2001). Phylogenetic analysis was conducted using maximum likelihood (ML) analysis using the best-fitting evolutionary model in PAUP*. Bootstrapping was performed using either neighbor joining or ML (1000 replicates) to determine the reliability of obtained topologies. Unweighted parsimony (MP) analysis on the alignments were conducted using PAUP* 4.0b10 (Swofford 2001). Gaps were treated as missing data and a random addition sequence was used. A bootstrap test was used to test the reliability of trees (Felsenstein 1985). Parsimony bootstrap analysis included 1000 re-samplings by using the Branch and Bound algorithm of PAUP*.

Results

The PCR amplification of the D1-D3 expansion segments of the 28S gene of all species tested produced a single PCR amplicon approximately 1.03 kb in size, suggesting a lack of D1-D3 expansion region size polymorphism among species. The average nucleotide frequencies in the D2-D3 region were 15.3% (A), 24.8% (C), 36.5% (G), 23.4% (T), 61.3% (G-C), and 38.7% (A-T). The D2 and D3 domain were determined by sequence similarity search in BLAST. The length of the D2 and D3 regions, except the core segment between the two regions, ranged from 528 bp for *Scutellonema brachyurum* to 538 bp for *Rotylenchus buxophilus*.

Pairwise Tajima-Nei distance (Tajima and Nei, 1984) among the D2-D3 expansion regions of Hoplolaiminae species revealed extensive genetic variation among species (Table 2). Sequence divergence within the ingroup ranged from complete identity between *Hoplolaimus columbus* and *Hoplolaimus seinhorsti* to 20.7% between *S. brachyurum* and *Hoplolaimus concaudajuvencus*.

The D2 and D3 expansion segments of the 28S gene were aligned for Hoplolaiminae species and examined with the outgroup *Globodera rostochiensis* for the D2-D3 region. The aligned D2-D3 expansion region showed a total of 558 characters and of these characters, 254 characters (45.5%) are variable and 198 characters (35.4%) are parsimony-informative.

Parsimony analysis of the D1-D3 expansion using equally weighted character states results in a single parsimonious tree (Fig. 1). This tree had a length of 724 steps, and a consistency index (CI) of 0.511 as documented using the branch and bound algorithm of PAUP 4.0b10.

Based on the molecular phylogenetic analysis, Hoplolaiminae consists of two distinct clades. Clade I is composed of *Hoplolaimus* species whereas clade II is composed of *Aorolaimus*, *Helicotylenchus*, *Rotylenchus*, and *Scutellonema* species. Clade I is supported by a high bootstrap value (97%) whereas clade II is supported by a relatively low bootstrap value (76%). According to maximum parsimony and maximum likelihood analysis, *Aorolaimus perscitus* is a sister taxon to all Hoplolaiminae species. Clade II is divided into two subclades in the maximum likelihood and neighbor-joining tree, labeled by group 1 and 2. Group 1 consists of *Scutellonema brachyurum*, *S. bradys*, and *Aorolaimus logistylus* in the maximum likelihood, whereas only *Scutellonema* species

TABLE 2. Pairwise genetic distance (%) of combined D2-D3 expansion segments of 28S gene among Hoplolaiminae.

	Speceis	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	*Hoplolaimus columbus*	-																						
2	*H. seinhorsti*	0	-																					
3	*H. magnistylus*	10.2	10.2	-																				
4	*H. concaudajuvenchus*	11.2	11.2	7.5	-																			
5	*H. galeatus* (FL184)	10.0	10.0	5.8	8.1	-																		
6	*Hoplolaimus sp.* 1	10.7	10.7	5.1	8.3	6.5	-																	
7	*Hoplolaimus sp.* 2	11.9	11.9	6.4	8.5	7.5	6.4	-																
8	*Hoplolaimus sp.* 3	11.3	11.3	5.6	8.7	7.3	4.7	1.9	-															
9	*Scutellonema brachyurum* (AL108)	17.9	17.9	20.3	20.8	20.5	19.6	21.1	21.1	-														
10	*Scutellonema bradys* (AR194)	18.1	18.1	15.6	16.8	16.9	17.3	16.7	16.7	16.9	-													
11	*Rotylenchus buxophilus* (VA191)	12.3	12.3	13.9	13.6	14.1	14.0	14.0	13.6	16.6	14.2	-												
12	*Rotylenchus laurentinus* (DQ328757)	12.2	12.2	14.3	14.0	13.9	13.7	13.9	14.3	17.1	16.7	4.1	-											
13	*Rotylenchus goodeyi* (DQ328756)	12.3	12.3	14.3	14.0	13.9	13.9	14.1	14.3	17.2	16.7	4.1	0.18	-										
14	*Rotylenchus uniformis* (DQ328735)	12.3	12.3	14.7	14.4	14.8	14.5	14.9	15.4	16.9	15.2	7.5	8.3	8.3	-									
15	*Rotylenchus eximinus* (DQ328741)	13.3	13.3	15.2	15.5	15.9	15.9	15.4	15.0	16.5	14.6	9.2	10.5	10.5	7.7	-								
16	*Helicotylenchus pseudorobustus* (IL171)	17.1	17.1	17.5	17.5	18.2	17.4	18.1	18.2	17.6	17.6	12.6	12.1	12.1	12.8	13.2	-							
17	*Helicotylenchus dihystera* (GA177)	15.7	15.7	17.2	17.3	17.8	17.4	17.3	17.1	17.5	16.3	12.8	12.5	12.4	12.8	12.8	4.3	-						
18	*Helicotylenchus microlobus* (FL180)	15.6	15.6	16.7	16.8	17.7	17.3	17.5	17.1	17.1	16.8	10.8	10.2	10.2	11.3	11.3	3.6	4.7	-					
19	*Helicotylenchus vulgaris* (KR210)	15.7	15.7	14.2	15.6	14.7	14.8	14.8	14.6	16.1	13.8	9.1	9.6	9.7	8.9	10.3	12.8	12.1	11.9	-				
20	*Helicotylenchus multicinctus* (DQ328745)	15.7	15.7	17.5	17.4	18.4	18.6	18.3	17.8	18.4	17.9	12.7	12.8	12.9	12.5	13.4	7.5	8.8	5.9	14.6	-			
21	*Helicotylenchus digonicus* (DQ328758)	14.3	14.3	14.6	15.0	15.2	15.3	15.2	14.8	18.4	14.3	8.9	9.5	9.5	10.0	11.7	12.5	12.3	11.4	6.0	13.9	-		
22	*Aorolaimus logistylus* (AR160)	14.6	14.6	15.2	15.3	15.5	15.3	16.4	16.4	16.0	15.1	11.8	12.4	12.4	11.4	12.7	13.5	12.4	13.0	11.4	15.8	12.1	-	
23	*Aorolaimus perscitus* (DQ328744)	13.8	13.8	13.0	13.4	13.9	14.1	15.3	15.1	17.5	16.3	13.0	13.5	13.5	11.9	13.4	15.4	15.0	13.5	15.0	14.3	15.9	12.9	-

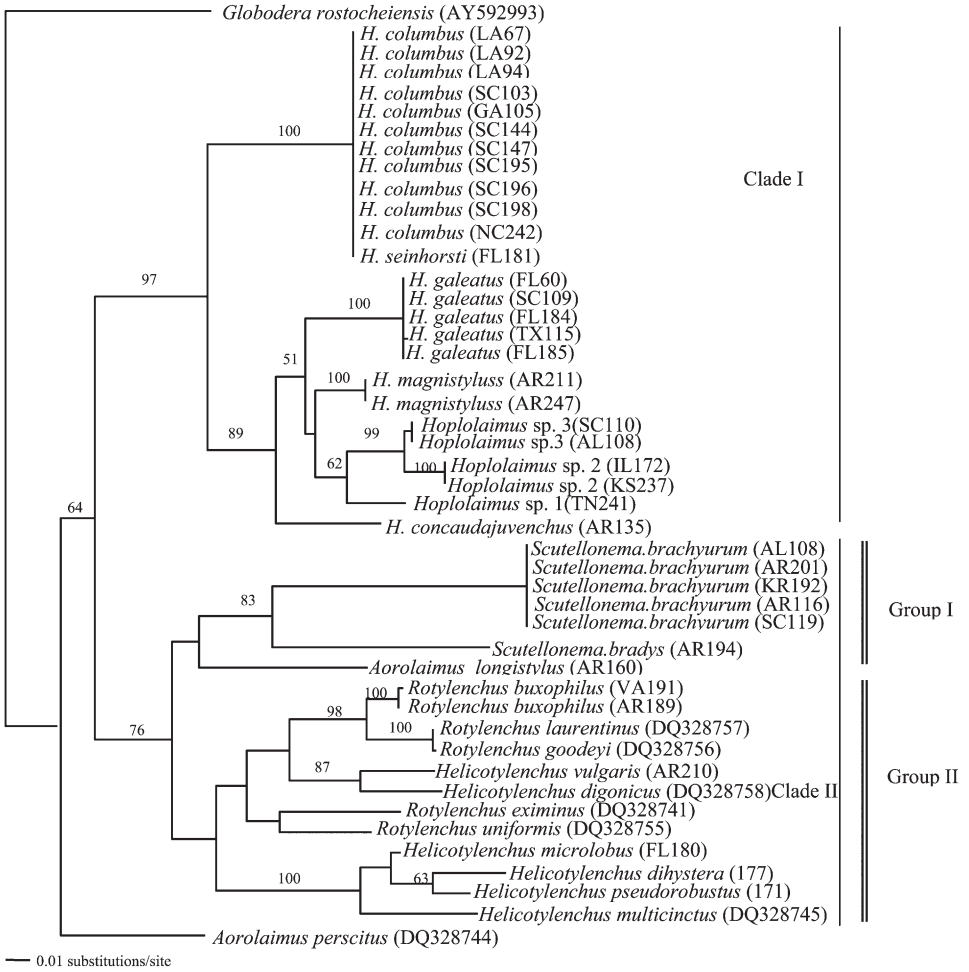

FIG. 1. A phylogenetic analysis for the Hoplolaiminae species based on 28S D2-D3 sequences, derived from maximum likelihood analysis. Maximum parsimony values (>50%) are provided at each node.

consist of group 1, supported by bootstrap value (83%) in the maximum parsimony tree. Group 2 consists of all *Helicotylenchus* and *Rotylenchus* species in the maximum likelihood. In the maximum parsimony and maximum likelihood trees, *Helicotylenchus* species is divided into two subclades. One subclade consists of *Helicotylenchus* species, including *Helicotylenchus microlobus, Helicotylenchus dihystera*, *Helicotylenchus pseudorobustus*, and *Helicotylenchus multicinctus*. The other subclade consists of *Helicotylenchus vulgaris* and *Helicotylenchus digonicus*. Three *Rotylenchus* species including *R. buxophilus*, *R. laurentinus*, and *R. goodeyi* consist of one subclade, supported by bootstrap value (98%) in the maximum parsimony. Two other *Rotylenchus* species, *R. eximinus* and *R. uniformis* were scattered in the maximum parsimony tree.

The results, presented in Fig. 1, demonstrate that *Hoplolaimus* species is monophyletic. *Hoplolaimus* species is subdivided two groups: One group comprises of *H. columbus* and *H. seinhorti* while another group comprises of the rest of *Hoplolaimus* species. The monophyly of *Hoplolaimus* species is supported by the parsimony tree (97%).

DISCUSSION

DNA sequence-based phylogenetic analyses among nematode species has recently been pursued vigorously from a wide range of taxonomic groups. Ribosomal DNA genes encoding the small subunit (SSU or 18S) and the large subunit (LSU or 28S) have been used to infer phylogentic relationships among closely or distantly related taxonomic lineages. Several studies have used the 18S genes of rDNA as phylogenetic markers to reconstruct phylogenetic relationships among the family or higher levels and the ITS region for the genus or closely related species level (Subbotin et al., 2001; Kanzaki and Futai. 2002; Ferris et al., 2004: Olivier et. al, 2004). With two regions, the D expansion domain also provided meaningful information for reconstructing a phylogeny among higher taxonomic lineages such as Tylenchida, Criconematina, Longidoridae, and also lower taxonomic groups such as *Pratylenchus* and *Longidorus* (AL-Banna et al., 1997; De Luca et al., 2004; He et al., 2005; Subbotin et al., 2005; Subbotin et al., 2006).

Our result suggests that the D2 and D3 expansion regions are useful to resolve deeper relationships

within Hoplolaiminae. Two clades are strongly supported: Clade I consists of *Hoplolaimus* species and clade II consists of *Aorolaimus*, *Sctellonema*, *Helicotylenchus*, and *Rotylenchus* species. According to Fortuner (1987), the genus *Hoplolaimus* consists of groups having several phenotypic traits derived from evolution. Depending on these characters, *Hoplolaimus* species can divide into two groups: The first has ancestral characters such as three gland nuclei, four lateral lines, and the position of excretory pore is below hemizonid and the second group has derived characters such as six gland nuclei, less than four lateral lines, and the position of excretory pore is above hemizonid. Geraert (1991) also argued that structure of lateral lines became reduced and finally disappeared. This view is accorded with Fortuner that four lateral lines are ancestor characters. In our phylogentic analysis, parthenogenetic species, *H. Columbus* and *H. seinhorsti*, having six nuclei and one lateral line, consist of one subclade whereas other amphimictic species having three nuclei and four lateral lines consist of another subclade. This phylogenetic analysis suggests that these phenotypic characters are phylogenetically informative characters for the species level and are also characters to delimit species. From phylogenetic analysis using ITS1, *Hoplolaimus* species are monophyletic (Bae et al., unpublished).

Germani et al. (1985) suggested that *Pararotylenchus*, having small opposite phasmid openings near the level of anus, might be considered as an ancestor of *Scutellonema* since other genera having large phasmid openings. A small phasmid, which was found in *Helicotylenchus* and *Rotylenchus*, is an ancestor character whereas a large phasmid is a derived character. Geraert (1990) also argued that from *Rotylenchus*, a new apomorphic character (scutella) arose relatively late in *Sctellonema* species and another transformation of this character occurred in *Aorolaimus* and *Hoplolaimus* species, which exhibit this character anterior and posterior to the vulva. In previous studies, *Helicotylenchus* and *Rotylenchus* species are paraphyletic and *Scutellonema* is clustered with *Hoplolaimus* (Subbotin et al., 2006). This result was obtained from phylogenetic analysis using several family species and thus the phylogenetic positions of some genera were not well resolved. In our phylogenetic analysis using the ITS1 sequence, *Hoplolaimus* species are clustered with *Aorolaimus* species (data not shown). Phylogenetic analysis using the D2-D3 region showed that *Scutellonema* is clustered with *Rotylenchus*, *Helicotylenchus*, and *Aorolaimus* instead of *Hoplolaimus*. This result suggests that position of phasmids is more phylogenetically informative than the size of phasmids because *Rotylenchus* and *Helicotylenchus* have small phasmids located at the level of anus, though the position of phasmids on *Aorolaimus* is still not clear and *Helicotylenchus* and *Rotylenchus* are paraphyletic. Seinhorst (1971) argued that genera having an asymmetrical esophagus and elongated subventral glands are closely related to those with a symmetrical esophagus. Therefore, *Helicotylencus* is related to *Rotylenchus*.

Selecting the proper target region of DNA within the genome of taxonomic units is an important step to reconstruct reliable phylogenetic history. Though phylogenetic analysis using D expansion segments of the 28S gene has not resolved the phylogenetic position of each genus under Hoplolaiminae clearly, this region is highly divergent within the genus and also has large variation among Hoplolaiminae species, thus this LSU locus has a good signal for reconstructing the phylogenetic history of deeper relationships. Different phylogenetic approaches, such as secondary structure information-based phylogenetic analysis and phylogenetic analysis using morphological data, are needed to resolve the phylogenetic position of *Aorolaimus* and paraphyly of *Helicotylenchus* and *Rotylenchus*. An extensive phylogenetic analysis with different phylogenetic markers including more species would illuminate the diversity of species in this subfamily and may provide more reliable information which is in accorded with morphological based view.

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