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Secondary structure models of D2-D3 expansion segments of 28S rRNA for Hoplolaiminae species

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Abstract: The D2-D3 expansion segments of the 28S ribosomal RNA (rRNA) were sequenced and compared to predict secondary structures for Hoplolaiminae species based on free energy minimization and comparative sequence analysis. The free energy based prediction method provides putative stem regions within primary structure and these base pairings in stems were confirmed manually by compensatory base changes among closely and distantly related species. Sequence differences ranged from identical between *Hoplolaimus columbus* and *H. seinhorsti* to 20.8% between *Scutellonema brachyurum* and *H. concaudajuvencus*. The comparative sequence analysis and energy minimization method yielded 9 stems in the D2 and 6 stems in the D3 which showed complete or partial compensatory base changes. At least 75% of nucleotides in the D2 and 68% of nucleotides in the D3 were related with formation of base pairings to maintain secondary structure. GC contents in stems ranged from 61 to 73% for the D2 and from 64 to 71% for the D3 region. These ranges are higher than G-C contents in loops which ranged from 37 to 48% in the D2 and 33-45% in the D3. In stems, G-C/C-G base pairings were the most common in the D2 and the D3 and also non-canonical base pairs including A•A and U•U, C•U/U•C, and G•A/A•G occurred in stems. The predicted secondary model and new sequence alignment based on predicted secondary structures for the D2 and D3 expansion segments provide useful information to assign positional nucleotide homology and reconstruction of more reliable phylogenetic trees.

Key words: 28S, D2-D3, Hoplolaiminae, *Hoplolaimus,* nematode.

The subfamily Hoplolaiminae Filipjev, 1934 belongs to the family Hoplolaimidae Filipjev, 1934 and is divided into two subfamilies; Hoplolaiminae Filipjev, 1934 and Rotylenchulinae Husain & Khan, 1967 (Fortuner 1987). Hoplolaiminae consists of eight genera; *Antarctylus* Sher, 1973, *Aorolaimus* Sher, 1963, *Aphasmatylenchus* Sher, 1965, *Helicotylenchus* Steiner, 1945, *Hoplolaimus* von Daday, 1905, *Pararotylenchus* Baldwin and Bell, 1981, *Scutellonema* Andrássy, 1958, *Rotylenchus* Filipjev, 1936. Some species, *Hoplolaimus*, *Scutellonema* and *Helicotylenchus* are distributed worldwide and cause economic damage to crops whereas other species such as *Aphasmatylenchus* and *Antarctylus* are each distributed in few sites of Africa and limited areas of Antarctic, respectively (Germani and Luc, 1984; Fortuner, 1991: Sher, 1973).

Ribosomal RNA genes encoding 5.8S, small subunit (SSU) or 18S, and large subunit (LSU) or 28S have been widely used to infer phylogenetic relationships among closely and distantly related taxonomic lineages. D expansion segments of the 28S ribosomal RNA molecule have been used as meaningful genetic markers for resolving phylogenetic relationship at lower and higher taxonomic levels and developing species- specific primers (Al-Banna et al., 1997; Al-Banna et al., 2004; Duncan et al., 1999; Subbotin et al., 2005, 207, 2008; Vovlas et al., 2008). The LSU ribosomal RNA, 28S gene, consists of core segments that are highly conserved structurally across broad taxonomic levels and variable regions, which are described as divergent D domains or expansion segments (Hillis and Dixon 1991). D domains vary greatly in nucleotide composition as well as length among species (De Rijk et al., 1995; Hassouna et al., 1984). Coexistence of variability and conservation within the 28S gene make this region suitable for estimation of phylogenetic relationships among species because sequence variation provides phylogenetic information while the conserved structure makes it easier to identify homologous positions (Hillis and Dixon. 1991; Gillespie et al., 2004).

The RNA molecule is important to study since it is involved in protein synthesis and its function is determined by structure (Noller 1984). The structural conservation of rRNA among closely and distantly related species has been revealed from extensive experimental and comparative sequence analyses using different target regions (Chilton et al., 1998; Gillespie et al., 2005; Hickson et al., 1996; Hung et al., 1999). Ribosomal RNA (rRNA) consists of paired stems and unpaired loop regions. rRNA folds onto itself to form complex secondary structures and maintains these structures by Watson-Crick base pairing patterns between close or distant regions of the rRNA molecule. This double strand rRNA region consists of traditional pair bonds, that is canonical base pairing which are Watson-Crick base pairs (G-C, and A-U), and wobble pairs (G○U).

The application of rRNA secondary structure to reconstruct phylogenetic history is reliable because structure based on sequence alignments facilitates accurate assessment of nucleotide homology which came from the same evolutionary origin (Chilton et al., 2001; Dixon and Hillis, 1993; Kjer. 1995). The characters used to infer phylogenetic relationship must be homologous, but if a high level of sequence variation in length and nucleotide composition exists, multiple sequence alignment becomes difficult (De Rijk et al., 1995; Hung et al., 1999). Reconstruction of phylogeny is dependent on the results of automated sequence alignments produced by computer programs (Kjer 1995). However, confidence of sequence alignment is sometimes questionable in length and nucleotide heterozygous taxonomic units owing

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to gaps added to increase sequence similarity. According to previous studies, the structure-based sequence alignments provided more reliable positional homology assignment than computer algorithms based on automated alignment and thus yield a more accurate phylogenetic tree (Hung et al., 1999; Kjer, 1995; Morrison and Ellis 1997). The structure-based sequence alignment considers each nucleotide character as a dependent character because the nucleotides that consist of stems affect another nucleotide forming base pairings to maintain their structure. However, automated sequence alignments consider each nucleotide character as an independent character. The structure conservation among distantly related species allows detecting homologous positions among sequencesand reconstructing phylogenetic analyses of broad taxonomic lineages (Goertzen et al., 2003; Kjer 1995).

In previous studies, Hung et al. (1999) found high levels of interspecific sequence variation (2-56%) in the ITS2 region among strongyloid nematodes. However, sequence alignment based on secondary structure increased positional homology, resulting in reconstruction of a more reliable phylogenetic tree. He et al. (2005) studied a molecular phylogenetic approach to the family Longidoridae by two different sequence alignments of the D2 and D3 region and they found that phylogenetic analysis based on secondary structure was not in accord with computer-based phylogenies.

Many studies have shown that covariation-based comparative sequence analysis successfully predicts secondary structure (Gomez-Zurita et al., 2000; Goertzen et al., 2003; Mai and Coleman 1997; Shinohara et al., 1999). Comparative sequence analysis shows that the most dominant interaction was composed of G:C and A:U base pairs in regular secondary structure helices (stems) but non-canonical base pairs also were detected from covariation analysis (Gutell et al., 2000). The secondary structure of the LSU rRNA in parasitic nematode was proposed by Chilton et al. (2003). They obtained the first complete LSU rRNA sequence and determined secondary structure for the parasitic nematode *Labiostrongylus bipapillosus* and revealed that sequence variability was located at D domains in a comparison between *L. bipapillosus* and *C. elegans*. Subbotin et al. (2005, 2007, 2008) proposed secondary structure models of D2 and D3 expansions segments of 28S rRNA gene for Criconematina, Hoplolaimidae and *Pratylenchus*, respectively, and applied these models to optimize sequence alignments and reconstruct phylogenetic relationships using the complex model of DNA evolution.

In this study, we have compiled 18 species of Hoplolaiminae along with two other taxa, *Globodera rostochiensis* and *Rotylenchulus reniformis*, to evaluate and refine previously described secondary structure models (i.e., *Labiostrongylus bipapillosus* by Clilton et al., 2003, longidorids by He et al., 2005, and Hoplolaimidae by Subbotin et al., 2007) and construct secondary structure of the D2 and D3 expansion segments of 28S rRNA of some species of Hoplolaiminae to approach accurate sequence alignment based on positional homology.

Materials and Methods

The species name and geographical origin of the nematode populations used in this study are presented in Table 1. Nematode samples were acquired from soil field samples or living specimens in water from 2002 to 2006 and adult females were selected for extraction of total DNA. Forty-five populations representing 18 species of the subfamily Hoplolaiminae were obtained from a wide range of geographical locations and various hosts. Two outgroup species, *Rotylenchulus reniformis* (GenBank: DQ328713), and *Globodera rostochiensis* (GenBank: AY 592993) were used.

Table 1. Populations and species of the Hoplolaiminae in this study.

Sample code	Collection year	Species	Host	Location	GenBank accession numbers
LA 67	2003	*Hoplolaimus columbus*	Corn	Pointe Coupee County, LA	EU554665
TX 115	2003	*H. glaeatus*	Corn	Texas City, TX	EU626788
FL181	2004	*H. seinhorsti*	Peanut	Experiment Station, Jay, FL	EU626791
AR221	2005	*H. magnistylus*	Cotton	Ashley County, AR	EU626789
AR135	2005	*H. concaudajuvenchus*	Hackberry	Perry County, AR	EU626792
TN241	2006	*Hoplolaimus* sp. 1	?	Smoky Mountains, TN	EU626793
IL172	2004	*Hoplolaimus* sp. 2	Turfgrass	University of Illinois	EU626794
SC110	2004	*Hoplolaimus* sp.3	Birch tree	Clemson Univ., SC	EU586798
AL108	2004	*Scutellonema brachyurum*	Cotton	Limestone County, AL	FJ485641
AR194	2005	*S. bradys*	Tomato	University of Arkansas	FJ485652
VA191	2005	*Rotylenchus buxophilus*	Cotton	Virginia Tech	FJ485646
FL180	2005	*Helicotylenchus microlobus*	Floratam St. Augustinegrass	Ft. Lauderdale, FL	FJ485648
GA177	2005	*H. dihystera*	Cotton	Research station, Midville, GA	FJ485651
IL171	2005	*H. pseudorobustus*	Turfgrass	University of Illinois	FJ485649
KR210	2005	*H. vulgaris*	Apple	University of Arkansas	FJ485650
AR160	2004	*Aorolaimus longistylus*	Black walnut	Devil's Den State Park, AR	FJ485640

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

Amplification and sequencing of the D1-D3 expansion segments of the 28S gene: The primer sequences used to amplify the D1 to D3 expansion segments of the 28S gene were primers LSUD-1f (5'- ACCCGCTGAACTT AAGCATTA-3') which was designed using comparative sequence alignment of *Globodera tabacum* sequence found in the GenBank (DQ 097515) and LSUD-2r (5'-TTTC GCCCCTATACCCAAGTC-3') which were designed using comparative sequence alignment of *G. rostochiensis* sequence found GenBank (AY 592993). Amplification was carried out in a thermal cycler with the following protocol: after initial denaturation of 95°C for 3 min, there was 35 cycles of 95°C for 45 s, 57°C for 1 min 30 s, 72°C for 2 min, and final extension step of 72°C for 10 min. Each reaction included negative control without DNA template. After amplification, six µl of each reaction were loaded onto 1.5% agarose gel (120V, 50 min) and photographed under UV light. This amplified fragment was purified using the Quantum Prep PCR Kleen Spin Columns (BIO-RAD) and directly sequenced in both directions. The University of Arkansas DNA sequencing and Synthesis Facility (Little Rock, AR) sequenced PCR products of D1-D3 expansion segments using an ABI Prism 377 DNA sequencer (PE Applied biosystems, Foster City, CA).

Secondary structure prediction and sequence alignment based on secondary structure: The secondary structure model of the D2 and D3 region of rRNA was predicted using Mfold (Zuker et al., 1999) based on an energy minimization approach. This free energy based prediction method is especially useful to *infer* position showing potential base pairings and these putative stems (helices) are confirmed by compensatory mutations which occur in the form of covariance. The 28S-D2 and D3 sequences were aligned manually based on predicted secondary structure and each aligned sequence was notated by following the method of Kjer (1995) and compared with secondary structures of Hoplolaimidae reconstructed by Subbotin et al. (2008).

Results

Sequence analysis of the D1-D3 expansion segments of the 28S gene: The amplification of D1-D3 expansion segments of eighteen Hoplolaiminae species yielded a single product approximately 1.03kb long and did not reveal length polymorphism among the species that were analyzed. The determination of each D1, D2, and D3 expansion domain was conducted by sequence similarity search using BLAST and the apparent PCR product length of the D1-D3 expansion regions excluding the core segments between D1 and D3 domain ranged from 681 bp for *Scutellonema brachyurum* to 692 bp for *Helicotylenchus microlobus*; the length of the D1 is 153-156 bp with 56.2-64.7% GC content. The length of the D2 is 359-371 bp with 57.6-67.7% GC content, and the length of the D3 is 167-169 bp with 55.6-64.2% GC content.

D2 expansion domain secondary structures for individual species of Hoplolaiminae: A secondary structure model of the D2 region was proposed for Hoplolaiminae species with outgroup species (*Globodera rostochiensis* and *Rotylenchulus reniformis*) by comparison of structure models predicted from each species. First, closely related species showing similar length and less genetic divergence were used to predict secondary structure. Second, structure models predicted from each species were compared with distantly related species by comparative sequence analysis to confirm nucleotide positions which form stems. Covariation-based comparative sequence analyses detected positions which showed significant amount of covariation and invariant Watson-Crick base-pairs and also positions showing no covariation. Stems (helices) were given a different number according to Van de Peer et al. (1994) if separated by a loop (multibranched loop, hairpin loop, and interior loop) or by a single strand area that does not form a loop. Therefore, the D2-28S segment consisted of 9 stems in all examined species. The nucleotides related with base pairings ranged from 75.2% of *Scutellonema brachyurum* to 79.7% of *Helicotylenchus pseudorobustus* The predicted secondary structure models for *Hoplolaimus columbus* were proposed (Fig. 1). Overall, G (35%) was the most common nucleotide, followed by U (26%), C (23%) and A (14%). G was also the most common nucleotide in stems (39.2%) whereas A(10%) showed the lowest frequency in paired region. The GC content in stem regions ranged from 61.6% in *H. magnistylus* to 73.1% in *Scutellonema brachyurum,* whereas GC content in the loop region ranged from 37.7% in *S. brachyurum* to 50% in *S. bradys.* Positions of complementary base changes found in the D2-28S gene secondary structure model for all Hoplolaiminae species are presented in Table 2.

In the 9 stems of the D2 region, most base pairings consisted of canonical base pairings which were Watson-Crick base pairs (G-C, and A-U), and also wobble pairs (G-U) (Table 2). Several conserved nucleotides were identified in unpaired region (e,g., in the terminal (CAGAUU) and internal bulge (UUCA: GCAUU) of stem c1-a and in the terminal (GCAA) and internal bulge (AG: AC) of stem c2-b) (Fig. 1). Most variable nucleotide polymorphisms concentrated on stems rather than loop. Among stems, stem c1-a was recognized as the most variable site.

The stem c1 of the predicted secondary structure of D2 expansion domain for all species of Hoplolaiminae was formed by complementary base pairings of the 3' and 5' end of the D2 region. The sequences of stem c1 consisted of 28 nucleotides and was highly conserved

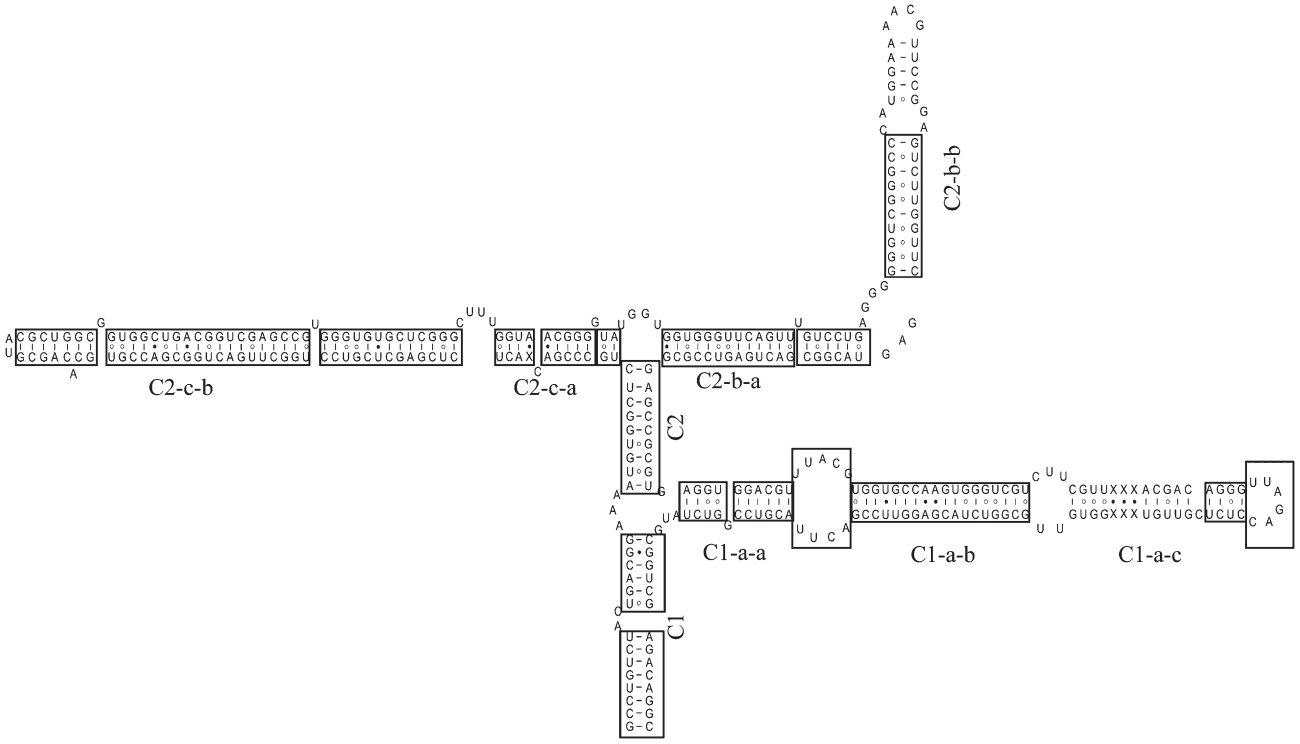

FIG. 1. Predicted secondary structure model of the D2 expansion domain for *Hoplolaimus columbus*.

across all species, including outgroup species, *Rotylenchulus reniformis* (GenBank; DQ328713) and *Globodera rostochiensis* (AY592993). However, one position showing complete and partial complementary base changes (transitional substitution) in stem c1 was detected at position 1, where the base pairing was U-A for *Hoplolaimus columbus* and C-G for *Scutellonema brachyurum* and *S. bradys,* but U-G for the other species examined (Table 6). This result reflects the possibility that covariation existed in this stem.

Stem c1-a is subdivided into three stems by two lateral bulges (stem c1-a-a, stem c1-a-b, and stem c1-a-c). Stem c1-a had the highest number of positional covariation among all stems. The stem c1-a-b and stem c1-a-c are well supported by complete or semi-conservative base changes. Stem c1-a-a consists of constant 10 base parings

TABLE 2. Positions of complementary base changes found in the D2-28S gene secondary structure model for Hoplolaiminae.

	Base pairing at position											
	c1	c1-a										
species	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8	No. 9	No.10	No.11	No.12
Hoplolaimus columbus	U-A	U-A	G○U	C-G	U○G	G-C	A●A	G●A	A-U	U○G	G○U	G-C
H. seinhorsti	U-A	U-A	G○U	C-G	U○G	G-C	A●A	G●A	A-U	U○G	G○U	G-C
H. magnistylus	U○G	U-A	G○U	C-G	U-A	G-C	G○U	C●A	U○G	U○G	G○U	A-U
H. concaudajuvenchus	U○G	U-A	G○U	C-G	U-A	G-C	G○U	C-G	C-G	U○G	G○U	G-C
H. galeatus	U○G	U-A	G○U	U○G	U-A	G-C	G○U	A-U	U○G	U-A	G○U	U-A
Hoplolaimus sp. 1	U○G	U-A	G○U	C-G	U○G	G-C	G○U	C-G	C-G	U●U	A-U	A-U
Hoplolaimus sp. 2	U○G	U-A	G○U	C-G	U○G	G-C	G○U	U-A	C-G	U○G	A-U	G○U
Hoplolaimus sp. 3	U○G	U-A	G○U	C-G	U○G	G-C	G○U	U-A	C-G	U○G	A-U	A-U
Scutellonema brachryrum	C-G	C-G	G○U	U-A	U○G	G○U	G-C	C-G	C-G	G-C	A-U	G-C
Scutellonema bradys	C-G	U-A	G○U	C-G	C-G	G-C	G○U	C-G	C-G	U○G	G-C	U○G
Aorolaimus longistylus	U○G	U-A	G○U	U○G	C-G	G-C	G-C	C-G	C-G	G-C	G○U	G-C
Helicotylenchus pseudorobustus	U○G	U-A	G○U	C-G	C-G	C-G	X -C	C-G	C-G	G-C	G○U	G-C
Helicotylenchus dihystera	U○G	U-A	G○U	C-G	C-G	U○G	X -U	C-G	C-G	G-C	G○U	G-C
Helicotylenchus microlobus	U○G	U-A	G○U	C-G	C-G	U-A	X -U	C-G	C-G	G-C	G○U	G-C
Helicotylenchus vulgaris	U○G	U-A	G○U	Y-G	U○G	G○U	X-U	C-G	C-G	C-G	G-C	G-C
Rotylenchus buxophilus	U○G	U-A	G○U	C-G	U○G	G○U	G○U	C-G	C-G	G○U	G-C	G-C
Globodera rostochiensis	U○G	U-A	U-A	C-G	U-A	G○U	G-C	U○G	U○G	U○G	G-C	G-X
Rotylenchulus reinformis	C-G	U-A	A-U	U-A	G●A	A-U	A-U	C-G	C-G	C●A	C●U	A-U

TABLE 2. Continued.

species	Base pairing at position											
	c1-a									c2-b		
	No.13	No.14	No.15	No.16	No.17	No.18	No.19	No.20	No.21	No.22	No.23	No.24
Hoplolaimus columbus	G○U	G-C	U○G	G○U	G○U	G-C	U○G	U-A	G-C	C-G	C-G	G○U
H. seinhorsti	G○U	G-C	U○G	G○U	G○U	G-C	U○G	U-A	G-C	C-G	C-G	G○U
H. magnistylus	G○U	A-U	U○G	G○U	G-C	G○U	G○U	U-A	G-C	C-G	U○G	G○U
H. concaudajuvenchus	G○U	A-U	U○G	G○U	G-C	G-Y	G○U	U-A	G-C	U-A	U-A	G○U
H. galeatus	G○U	G○U	U○G	G○U	G-C	G○U	G○U	U-A	G-C	U-A	U-A	G○U
Hoplolaimus sp. 1	G○U	G○U	U○G	G-C	G-C	G○U	A-U	U-A	G-C	C-G	C-G	A-U
Hoplolaimus sp. 2	G○U	A-U	A○U	G-C	G-C	G○U	G○U	U○G	G-C	C-G	U-A	A-U
Hoplolaimus sp. 3	G○U	A-U	U○G	G-C	G-C	G○U	G○U	U○G	G-C	C-G	U○G	A-U
Scutellonema brachryrum	X-U	C-G	G-C	G-C	C-G	G○U	C-G	C-G	G-C	C-G	U○G	G○U
Scutellonema bradys	A-U	G-C	C-G	G-C	U-A	G○U	C-G	C-G	U-A	U- X	C-G	G-C
Aorolaimus longistylus	A-U	C-G	A-U	G-C	U-G	G○U	C-G	C-G	G-C	C-G	C-G	G○U
Helicotylenchus pseudorobustus	G○U	C-G	C-G	C-G	G○U	G○U	C-G	C-G	G●A	C-G	U-A	G○U
Helicotylenchus dihystera	G○U	C-G	C-G	C-G	G○U	G○U	C-G	C-G	G●A	C-G	C-G	G○U
Helicotylenchus microlobus	G-C	C-G	C-G	C-G	G○U	G○U	C-G	C-G	G●A	C-G	U○G	G○U
Helicotylenchus vulgaris	U○G	A-U	U○G	G-C	U-G	A-U	C-G	C-G	G-C	U-A	U○G	G○U
Rotylenchus buxophilus	G○U	C-G	A-U	G-C	G-C	G○U	C-G	C-G	G-C	C-G	U○G	G○U
Globodera rostochiensis	G○U	U●U	C-G	A-U	G-C	G○U	C-G	C-G	G-C	C-G	U○G	G○U
Rotylenchulus reinformis	G○U	U●U	C-G	A-U	G-C	G○U	C-G	C-G	G-C	C-G	U○G	G○U

TABLE 2. Continued

species	Base pairing at position											
	Stem IV						Stem V					
	No.25	No.26	No.27	No.28	No.29	No.30	No.31	No.32	No.33	No. 34	No.35	No.36
Hoplolaimus columbus	A-U	C-G	U○G	C-G	U○G	G○U	U○G	A-U	G-C	G-C	C-G	A●A
H. seinhorsti	A-U	C-G	U○G	C-G	U○G	G○U	U-G	A-U	G-C	G-C	C-G	A●A
H. magnistylus	A-U	C-G	U○G	U-A	U-A	G-C	C-G	A-U	G-C	G-C	C-G	A●A
H. concaudajuvenchus	A-U	C-G	U○G	U-A	U○G	G-C	C-G	G○U	G-C	G-C	C-G	A●A
H. galeatus	A-U	U-A	U○G	U-A	U○G	G-C	C-G	A-U	G-C	G-C	C-G	A●A
Hoplolaimus sp. 1	A-U	C-G	U-A	U-A	U-A	G-C	C-G	A-U	G-C	G-C	C-G	A●A
Hoplolaimus sp. 2	A-U	C-G	U○G	U-A	U-A	G●A	C-G	A-U	G-C	G-C	C-G	A●A
Hoplolaimus sp. 3	A-U	C-G	U○G	U-A	U-A	G●A	C-G	A-U	G-C	G-C	C-G	A●A
Scutellonema brachryrum	G-C	C-G	C-G	C-G	C-G	G-C	U○G	U●U	G-C	G-C	C-G	G-C
Scutellonema bradys	G-C	C-G	U○G	C-G	C-G	G-C	G-C	G○U	G-C	G-C	A-U	A-U
Aorolaimus longistylus	G-C	C-G	U○G	C-G	C-G	G-C	C-G	A-U	G-C	G-C	C-G	A-U
Helicotylenchus pseudorobustus	G-C	C-G	U○G	C-G	C-G	G-C	C-G	C-G	G-C	G-C	C-G	A-U
Helicotylenchus dihystera	G-C	C-G	U○G	C-G	C-G	G-C	C-G	C-G	G-C	G-C	C-G	A-U
Helicotylenchus microlobus	G-C	C-G	U○G	C-G	C-G	A-U	C-G	C-G	G-C	G-C	C-G	A-U
Helicotylenchus vulgaris	G-C	C-G	U○G	C-G	C-G	G-C	C-G	U○G	G-C	G-C	C-G	G-C
Rotylenchus buxophilus	G-C	C-G	U○G	C-G	C-G	A-U	U-A	A-U	G-C	G-C	C-G	A-U
Globodera rostochensis	G-C	C-G	U○G	C-G	U-A	G-C	C-G	C-U	G-U	U-A	C-G	A-U
Rotylenchulus reinformis	G-C	C-G	U○G	G○U	C-G	A-U	U-A	C-U	U-A	G-C	C-G	A-U

across all species. The number and composition of nucleotides were also highly conserved across all species including the two outgroup species. One complementary base change (transitional substitution) was detected at position 2, where the base pairing is U-A for all species except it is C-G for *S. brachyurum*. The numbers of nucleotides for stem cl-a-b composed of from 34 nt to 36 nt (nucleotide). All base pairs are supported with complete or partial complementary base changes except two consecutive GC residues at 5' of c1-a-b stem without consideration of outgroup species. Among them, 19 positions (No.3 to No. 21) have complete complementary base changes which included substitutions of both side of the stem to maintain base pairing interaction and there are also non-canonical base pairings, A●A (No. 7), G●A (No. 8), C●A (No. 8, and No. 10), U●U (No. 10), and C●U (No. 11) in these stems. For example, complete or partial complementary substitutions were found at No. 4, No. 5, No. 7, No. 8, No. 11, No. 12, and No, 13 showing transitional changes (CG ↔UA, CG↔UG, UG↔UA) and No. 3, No. 6, No. 9 and No. 10, showing transversional substitutions (AU↔UA, AU↔CG, and GC↔UA). The number of base pairings for stem c1-a-c ranged from 13 (27 nt) in *Hoplolaimus columbus* to 15 (32 nt) in *Helicotylenchus pseudorobustus*. Six positions (No. 14 to No. 19) consisted of complete and partial complementary base

TABLE 2. Continued.

species	Base pairing at position						
	No.37	No.40	No.41	No.42	No.43	No.44	No.45
Hoplolaimus columbus	A-X	U-A	U●U	U-A	G-C	G-C	U-G
H. seinhorsti	A-X	U-A	U●U	U-A	G-C	G-C	U-G
H. magnistylus	A-U	U-A	A-U	U-A	G-C	G-C	U-G
H. concaudajuvenchus	A-U	U-A	A-U	U-A	G-C	G-C	Y-G
H. galeatus	A-U	U-A	A-U	U-A	G-C	G-C	U-G
Hoplolaimus sp. 1	A-U	U-A	A-U	U-A	G-C	G-C	U-G
Hoplolaimus sp. 2	A●A	U-A	A-U	U-A	G-C	G-C	U-G
Hoplolaimus sp. 3	A-U	U-A	A-U	U-A	G-C	G-C	U-G
Scutellonema brachryrum	G●A	C●A	G○U	C-G	G-C	G-C	U-A
Scutellonema bradys	A-U	C-G	G-C	U-A	A-U	A-U	U-A
Aorolaimus longistylus	A-U	C●A	G○U	U-A	G-C	G-C	U-G
Helicotylenchus pseudorobustus	C○C	C●A	G○U	U-A	G-C	G-C	C-G
Helicotylenchus dihystera	C○C	C●A	G○U	U-A	G-C	G-C	C-G
Helicotylenchus microlobus	A-U	C●A	G○U	U-A	G-C	G-C	C-G
Helicotylenchus vulgaris	A●A	C-G	G○U	U-A	G-C	G-C	C-G
Rotylenchus buxophilus	A-U	C●A	G○U	U-A	C●C	G-C	A-G
Globodera rostochiensis	A-U	U-A	U●U	U-A	G-C	G-C	C-G
Rotylenchulus reinformis	A-U	U-A	U●U	U-A	G-C	G-C	A-G

changes. When compared with *H. pseudorobustus*, other species have nucleotide deletions in the middle of the stem ranging from 3 nt for *Rotylenchus buxophilus* to 6 nt for *H. columbus* and all other *Hoplolaimus* species. However, three consecutive base pairs (CUC:GGG) laid adjacent to the terminal bulge of stem c1-a-c which showed constant nucleotide base pairs except CUC:AGG in *Scutellonema bradys*. This stem had a high level of substitutions with stem c1-a-b and also several insertion/deletion events. However, these variable sites maintained their structures by compensatory base changes. Secondary structures of stem cl-a for all species are presented in Fig. 2.

The number of base pairings for stem c2 is 18bp long and highly conserved nucleotides were observed with five consecutive identical base pairings at the base and also three base pairings at the top of this stem. The transitional change (T↔C) was detected in two positions.

For stem c2-b, two nucleotide deletions occurred at different sites across all species and the numbers of nucleotide consisting of stem c2-b-a ranged from 39 nt to 41 nt. Within stem c2-b-a, nine consecutive base pairs at the 3' end of the stem are highly conserved and few nucleotide substitutions were detected in this region. The six positions showed complete and partial compensatory base pairs (No. 22, to No. 27). Transitional base pairs were detected in all six positions (CG↔UA, CG↔UG). The number of base pairings for stem c2-b-b ranged from 14 (35 nt) for *Scutellonema brachyurum* to 19 (41nt) for *Helicotylenchus pseudorobustus*. The deletion of four base pairings (8nt) occurred in the terminal of stem c2-b-b of *S. brachyurum* (Fig. 3). The two noncanonical A●C and G●A formed an internal bulge in all examined species, including the two outgroup species. The complete compensatory base changes were detected in four positions (No. 28 to No. 32).

Stem c2-c consisted of two subdivided stems (c2-c-a and c2-c-b) and was much longer than the other stems (c1, cl-a, c2b). This stem is composed of at least 46 base pairings. For stem c2-c-a, species composed of 24 nucleotides except *H. columbus* which has one base deletion. In the Hoplolaiminae, one transversational substitution occurred in position 32 (AU:CG). In position 33, and 35, all Hoplolaiminae species have dinucleotides (GC) at position 32 and (CG) at position 33, but *Rotylenchulus reniformis* has (UA) at position 33 and (AU) at position 35. One position showing complete complementary base changes (transitional substitution) was observed at position 34, where the base pairing was G-C for all Hoplolaiminae species and *R. reniformis* but U-A for *G. rostochiensis*. The stem c2-c-b consisted of at least 37 base pairings and separated stem c2-c-a by four nucleotides lateral bulge which had high levels of nucleotide compositions among species. For stem c2-c-b, three consecutive base pairs (GGG:CUC) at 5' end and seven consecutive base pairs (CGGUCGC:GCGACCG) at the terminal of stem were well conserved among all species. One compensatory base change (transitional substitution) was detected at position 38, where *Hoplolaimus* species have U-A and other species have C●A or U-A but *R. reniformis* has C-G residues. When compared with other stems, stem c2-c-b is relatively conserved among all species examined even though it is longer than other stems. The complete transitional substitution was detected in four positions from position 42 to position 45. In position 42, *S. brachyurum* has C-G but other species have UA. In position 43, and 44, *R. reniformis* has AU whereas other species have G-C except C●C for *Rotylenchus buxophilus*.

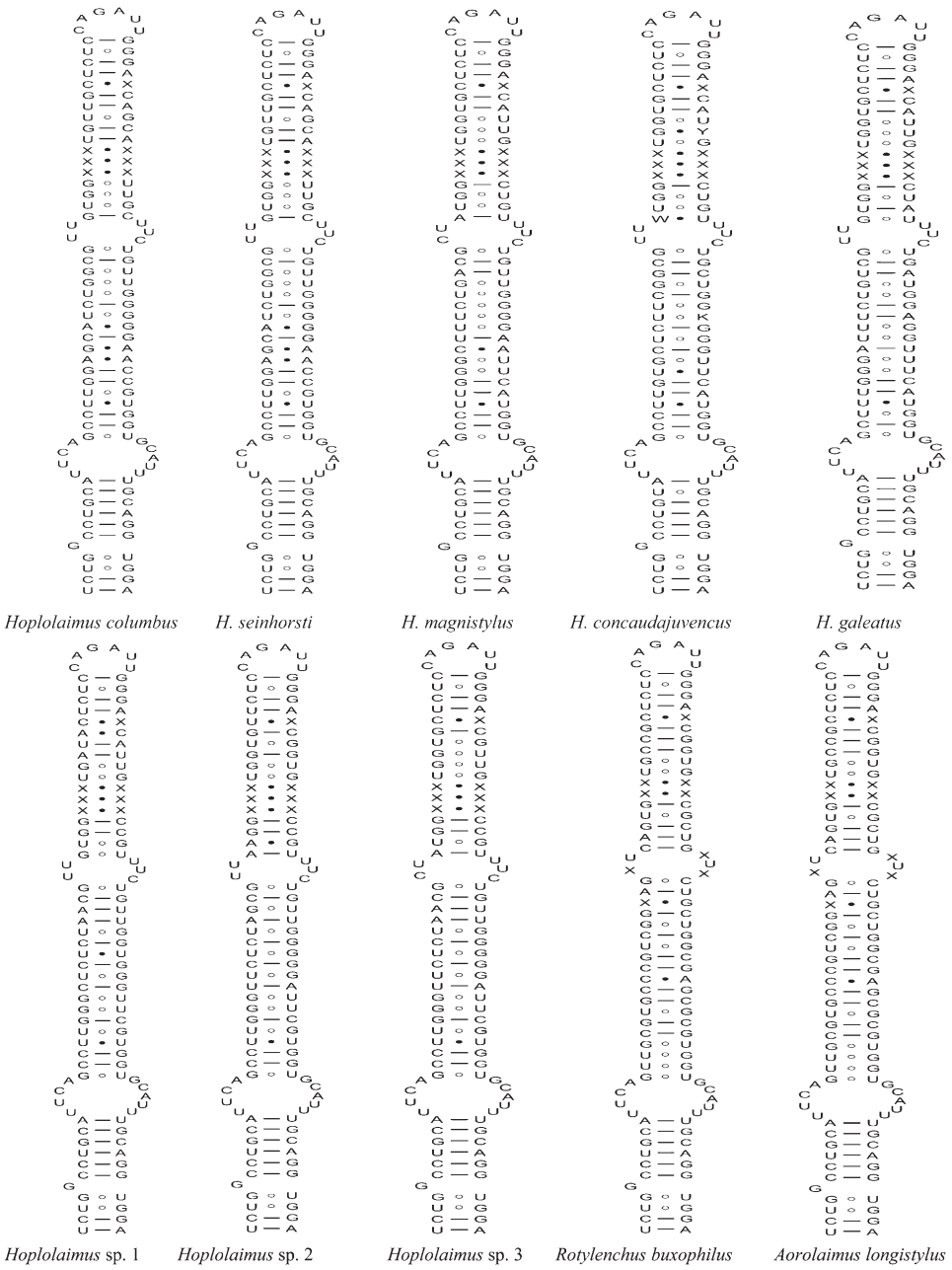

FIG. 2. Predicted secondary structure for stem C1 of D2 expansion domain for Hoplolaiminae and outgroup species.

D3 expansion domain secondary structures for individual species of Hoplolaiminae: The 28S-D3 which consists of 165 to 169 nucleotides, had six stems in all species, labeled " d2, d3, d4, d4_1, d5, and d5_1 following the notation of Chilton et al. (2003). The sequence and predicted secondary structures of D3 domain are showed in Fig. 4. The nucleotides related with base pairings ranged from 68.2% of *Hoplolaimus magnistylus* to 73.1% of *Rotylenchulus reniformis* (Table 2). The nucleotide composition of GC content in stem region ranged from 63.7% of *Hoplolaimus galeatus* to 70.9% of *Scutellonema brachyrum* whereas GC content in loop region ranged from 33.3% of *H. galeatus* to 45.4% *S. brachyrum.*

The predicted secondary structure consists of six stems. The stem d2 of the predicted secondary structure of D3 expansion domain for all species of Hoplolaiminae was formed by complementary base pairings of the 3' and 5' end of D3 region. Positions of complementary base changes found in the D3-28S gene secondary structure model for all Hoplolaiminae species are presented (Table 3). For stem d3, incomplete transitional base changes (A↔G, C↔U) occurred in three positions. Stem d4 is the shortest stem among D3 stems, consisting of one canonical base pair (A-U) and one wobble pair (G○U) from all species including three outgroup species. Stem d4_1 consists of 6 base pairings and has one complete

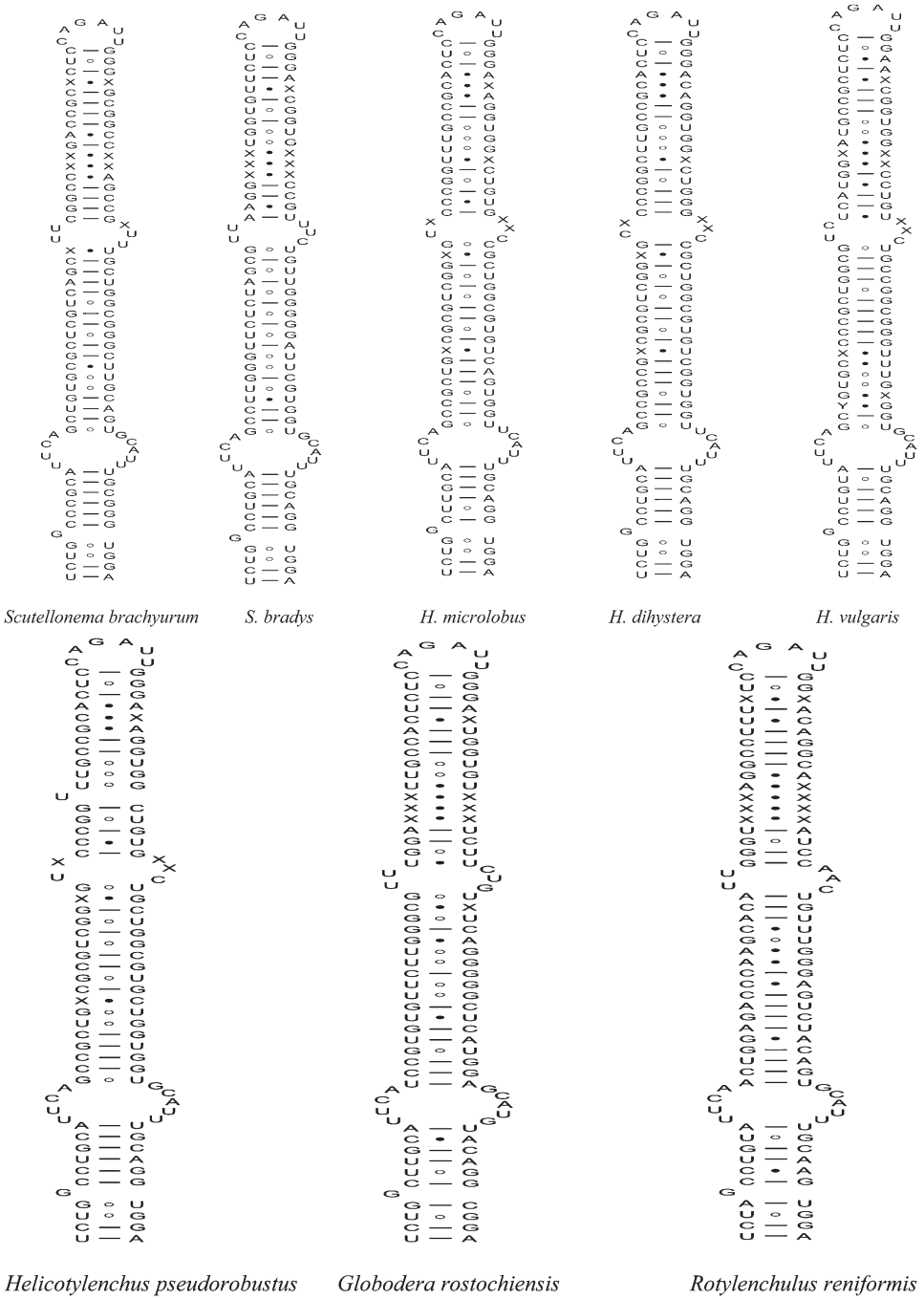

FIG. 2. Continued.

transitional compensatory base change at position No. 1, where the base pairing is C-G for *Hoplolaimus concaudajuvenchus,* but U-A for all other species. In position 2, the base pairing is A-U for *H. concaudajuvenchus* but C-G for all other species. For stem d5, position No. 3 and No. 4 show complete compensatory base pairs, where most species have UA in position No. 3 but *S. brachyrum* and *Helicotylenchus vugaris* have CG. The internal bulge composed of GAC:CGCA was found in all species. The stem d5_1 composed of 38-39 nucleotides and three complete compensatory base changes (transitional substitution) were discovered in position 4, 5 and 6. In position 4, *S. brachyrum* has GC whereas other species have AU residues. In position 5, complete and partial compensatory base changes are detected (CG↔UG↔UA).

DISSCUSSION

The reconstruction of reliable phylogenetic trees can be approached through accurate sequence alignments obtained from correct assignment of homologous characters. Many species show differences in sequence length

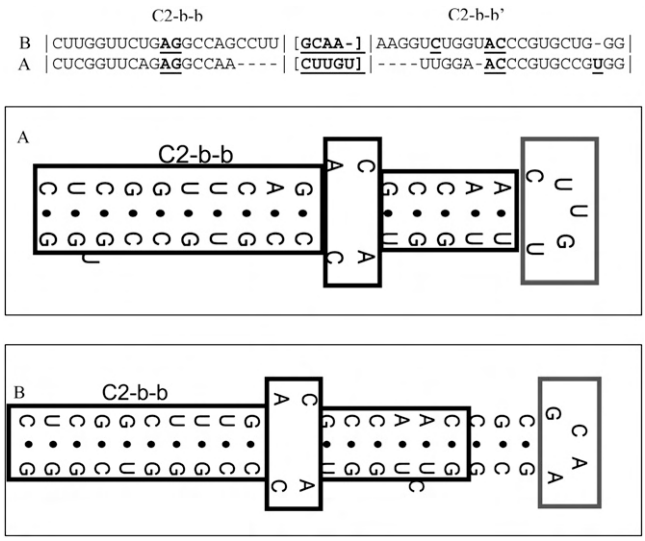

FIG. 3. Predicted secondary structure and sequence alignment based on secondary structure of stem C2-b-b of D2 expansion domain for *Sctutellonema brachyurum* (A) and *Helicotylenchus pseudorobustus* (B)

and composition and this discrepancy make sequence alignment more complicated and subjective due to gaps which were added to increase sequence identity. Sometimes, this sequence alignment produces different phylogeny history (Chilton et al., 1998; Hung et al., 1999; Kjer. 1995; Subbotin et al., 2005, 2007). Sequence alignments based on secondary structure has been used as meaningful tools to approach reconstruction of more reliable phylogenetic analyses by providing accurate sequence alignment. There are different evolutionary functional constraints between stem and loop sequences because of the need to preserve secondary structure in the stem region. Conserved secondary structure exists

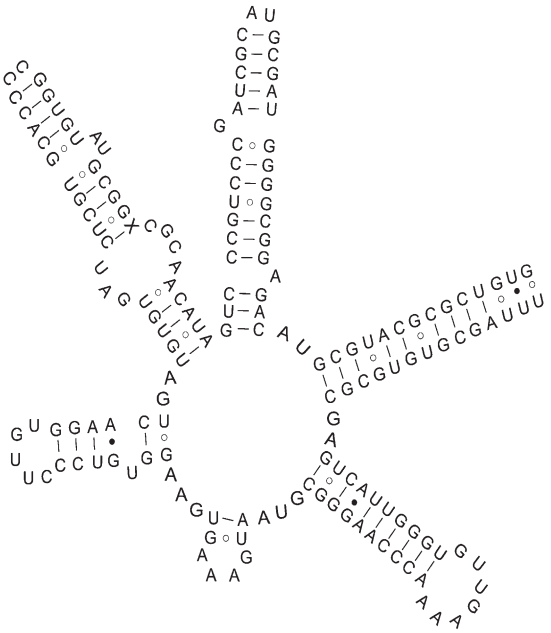

FIG. 4. Predicted secondary structure model of the D3 expansion domain for *Hoplolaimus columbus.*

TABLE 3. Positions of complementary base changes found in the D3-28S gene secondary structure model for Hoplolaiminae.

	Base pairing at position					
	d4_1		d5	d5_1		
species	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Hoplolaimus columbus	U-A	C-G	U-A	A-U	C-G	C-G
H. seinhorsti	U-A	C-G	U-A	A-U	C-G	C-G
H. magnistylus	U-A	C-G	U-A	A-U	U-A	C-G
H. concaudajuvenchus	C-G	A-U	U-A	A-U	U-G	C-G
H. galeatus	U-A	C-G	U-A	A-U	U-G	C-G
Hoplolaimus sp. 1	U-A	C-G	U-A	A-U	U-G	C-G
Hoplolaimus sp. 2	U-A	C-G	U-A	A-U	U-G	C-G
Hoplolaimus sp. 3	U-A	C-G	U-A	A-U	U-G	C-G
Scutellonema brachryrum	U-A	C-G	C-G	G-C	C-G	C-G
Scutellonema bradys	U-A	C-G	U-G	A-U	U-A	C-G
Aorolaimus longistylus	U-A	C-G	U-A	A-U	C-G	C-G
Helicotylenchus pseudorobustus	U-A	C-G	U-A	A-U	C-G	U-A
Helicotylenchus dihystera	U-A	C-G	U-A	A-U	C-G	U-A
Helicotylenchus microlobus	U-A	C-G	U-A	A-U	C-G	U-A
Helicotylenchus vulgaris	U-A	C-G	C-G	A-U	U-A	U-A
Rotylenchus buxophilus	U-A	C-G	U-A	A-U	C-G	C-G
Globodera rostochiensis	U-A	C-G	U-A	A-U	U-A	C-G
Rotylenchulus reinformis	U-A	C-G	U-G	A-U	U-A	C-G

across distantly related lineages for rRNA genes and therefore, alignment position was recognized as homologous if they located at the same position in the secondary structure model (Hickson et al., 1996; Hung et al., 1999). Comparative sequence analysis with minimum energy models has proven to be useful in predicting base pairings in stem and to confirm potential positional covariance to maximize sequences homology. Comparative sequence analysis of relatively closely related species provides important information for refining secondary structure features (Gillespie et al., 2004; Hung et al., 1999; Springer and Douzery 1996; Wang and Lee. 2002; Subbotin et al., 2007).

As a genetic marker, D expansion segments have been used in a wide variety of different taxonomic lineages (Al-Banna et al., 1997; Al-Banna et al., 2004; Duncan et al., 1999; Subbotin et al., 2005, 2007). Among twelve D domains in nematodes, D1, D2, and D3 domains are particularly important for resolving phylogenetic relationships within closely related taxonomic groups although other domains have also important information for species diagnostics and phylogenetic analysis (Al-Banna et al., 1997; Baldwin et al., 1977; de Bellocq et al., 2001; De Luca et al., 2004; Duncan et al., 1999; He et al., 2005; Kaplan et al., 2000; Subbotin et al., 2005).

Secondary structures of D2 and D3 expansion segments of 28S obtained in our study are in agreement with the consensus secondary structures of these segments earlier proposed for Hoplolaimidae and reconstructed for *H. seinhorsti, S. brachyurum, H. pseudorobustus* and *H. vulgaris* by Subbotin *et al.* (2007). In this study, *Hoplolaimus columbus* and *H. seinhorsti* showed identical sequences in the D2 and D3 domains and this may be

because these two species diverged very recently. Although these two parthenogenetic species have genetically identical sequences, this rRNA gene is considered a good target region for phylogenetic and species diagnostic markers. In the subfamily Hoplolaiminae, the 28S-D2 and D3 expansion segments shows similarity in length (359-371bp in the D2 and 167-169bp in the D3 region) and GC content (56.3-66.2%) from all species examined with outgroup species even though a high level of sequence divergence existed among species. Among the D domains examined, D2 had more genetic variation than other two regions, D1 and D3. When the size of the D2 was compared with other nematode species, the length of D2 domain (359-371bp) is shorter than *Longidorus* species (500bp) (De Luca et al., 2004) but longer than that of *Labiostrongylus bipapillosus* (224bp) and *C. elegans* (286bp) (Chilton et al., 2003; Ellis et al., 1986). In nucleotide composition analysis, GC content in D2+D3 region ranged from 56.3% of *Rotylenchulus reniformis* to 66.2% of *Scutellonema brachyurum.* A GC rich region exists in D domain of 28S gene of other nematodes, such as D3 of *Globodera rosotcheisis* (GC=55.1%, Genbank AF393842), D2-D3 of *Xiphinema index* (GC=55.4%, Genbank; AY601628) and *C. elegans* (D2; 56.2%, D3; 54.3%). However, other nematode species including Strongylida (bursate nematodes) showed that AT content was very rich (combined D1+D2: 61.1-65.5%; D2 alone: 64.8-70.4%) in D1 and D2 expansion regions (de Bellocq et al., 2001). Sequence comparison between stem and loop region of Hoplolaiminae species including outgroup species shows structure related GC content biases in base composition; 1) GC contents (61.7 to 71.9%) of 28S-D2 domain are higher than AU contents in stem region and GC contents (63.7 6 to 70.9%) in stem region of D3 are also higher than AU contents; 2) The frequency of adenine increases in loops when compared to that in stems (loops: 25.8-36.1% vs stem: 7.2-12.8%). Gillespie et al. (2004) observed that paired regions have about 40% guanine and this results in its crucial property to form hydrogen bonds with both cytosines and uracil. Most base pairings within stems in the D2 and D3 regions consist of A-U or C-G but a small percentage of base pairings composed of G-U which is thermodynamically less stable. Unlike high GC contents of rRNA gene, AT rich content can also form secondary structure in the ITS-2 region with lowest ΔG value in trichostrongylid nematodes (Chilton et al., 1998).

At least 75% of nucleotides from examined nematode species are involved in formation of base pairings in the stems. Chilton et al. (2003) proposed the complete sequence and secondary structure model of 28S for the parasitic nematode *Labiostrongylus bipapilosus* and compared it with that for *Caenorhabditis elegans.* They found that the total sequence difference between these two lineages is 14% by sequence alignment based on secondary structure, and among the total sequence differences, 36% sequence difference occurred in unpaired region. In structure comparison, Chilton et al (2003) showed stem c2 as 9-bp structure in the D2 and it is identical with secondary structure model of Hoplolaiminae. Other species, *Xiphinema brevicollum* and *Mesocriconema xenoplax* had 8-bp and 12-bp structures, respectively (He et al., 2005; Subbotin et al., 2005). Our 28S-D2 and D3 domain model is similar to those of Chilton et al. (2003), He et al. (2005), and Subbotin et al. (2005, 2007). However, an important difference in the D2 model is that the number of base pairings and nucleotides in stem c2-c in Chilton's model are much shorter than those of other models; 14bp (5 base pairings) in *Labiostronggylus bipapilosus* and therefore, a sequence length difference at least 90 bp in other species. In a D3 secondary structure model, Subbotin et al. (2005) found that D3 structure is relatively conserved in studied Longidoridae species except the D4_1 stem and loop region that shows variations that some *Longidorus* species did not have this region. In our study, all species have this region and are structurally conserved in all species studied. The predicted secondary structure model for Hoplolaimids consists of relatively long helix (c1-a, c2-b and c2-c), and the inner most helix (c1) which is composed of compensatory base pairings of 3' and 5' end of D2 domain. Among stems, stem c1-a showed to be the most variable in the number of base pairings and nucleotide composition. Among stems in the D2 region, stem c2 and stem c2-c are more conserved than stem c1-a and c2-b. The conserved stems showed less frequency of positional covariation than more variable stems. Unlike D2 expansion domain, D3 domain is structurally more conserved than D2.

According to previous studies, different mutation rates may accumulate in between double stranded and single stranded regions (Vawter and Brown 1993). They suggested that stem, loop, and bulge regions show the same evolution rate whereas single-stranded region show the slowest rate among them due to interaction with proteins (Woese et al. 1983). In double stranded regions, one base mutation repaired another corresponding base in the manner of compensatory base changes whereas mutation occurred in single stranded region was generated independently.

In the statistical analysis, the ratio between transitions to transversions shows that more transitions in stem region were observed than loop region because of structure constraints to maintain paired regions (Chilton et al. 2003; Gillespie et al., 2004; Springer and Douzery 1996; Vawter and Brown 1993). A certain transition (C↔T) occurs in higher frequency than another transition (A↔G) in stems and loop whereas transversions (A↔T and A↔C) in loop region occurs at a higher rate than transitions (A↔G) (Vawter and brown 1993). In our study, single transitional base changes (A:U↔G:U and G:U ↔G:C) are very common. Two transitional changes (A:U↔G:C or U:A↔C:G) also frequently occurred. However, changing from A:U↔U:A and G:C↔C:G

occurred less because these changes need two direct changes to decrease the possibility of unpaired transitional events in base pairings.

Ribosomal RNA (rRNA) array consists of tandemly repeated copies of the transcription unit for 18S, 5.8S, and 28S rRNA with two internal transcribed spacers, ITS1, and ITS2 (Hillis and Dixon, 1991). In most cases, multiple copies are similar or the same by concerted evolution, which results in homogenization among both homologous and non-homologous chromosomes (Hillis and Dixon 1991). However, several researchers have found heterogeneity of rRNA among copies within an individual (Carranza et al., 1996; Hosny et al., 1999). Heterogeneity was detected from the D2 and D3 domain of *Hoplolaimus concaudajuvencus* and D2 from *Helicotylenchus vulgaris*.

Our prediction of secondary structure for five different genera in Hoplolaiminae and two different outgroup genera provides important suggestions, clues and explanations for studying their phylogeny. Many previous studies that performed phylogenetic analysis using different loop and stem weightings and different root-stem weighting schemes are still being debated (Dixon and Hillis, 1993; Springer and Douzery, 1996; Wang and Lee, 2002). The subfamily Hoplolaiminae is an important group, systemically related to the subfamily Heteroderinae in some morphological aspects. In our study, secondary structure of *Globodera rostochienesis* was proposed and aligned with Hoplolaiminae species based on secondary structure. This sequence alignment provided a more reliable sequence alignment with confidence and will improve positional homology among more distantly related species. In genetic analysis, the D2 and D3 expansion segments of the 28S gene shows significant interspecific sequence differences among Hoplolaiminae species, suggesting each domain has informative information as phylogenetic and species diagnostic markers.

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