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Molecular Analysis of the Lance Nematode, *Hoplolaimus* spp., Using the First Internal Transcribed Spacer and the D1-D3 Expansion Segments of 28S Ribosomal DNA[1]

C. H. Bae,[2,3] A. L. Szalanski,[4] R. T. Robbins[2]

Abstract: DNA sequence analyses of the nuclear ribosomal ITS1 region of the ribosomal DNA and D1-D3 expansion segments of the 28S gene were conducted to characterize the genetic variation of six amphimictic *Hoplolaimus* species, including *H. magnistylus*, *H. concaudajuvencus*, *H. galeatus*, *Hoplolaimus* sp. 1, *Hoplolaimus* sp. 2 and *Hoplolaimus* sp. 3, and two closely related parthenogenetic species, *H. columbus* and *H. seinhorsti*. PCR amplifications of the combined D1-D3 expansion segments and the ITS1 region each yielded one distinct amplicon. In the D1-D3 region, there was no nucleotide sequence variation between populations of *H. columbus*, *H. magnistylus*, *Hoplolaimus* sp. 2 and *Hoplolaimus* sp. 3, whereas the ITS1 sequences had nucleotide variation among species. We detected conserved ITS1 regions located at the 3' and 5' end of ITS1 and also in the middle of the ITS1 among *Hoplolaimus* species. These regions were compared with sequences of distantly related *Heterodera* and *Globedera*. PCR-RFLP and sequence analysis of ITS1 and 28S PCR products revealed that several haplotypes existed in the same genome of *H. columbus*, *H. magnistylus*, *H. seinhorsti*, *H. concaudajuvencus* and *Hoplolaimus* sp. 1. Maximum likelihood and maximum parsimony analysis using the combined ITS1 and D1-D3 expansion segment sequences always produced trees with similar topology; *H. columbus* and *H. seinhorsti* grouped in one clade and the other six species (*H. galeatus*, *H. concaudajuvencus*, *H. magnistylus*, *Hoplolaimus* sp. 1, *Hoplolaimus* sp. 2, *Hoplolaimus* sp. 3) grouped in another. Molecular analysis supports morphological schemes for this genus to be divided into two groups based on several phenotypic traits derived from morphological evolution.

Key words: 28S gene, clades, D1-D3 region genome, haplotypes, *Hoplolaimus,* lance, ITS1, nematode

The genus *Hoplolaimus* von Daday, 1905 belongs to the subfamily Hoplolaiminae Filipjev 1934 and includes endo-, ecto- and semi-endoparasitic nematodes. Their stylet knobs are tulip-shaped, and they have a relatively large body length (0.9 - 2 mm) when compared to other Hoplolaiminae genera (Lewis et al., 1976). Twenty-nine species of this genus have been described, of which three species are economically important nematode pests on various agronomic crops: *H. columbus* Sher, 1963, *H. galeatus* (Cobb, 1913), Thorne, 1935 and *H. magnistylus* Robbins, 1982 (Fassuliotis, 1974; Nyczepir and Lewis, 1979; Robbins et al., 1987; Henn and Dunn, 1989; Robbins et al., 1989; Handoo and Golden, 1992; Noe, 1993; Koenning et al., 2004). Species identification of this genus usually relies on morphological and morphometric characters, such as the number of lines in the lateral field, the number of esophageal gland nuclei, stylet length and body length. Based on morphological characteristics, *H. columbus* is grouped with *H. seinhorsti* Luc, 1958, both having a single lateral line and six esophageal gland nuclei, whereas *H. galeatus* is grouped with *H. tylenchiformis* Daday, 1905, *H. californicus* Sher, 1963, *H. concaudajuvencus* Golden and Minton, 1970 and other species having four later lines and three esophageal gland nuclei (Fortuner, 1991).

Traditional identification of species has been determined by interspecific variation of phenotypic traits that rely on morphological and morphometric characters. However, these taxonomic criteria are sometimes not practical because of their limited ability to delimit closely related species due to overlap of key descriptive characters (Hyman, 1990).

DNA-based molecular diagnostics of nuclear ribosomal DNA (rDNA) using PCR techniques have been commonly accepted as an alternative method to solve taxonomic problems. Recently, a comparative sequence analysis that utilizes detectable nucleotide difference in ribosomal DNA has become a useful and accurate tool for phylogenetic analysis and species diagnostics (Subbotin et al., 2001). As a genetic marker, the ribosomal DNA (rDNA) array consists of tandem repeated copies of the transcription units for 18S, 5.8S and 28S rDNA with two internal transcribed spacers, ITS1, and ITS2 (Hillis and Dixon, 1991). The18S and 28S genes are slowly evolving, thus these genes permit construction of universal primers and are useful to infer phylogenetic relationships between higher groups of organisms. The ITS1 and ITS2 region of rDNA can be appropriate to infer phylogeny among closely related species and for species identification (Hillis and Dixon, 1991; Cherry et al., 1997; Szalanski et al., 1997; Ye et al., 2004). The large subunit rDNA (LSU rDNA) 28S gene is the largest region of the rRNA. D expansion segments within LSU rDNA provide highly variable domains, and D expansion sequences can be used to infer phylogenetic relationships among more closely related species (Al-Banna et al., 1997; Duncan et al., 1999; Kaplan et al., 2000; Bellocq et al., 2001; De Luca et al., 2004).

Difficulty in using these sequences as phylogenetic and taxonomic markers may arise from intra-individual and intraspecific variation. It is commonly assumed that multiple copies evolve independently and each copy of rDNA is very similar to other copies within individuals and species due to concerted evolution. Concerted evolution results in homogenization among both homologous and nonhomologous chromosomes (Hillis and Dixon,

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[1]A portion of a PhD dissertation by the first author. Department of [2]Plant Pathology and [4]Entomology, University of Arkansas, Fayetteville, AR 72701.

[3]Current address: National Institute of Biological Resources, Incheon, Korea. E-mail: rrobbin@uark.edu

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1991). However, several researchers have found heterogeneity of the ITS region among several nematode taxa (Cherry et al., 1997; Powers et al., 1997; Szalanski et al., 1997; Body et al., 2000; Subbotin et al., 2000; Olivier et al., 2001).

With regard to mode of reproduction, *H. columbus* and *H. seinhorsti* are parthenogenetic. Parthenogenetic lineages of animals are generally recognized to be genetically identical and show a short-term evolutionary life span with a distribution pattern known as 'geographic parthenogenesis' when compared to sexual lineages (Kearney, 2003). In general, it is believed that parthenogenetic species lack the ability to produce new genotypes for adaptation to new environmental conditions, but have the advantage of colonization and long-term preservation of their own genotype (Little and Hebert, 1997; Maraun et al., 2003; Castagnone-Sereno, 2006). Other parthenogenetic species, however, have 'ancient asexual status' that has survived from a long time ago with proof of fossils (Poinar and Ricci, 1992). In the case of nematodes, some mitotic parthenogenetic species of root-knot nematode (RKN), *Meloidogyne* spp., including *M. incognita* (Kofield and White, 1919) Chitwood, 1949, *M. javanica* Treub, (1885) Chitwood, 1949 and *M. arenaria* (Neal, 1889) Chitwood, 1949 are recognized as exceptions to the general concept of parthenogenetic species. These species show a high level of clonal diversity within an individual, a wide host range, wide geographical distribution and also have good ability to adapt to new and unfavorable environmental conditions such as resistant hosts (Trudgill, 1997; Hugall et al., 1999; Castagnone-Sereno, 2006).

It is important to investigate basic characteristics of these sequences among *Hoplolaimus* species to develop phylogenetic and taxonomic markers. Genetic studies which evaluate interspecific genetic variation of ribosomal DNA in *Hoplolaimus* species are lacking.

The objectives of this study were to: (i) molecularly characterize the ITS1 region and D1-D3 expansion domain of the 28S to investigate the use of a species marker for *Hoplolaimus* species; (ii) infer phylogenetic relationships among eight *Hoplolaimus* species; and (iii) study heterogeneity of the ITS sequence and D expansion region within populations of *Hoplolaimus* species using PCR-RFLP.

Materials and Methods

Hoplolaimus spp. populations: The species name and geographical origin of nematode populations used in this study are presented in Table 1. Nematode samples were acquired between 2002 and 2006 from field soil samples or living specimens in water. Adult females were selected for extraction of total DNA. Eleven populations of *H. columbus*, one population of *H. seinhorsti*, five populations of *H. galeatus*, two populations of *H. magnistylus*, one population of *Hoplolaimus* sp. 1, two populations of *Hoplolaimus* sp. 2, two populations of *Hoplolaimus* sp. 3 and one population *of H. concaudajuvencus* were collected from seven different states.

DNA Extraction: One or two individuals from each population were hand picked and transferred into a 1.5 ml microcentrifuge tube with 0.5 μl RNA-free water. DNA was extracted from individual nematodes with the RED Extract-N-Amp Tissue PCR Kit (Sigma-Aldrich Co., St. Louis, MO).

Amplification, cloning and sequencing of the ITS region and D1-D3 expansion segments of 28S gene: The ITS 1 region and D1-D3 expansion segments of 28S gene were amplified and sequenced to characterize intra- and interspecies variation. The primer sequences used for the ITS1 region, Hoc-1f primer (5'- AACCTGCTGCTG-GATCATTA-3') and Hoc-2r primer (5'-CCGAGTGAT-CCACCGATAA-3'), were designed using comparative sequence alignment with a *Scutellonema bradys* (Steiner and LeHaw, 1933) Andrassy, 1958 sequence from GenBank (AY 271722). The primer sequences used to amplify the D1-D3 expansion segments of 28S gene were LSUD-1f primer (5'- ACCCGCTGAACTTAAGCA-TAT-3'), which was designed using comparative sequence alignment with a *Globodera tabacum* (Lownsbery and Lownsbery, 1954) Behrens, 1975 sequence from GenBank (DQ 097515), and LSUD-2r PCR primer (5'-TTTCGCCCCTATACCCAAGTC-3'), which was designed using comparative sequence alignment with a *Globodera rostochiensis* (Wollenweber, 1923) Behrens, 1975 sequence from GenBank (AY 592993). PCR amplification was conducted with the following protocol: initial denaturation of 95°C for 3 min, followed by 35 cycles of 95°C for 45 sec, 57°C for 1 min 30 sec, 72°C for 2 min, and final extension at 72°C for 10 min. For each PCR run, a negative control without DNA template was included. After amplification, 6 μl of each reaction was loaded onto a 1.5% agarose gel (120V, 50 min) and photographed under UV light.

The PCR product included the 3' portion of 18S gene, the entire ITS1, and 5' portion of the 5.8S gene. Amplicons were resolved on 1% agarose gel, and amplified fragments were purified using the Quantum Prep PCR Kleen Spin Columns (BIO-RAD) and cloned into pDrive cloning vectors (Qiagen, Valencia, CA). The plasmids were transformed into *Escherichia coli* Qiagen EZ Competent cells according to the manufacturer's protocols. Positive clones (white) were obtained through blue/white color selection and further identified by EcoRI restriction digestion at 37°C for 1 hr. The plasmid preparation was extracted using QIAquick Spin Miniprep Kit (Qiagen, Valencia, CA). The University of Arkansas DNA sequencing and Synthesis Facility (Little Rock, AR) sequenced PCR products of ITS region using an ABI Prism 377 DNA sequencer (PE Applied Biosystems, Foster City, CA). DNA

TABLE 1. Populations and species of the lance nematode, *Hoplolaimus* species, selected for comparative sequence analysis.

Sample code	Collection year	Species	Host	Location	GenBank Accession number	
					ITS1	D region
LA67	2003	*H. columbus*	Corn	Pointe Coupee County, LA	EU443781	EU554665
LA92	2003	*H. columbus*	Cotton	Franklin County, LA	EU443782	EU554666
LA94	2003	*H. columbus*	Cotton	Pointe Coupee County, LA	EU443783	EU554667
SC103	2003	*H. columbus*	Cotton	Lee County, SC	EU443784	EU554668
GA105	2003	*H. columbus*	Cotton	UGA research station Midville, GA	EU515313	EU554669
SC144	2004	*H. columbus*	Corn	Dorchester County, SC	EU515314	EU554670
SC147	2004	*H. columbus*	Soybean	Dorchester County, SC	EU515315	EU554671
SC195	2005	*H. columbus*	Cotton	Blackville, SC	EU443780	EU554672
SC196	2005	*H. columbus*	Cotton	Florence, SC	EU515316	EU554673
SC198	2005	*H. columbus*	Soybean	Blackville, SC	EU515317	EU554674
NC242	2006	*H. columbus*	Cotton	Johnston, NC	EU515318	EU554676
GA177	2005	*H. columbus*	Cotton	UGA research station Midville, GA	EU515319	EU554675
TX115	2003	*H. galeatus*	Corn	Texas City, TX	EU515320	EU626784
SC109	2003	*H. galeatus*	Cotton	Colleton County, SC	EU515321	EU626785
FL60	2003	*H. galeatus*	Cotton	B.P.I., FL	EU515322	EU626786
FL184	2005	*H. galeatus*	Bermuda grass	Fort Lauderdale Research and Education center, FL	EU515322	EU626787
FL185	2005	*H. galeatus*	Augustine grass	Fort Lauderdale Research and Education center, FL	EU515324	EU626788
AR221	2005	*H. magnistylus*	Cotton	Portland Ashley County, AR	EU515325	EU626789
AR248	2006	*H. magnistylus*	Willow tree	Hope County, AR	EU515326	EU626790
FL181	2004	*H. seinhorsti*	Peanut	IFAS Experiment Station, Jay, FL	EU515327	EU626791
AR135	2005	*H. concaudajuvencus*	Hackberry	Perry County, AR	EU515328	EU626792
TN241	2006	*Hoplolaimus* sp. 1	?	Smoky Mountain, TN	EU515329	EU626793
IL172	2004	*Hoplolaimus* sp. 2	Turfgrass	University of Illinois, IL	EU515331	EU626794
KS237	2006	*Hoplolaimus* sp. 2	Turfgrass	Manhattan, KS	EU515330	EU626795
SC110	2004	*Hoplolaimus* sp.3	Birch tree	Clemson University, SC	EU515332	EU586798
AL108	2004	*Hoplolaimus* sp.3	Cotton	Belle Mina, Limestone County, AL	EU515333	EU586797

sequences have been submitted to GenBank as accession numbers EU554665 to EU586798.

Restriction enzyme digestion: The ITS region was amplified and digested with restriction enzymes to determine if microheterogenity existed in the ITS and 28S gene. Restriction enzymes were predicted from NEB cutter Software (http://www.neb.com), using available restriction enzymes. Eight microliters of PCR product was digested from 1 hr to overnight in a total volume of 20 μl using each of following restriction enzymes according to manufacturer's recommendations: Hha I, Msp I and Rsa I. Restriction fragments were separated using a 3% TBE agarose gel (Sigma) or 6% polyacrylamide gel (Invitrogen) along with a 100-bp DNA ladder (Invitogen) to measure fragment size and stained with ethidium bromide. Gels were photographed under UV light.

Alignment and Phylogenetic analysis: Consensus DNA 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. A sequence of *Globodera tabacum* (Genebank AF 339502) was used as the outgroup taxon for the ITS1 region, and a sequence of *G. rostocheiensis* (AY592993) for the 28S region. For phylogenetic analysis, DNA sequences of both the ITS1 and 28S D1-D3 regions were aligned and combined using Clustal W (Thompson et al., 1994). A best fit model of nucleotide substitution was selected using the GTR+G model among 64 different models using ModelTest v 3.7 (Posada and Crandall, 1998) (with the AIC criterion for both the 28S and ITS1 datasets) and PAUP* 4.0b10 (Swofford, 2001). "PAUP* was utilized to conduct a maximum likelihood analysis using the GTR+G model of nucleotide substitution. Bootstrapping was performed using either neighbor joining or ML (1,000 replicates) to determine the reliability of obtained topologies. Unweighted parsimony (MP) analysis on the alignments was conducted in PAUP* 4.0b10 (Swofford, 2001) using 10 random addition of sequences with a heuristic search strategy (stepwise starting tree), and gaps were treated as missing data. A bootstrap test was used to test the recoverability of relationships within the tree (Felsenstein, 1985). Parsimony bootstrap analysis included 1,000 resamplings using the Branch and Bound algorithm of PAUP*.

RESULTS

Sequence analysis of the D1-D3 expansion region of 28S: PCR amplification of the D1-D3 expansion region of eight *Hoplolaimus* species yielded a single amplicon approximately of 1.03 kb. Determination of each D1, D2 and D3 expansion domain was conducted by

sequence similarity search using BLAST. The PCR product length of combined D1-D3 expansion regions is 685 bp to 688 bp with 58.8% average GC content for *Hoplolaimus* species; the length of D1 is 153 bp with 56.8 to 62.7% GC content; the length of D2 is 364 to 366 bp with 57.6 to 61.2% GC content; and the length of D3 is 168 bp to 169 bp with 53.9 to 57.1% GC content. Sequence length and GC content in each domain of *Hoplolaimus* species are presented in Table 2. Sequence analysis revealed that each domain of D1, D2 and D3 of the 28S gene was constant in length in all eight species and more conserved than their ITS1 sequences. Among three expansion domains, the highest nucleotide substitution rate was concentrated in the D2 domain, followed by the D1 domain which was more variable than the D3 domain.

For all of the *H. columbus*, two populations (AR211 and AR248) of *H. magnistylus*, *Hoplolaimus* sp. 2 and *Hoplolaimus* sp. 3, no intra-specific variation was observed. The result of sequence comparisons from the five *H. galeatus* populations (FL60, SC109, TX115, FL184 and FL185) revealed four identical populations (FL60, SC109, FL184 and FL185) and one population (TX115) that differed by a single nucleotide in the D2 region.

Pairwise Tajima-Nei distance (Tajima and Nei, 1984) among combined D1-D3 expansion regions of *Hoplolaimus* species showed extensive variation among species (Table 3). Within the genus, genetic variation of combined D1-D3 expansion domain ranged from 0.3% between *H. columbus* and *H. seinhorsti* to 11.6% between *H. seinhorsti* and *H. concaudajuvencus*.

Sequence analysis of the ITS1 region of rDNA: The amplification of the ITS1 using primers Hoc-1f and Hoc-2r yielded one distinct amplicon approximately 650 bp in size for all species. The amplified PCR product included 29 bp of 18S and 29 bp of 5.8S with the ITS1 sequence. The size of ITS1 ranged from 564 to 572 bp (Table 2). The average nucleotide composition of ITS1 was 20.2% (A), 22.9% (C), 28.9% (G), 28.0% (T), 51.8% (G+C) and 48.2% (A+T).

Pairwise Tajima-Nei distance among ITS1 of *Hoplolaimus* species showed high genetic variation among species. Within *Hoplolaimus* species, genetic variation ranged from 3.9 to 5.4% between *H. columbus* and *H. seinhorsti* to 28.8% between *H. seinhorsti* and *Hoplolaimus* sp. 2 (Table 4).

DNA sequence similarity between ITS1 regions: Multiple sequence analysis revealed that both conserved sequence regions and highly variable regions occurred in the ITS1 of *Hopolaimus* species. In sequence comparison with *Globodera tabacum* (Genbank AF 339502) as the outgroup for phylogenetic analysis, conserved blocks (I-IV) including 5', 3' end and in the middle of the ITS1 region ranging in size from 8 bp to 43 bp bases were detected: (i) 15 bp at 5' end of ITS1, (ii) two conserved blocks (II-III) having about 30-43 bp in the middle of the ITS1, and (iii) 9 bp at 3' end of the ITS1. When we added distantly related species such as *G. rostochiensis* (Genbank; AB207271), *G. pallida* (Stone, 1973) Behrens, 1975 (Genbank; AF016871), *Heterodera glycines* Ichinohe, 1952 (Genbank; AF156266) and *Heterodera zeae* Koshy, Swarup, and Sethi, 1971 (Genbank; U89390) to the analysis, a high level of sequence similarity was also detected in restricted regions within the ITS1 sequences.

Heterogeneity of the ITS1 and D1-D3 expansion region of 28S: Heterogeneity with multiple bases at some positions of the D expansion regions within individuals of *H. magnistylus*, *H. concaudajuvencus*, *Hoplolaimus* sp. 1 and *Hoplolaimus* sp. 3, and the ITS1 region within individuals of *H. columbus* has been observed. Intraspecific variation within these species was detected in sequences obtained by direct analysis of the D1-D3

TABLE 2. Nucleotide length (L) and G+C composition (%) of the ITS1 and D1-D3 expansion segments of 28S gene from eight *Hoplolaimus* species.

	D1		D2		D3		D1-D3		ITS1	
Species	L (bp)	G+C (%)	L (bp)	G+C (%)	L (bp)	G+C (%)	L (bp)	G+C (%)	L (bp)	G+C (%)
H. columbus (195)	153	62.7	366	61.2	169	56.7	688	60.7	569	52.9
H. galeatus (FL60)	153	58.8	366	57.9	169	53.9	688	57.6	570	50
H. galeatus (SC109)	153	58.8	366	57.9	169	53.9	688	57.6	570	50
H. galeatus (FL184)	153	58.8	366	57.9	169	53.9	688	57.6	569	49.9
H. galeatus (TX115)	153	58.2	366	57.6	169	53.9	688	57.4	572	50
H. galeatus (FL185)	153	58.8	366	57.9	169	53.9	688	57.6	570	50.1
H. seinhorsti (FL181)	153	62.1	366	61.2	169	56.0	688	60.4	570	54.6
H. magnistylus (AR247)	153	56.8	366	58.2	169	57.1	688	57.7	569	52.2
H. magnistylus (AR211)	153	56.8	366	58.2	169	57.1	688	57.7	569	51.3
H. concaudajuvencus (AR135)	153	58.8	364	58.5	168	57.1	685	58.5	570	52.6
Hoplolaimus sp. 1 (TN241)	153	57.5	365	59.1	169	56.7	687	58.4	564	51.2
Hoplolaimus sp. 2 (IL172)	153	57.5	366	57.6	169	56.7	688	57.7	564	50.3
Hoplolaimus sp. 2 (KS237)	153	57.5	366	57.6	169	56.7	688	57.7	564	49.6
Hoplolaimus sp. 3 (SC110)	153	56.2	366	58.4	169	57.4	688	57.9	568	51.2
Hoplolaimus sp. 3 (AL108)	153	56.2	366	58.2	169	57.4	688	57.9	566	50.7

TABLE 3. Pairwise genetic distance (%) of combined D1-D3 expansion segments of 28S gene among *Hoplolaimus* species (D1/D2/D3/D1-D3).

	Species	1	2	3	4	5
1	*H. columbus*	-				
2	*H. seinhorsti* (FL181)	0.6/0/0/0.3	-			
3	*H. galeatus* (FL60) (SC109) (FL184) (FL185)	4.5/13.2/4.2/9.4	5.2/13.2/4.2/9.7	-		
4	*H. galeatus* (TX115)	4.5/13.2/4.2/9.4	5.2/13.2/4.2/9.7	0.6/0/0/0.15	-	
5	*H. concaudajuvencus* (AR135)	7.2/13.6/7.8/11.3	7.8/13.6/7.8/11.6	9.2/8.8/7.8/8.5	9.2/9.0/7.8/8.6	-
6	*H. magnistylus* (AR221, AR248)	6.5/13.2/4.9/10.0	7.1/13.2/4.9/10.3	4.5/7.1/3.5/5.7	4.5/7.3/3.5/5.9	8.5/8.8/5.7/7.9
7	*Hoplolaimus* sp. 1 (TN241)	6.5/13.6/4.9/10.4	7.1/13.6/5.6/10.6	5.8/8.2/3.5/6.6	5.8/7.9/3.5/6.5	10.5/9.9/5.7/9.1
8	*Hoplolaimus* sp. 2 (IL172, KS237)	7.2/15.4/4.9/11.2	7.8/15.4/5.6/11.5	5.2/9.5/3.5/7.2	5.2/9.8/3.5/7.4	9.2/10.2/5.7/8.8
9	*Hoplolaimus* sp. 3 (SC 110, AL108)	7.2/14.3/5.6/10.8	6.5/14.3/6.3/10.8	5.2/8.2/4.2/6.9	5.2/9.0/4.2/7.1	10.5/9.9/6.4/9.1
1	*H. columbus*					
2	*H. seinhorsti* (FL182)					
3	*H. galeatus* (FL60)					
4	*H. galeatus* (TX115)					
5	*H. concaudajuvencus* (AR135)					
6	*H. magnistylus* (AR 221, AR248)	-				
7	*Hoplolaimus* sp. 1 (TN241)	6.5/6.8/1.4/5.6	-			
8	*Hoplolaimus* sp. 2 (IL172, KS237)	7.1/8.2/2.8/6.8	4.5/8.2/2.1/6.1	-		
9	*Hoplolaimus* sp. 3 (SC110, AL108)	7.1/6.5/3.5/6.1	4.5/6.8/2.1/5.0	1.3/2.1/0.7/1.6	-	

expansion segments and cloning of ITS1. In DNA sequence electropherograms, several minor but clear double peaks in both directions were detected in *H. magnistylus*, *H. concaudajuvencus*, *Hoplolaimus* sp. 1 and *Hoplolaimus* sp. 3. In the case of *H. magnistylus*, restriction maps of the D1-D3 expansion region showed that the restriction sites for Hha I recognized the polymorphism at nucleotide site 57 (C↔T) of D2 expansion region. Digestion with the enzyme Hha I showed two different fragment length patterns corresponding to two haplotypes; the first haplotype is represented by fragments of 437 bp, 271 bp, 100 bp, 80 bp and 60 bp; the second haplotype is represented by fragments of 437 bp, 151 bp, 120 bp, 100 bp, 80 bp, and 60 bp (Fig. 1). Both types occurred in all tested individuals of two *H. magnistylus* populations.

Phylogenetic analysis: Analysis on the number of variable character states and parsimony informative and uninformative characters was conducted on the IT1, D1-D3 and combined data sets (Table 5). Based on maximum likelihood and maximum parsimony analysis of the combined ITS1 D1-D3 dataset, *Hoplolaimus* species formed two distinct groups (Fig. 2). The well-supported clade I included *H. columbus* and *H. seinhorsti,* which reproduced by parthenogenesis, whereas *H. magnistylus*, *H. galeatus*, *Hoplolaimus* sp. 1, *Hoplolaimus* sp. 2 and *Hoplolaimus* sp. 3 are included in clade II, which consisted of amphimictic species.

Genetic variation of D1-D3 regions among species including clade I and clade II showed that within clade II, genetic variation ranged from 1.6% between *Hoplolaimus* sp. 2 and *Hoplolaimus* sp. 3, to 9.1% between *H. concaudajuvencus* and *Hoplolaimus* sp. 1 (Fig. 2, Table 3). The interspecies genetic variation between clade I and clade II ranged from 9.4% between *H. galeatus* and *H. columbus* to 11.6% between *H. seinhorsti* and *H. concaudajuvencus*. The genetic variation between three undescribed species and *H. galeatus* ranged from 6.5% between *Hoplolaimus* sp.1 (TN241) and *H. galeatus*, to 7.4% between *Hoplolaimus* sp.2 (KS237 and IL 172) and *H. galeatus*. Within undescribed species, genetic variation ranged from 1.6% between *Hoplolaimus*

TABLE 4. Pairwise genetic distance (%) of the ITS 1 of rDNA among *Hoplolaimus* species.

	Species	1	2	3	4	5	6	7	8	9	10	11	12
1	*H. columbus* (SC195)	-											
2	*H. seinhorsti* (FL182)	4.6	-										
3	*H. galeatus* (FL 60)	25.0	24.9	-									
4	*H. galeatus* (SC109)	25.0	25.1	0.5	-								
5	*H. galeatus*(TX115)	25.0	24.9	0.7	0.7	-							
6	*H. galeatus* (SC184)	24.7	24.9	0.5	0.5	0.8	-						
7	*H. galeatus* (SC185)	24.7	24.9	0.8	0.8	0.8	-						
8	*H. concaudajuvencus* (AR 135)	27.3	27.4	20.2	20.7	20.6	21.2	-					
9	*H. magnistylus* (AR 221)	23.1	24.5	14.3	14.1	14.5	14.1	14.1	20.9	-			
10	*Hoplolaimus* sp. 1(TN 241)	26.9	27.6	16.6	16.8	17.3	16.8	17.5	24.0	15.6	-		
11	*Hoplolaimus* sp. 2(KS 237)	27.3	28.8	19.0	19.2	19.4	19.2	19.5	23.7	17.1	15.5	-	
12	*Hoplolaimus* sp. 3(SC 110)	27.6	28.4	18.8	18.7	18.9	18.7	19.3	24.1	16.4	15.6	7.1	-

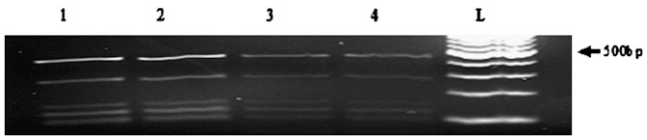

FIG. 1. Restriction enzyme digestion of the D expansion domain of *H. magnistylus* using Hha I. Lanes 1 and 2, AR 211; lanes 3 and 4, AR 247.

sp. 2 and *Hoplolaimus* sp. 3 to 6.1% between *Hoplolaimus* sp. 1 and *Hoplolaimus* sp. 2.

The genetic variation between clade I and clade II was higher than that within clade I and clade II; the genetic variation between *H. columbus* (clade I) and *H. galeatus* (clade II) ranged from 24.7% to 25.0%; the genetic variation between *H. galeatus* (clade II) and *H. magnistylus* (clade II) ranged from 14.1% to 14.5%. The genetic variation between the three undescribed species and *H. galeatus* ranged from 16.6 to 17.5% between *Hoplolaimus* sp. 1 (TN 241) and *H. galeatus* to 19.0 to 19.5% between *Hoplolaimus* sp. 2 (KS237 and 172) and *H. galeatus*. The level of intraspecific genetic variation of ITS1 from 11 populations of *H. columbus* ranged from 0% to 5.3%, whereas five populations of *H. galeatus* ranged from 0 to 0.8% (Table 4).

DISCUSSION

According to Fortuner (1991), the genus *Hoplolaimus* is divided into two groups (ancestral state and derived state) based on several phenotypic traits derived from evolution: first, the number of gland nuclei (ancestral state: three nuclei; derived state: six nuclei); second, the number of lateral lines (ancestral: four lines, derived: less than four lines); third, the position of the excretory pore (ancestral: below the hemizonid, derived: above the hemizonid); fourth, the presence of either regular or irregular striae on the basal lip annulus. In our phylogenetic analysis using a combined ITS1 D1-D3 data set, this morphological classification scheme is well supported by molecular data. Clade I consists of *H. columbus*, *H. seinhorsti* having derived morpholological characters, and clade II consists of *H. galeatus*, *H. magnistylus*, *H. concaudajuvenicus*, *Hoplolaimus* sp. 1, *Hoplolaimus* sp. 2 and *Hoplolaimus* sp. 3 having ancestral morphological characters. This result suggests that these phenotypic characters are informative of phylogenetic relationships.

All *Hoplolaimus* species have similarly sized ITS1 and D expansion regions of 28S rDNA. In nematodes, congeneric species that have similar ITS size length variation include *Heterodera*, *Globodera*, *Ditylenchus*, *Belonolaimus*, *Tricodorus* and *Hoplolaimus* (Powers et al., 1997). However, considerable size variation in the ITS1 can occur. For example, ITS1 can range from 870 bp to 1,354 bp in *Xiphinema* species (Ye et al., 2004) and 791 and 2,572 bp in Coccinelidae beetles due to the presence of repeated elements in the ITS1 sequences; these repeat elements showed variation of occurrence frequency among species (Von der Schulenburg et al., 2001).

The 28S rDNA molecule of nematodes contains relatively conserved core elements, as well as 12 variable expansion domains (Chilton et al., 2003). Among the variable expansion domains, the D domain is commonly used to resolve phylogenetic relationships at lower taxonomic levels and to develop species-specific primers to separate closely related species due to existence of high levels of genetic divergence rates between different lineages within this domain (Al-Banna et al., 1997; Duncan et al., 1999; Subbotin et al., 2000 Al-Banna et al., 2004). Our results show that the combined data of D1-D3 expansion region with each D domain (D1, D2 and D3) is, with the exception of *H. columbus* and *H. seinhorsti*, specific to species level and thus information of sequence variation at certain locations between species can be used to delimit most species. In sequence comparisons between populations of *H. columbus*, *H. magnistylus* and *Hoplolaimus* sp. 2, there was no intraspecific variation in the D1- D3 expansion domain, which is similar to the low levels of genetic variation observed among populations of *H. galeatus* (Subbotin et al., 2000). Maraun et al. (2003) found a lack of intra-individual and intraspecific variation of oribatid species, *Platynothrus peltifer*, in the D3 region of 28S gene, whereas the ITS1 region showed a high level of intra-individual variation (Heethoff et al., 2000). Thus, they argued that the D expansion region of nuclear genome is subject to a higher degree of concerted evolution than the internal transcribed spacer region (ITS). However, we found heterogeneity at D2 region (C↔T) in *H. magnistylus*, and this polymorphism was recognized by PCR-RFLP with Hha I and analysis of DNA sequence electropherograms. This result showed that these two genotypes existed in all individuals from

TABLE 5. Number of variable and constant characters in *Hoplolaimus* species used for phylogenetic analysis.

	ITS1	D1	D2	D3	D1+D2+D3	ITS1 & D1-D3
Total characters	626	155	376	171	716	1322
Constant characters	261	119	257	113	530	772
Variable but parsimony-uninformative	119	17	41	16	74	192
Parsimony-informative characters	246	19	78	12	112	358
CI	0.751	0.788	0.777	0.775	0.806	0.763
Length	666	155	376	40	273	942

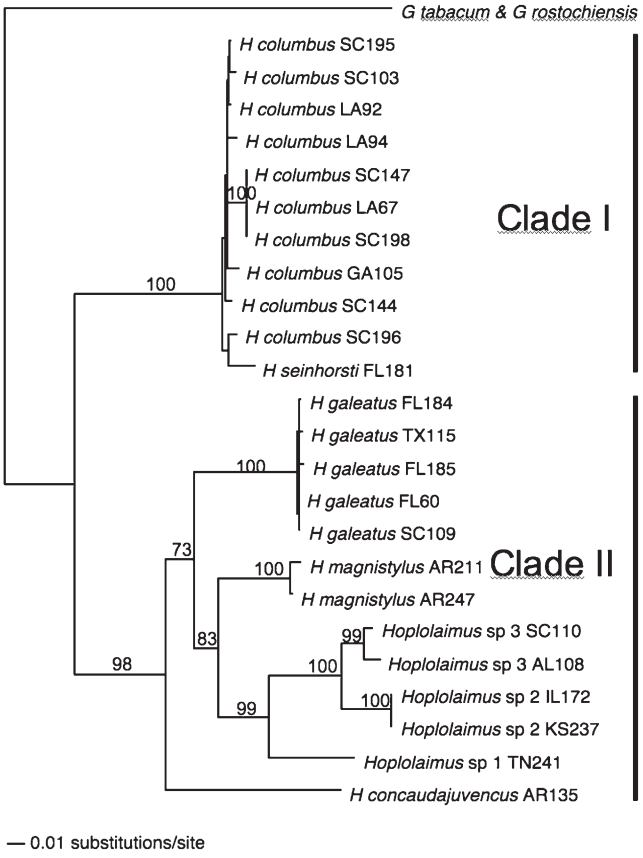

FIG. 2. A phylogenetic analysis for *Hoplolaimus* species based on ITS1 and 28S D1-D3 sequences, derived from maximum likelihood analysis (-ln L = 6255.50036). Bootstrap values from the MP analysis are provided.

both populations (AR221, AR347). However, we cannot conclude that this polymorphism is a result of variation among multiple copies derived from a single rDNA locus, the presence of different loci of the same chromosome, or that there is heterozygosity (Caranza et al., 1996 Roehrdanz et al., 2003). Intra-individual variation of the ITS1 and D expansion domain of 28S gene has been observed from many organisms through multiple sequence analysis and PCR-RFLP analysis (Ibrahim et al., 1994; Bulman and Marshall., 1997; Cherry et al., 1997; Szalanski et al., 1997; Hijri et al., 1999; Zheng et al., 2000; De Luca et al., 2004). This microheterogeneity was derived from amplification of rDNA fragments with two different sizes or the presence of more than one rDNA variant sequence, which can impede application of multigene families for species diagnostics and phylogenetic analysis and also lead to erroneous conclusions (Ibrahim et al., 1994; Powers et al., 1997; Kaplan et al., 2000). Genetic variation of multigene families can be generated based on several biological components including number of gene copies, mutation rate, concerted evolution, number and chromosomal location of loci and interbreeding with sibling species or closely related species (Volger and Desalle, 1994; Ibrahim et al., 1994; Appel and Gordon, 1996; Caranza et al., 1996; Campbell et al., 1997; Odorico and Miller, 1997; Van Herwerden et al., 1998; Hugall et al., 1999; Ko and Jung, 2002). In the case of the parthenogenetic species *H. columbus*, the third hypothesis was chosen as being more consistent with the available sequence information, although more rigorous evidence is needed to demonstrate that this genetic variation is derived from a single rDNA locus.

Among the D domains in this study, the D2 domain is more variable than the D1 domain, while the D3 domain was the least variable (Table 3). The length of the D2 domain is 364 to 366 bp, shorter than *Longidorus* species (500 bp) (De Luca et al., 2004) but longer than that of *Dictyocaulus noerneri* (314 bp, Genbank AF210032) and *C. elegans* (286 bp) (Ellis et al., 1986). In nucleotide composition analysis, GC content of the D domain (57.6-60.4%) was slightly greater than that of the ITS1 (49.6-54.6%). This GC-rich region also exists in the D domain of the 28S gene of other nematodes, such as the D3 of *Globodera rosotchiensis* (GC = 55.1%, Genbank AF393842) and the D2-D3 of *Xiphinema index* Thorne and Allen, 1950 (GC = 55.4%, Genbank; AY601628). However, other nematode species, including Stronglida (bursate nematodes), showed AT content that was very rich (combined D1+D2: 61.1-65.5%; D2 alone: 64.8-70.4%) in the D1 and D2 expansion regions, and this high AT content made the D expansion domain not useful for phylogenetic relationships among the major taxa of the relatively large group (Bellocq et al., 2001).

Maraun et al. (2003) investigated genetic divergence of the D3 region between amphimictic and parthenogenetic oribatid mites and observed that genetic divergence between some parthenogenetic species was larger than that of amphimictic species (parthenogenesis 0-6% vs. amphimictic species 0-1.6%), supporting the view that parthenogenetic species probably persisted for a long period of time (ancient asexual status) having a greater age than amphimictic species and also may radiate slower than amphimictic species. We observed that the gradient in sequence divergence of the D expansion and ITS1 region between *H. galeatus* and three undescribed *Hoplolaimus* species was less than that between *H. galeatus* and other described species. This result showed that amphimictic species contained taxa of different ages. However, limited parthenogenetic species and amphimictic species were used in this study, and thus it is difficult to say whether amphimictic and parthenogenetic lineages radiate in a similar way or not. We did not find putative sexual ancestors of *H. columbus* and *H. seinhorsti*.

The comparative sequence analysis of the ITS1 exhibited extensive sequence divergence among *Hoplolaimus* species as do other nematode species (Ye et al., 2004). Nevertheless, we observed several blocks that were highly conserved across the eight ITS1 sequences, located at (i) 15 bp of 5' end of the ITS1, (ii) two small

dispersed regions of 30 to 43 bp in the middle of the spacer, and (iii) 9 bp of 3' end of the ITS1. This region showed further supporting homology of the observed sequences. These conserved elements have been found at 3', 5' ends and in the middle of the ITS1 and also ITS2 by multiple sequence alignment from nematode, insect, snail and shellfish (Armbruster et al., 2000; Von der Schulenburg et al., 2001; Insua et al., 2003; De Luca., 2004). Armbruster et al. (2000) found six conserved blocks in sequence analysis of the ITS1 obtained from land snails and freshwater snails having Mesozoic origin which may have the possibility of strong divergence. One of the conserved blocks, especially, 3'end of the ITS1 was considered to have an important functional significance, such as ribosomal biogenesis and rRNA processing (Weaver et al., 1997; Insua et al., 2003). In the sequence analysis of the ITS1 blocks with other closely and distantly related species, we found that sequence similarity was distributed through restricted areas, indicating that the blocks have functional constraints, phylogenetically informative signals and also related with conserved secondary structure motifs which found in 5' and 3' end of ITS region (Henry et al., 1994; Von der Schulenburg et al., 2001).

Sequence comparison among the six amphimictic species and the two parthenogenic species illustrates that the ITS1 and D1-D3 expansion domain sequences are useful to delimit most *Hoplolaimus* species. Phylogenetic analysis using both ITS1 and D expansion domain proved that these two regions have accumulated much sequence variation. However, more taxon sampling within *Hoplolaimus* is needed to support the hypothesis that parthenogenetic lineages are diverged from amphimictic species. Without DNA sequence information from other *Hoplolaimus* species, we can not come to the evolutionary conclusion that this heterogeneity has arisen from a hybridization event, or whether amphimictic and apomictic species of this genus radiate with similar divergence rates.

Literature Cited

Al-Banna, L., Ploeg, A. T., Williamson, V. M., and Kaloshian, A. 2004. Discrimination of six *Pratylenchus* Species using PCR and Species-Specific Primers. Journal of Nematology 36 (2): 142–146.

Al-Banna, L., Willamson, V. M., and Gardner, S. L. 1997. Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. Molecular Phylogenetics and Evolution 7:94–102.

Appel, D. J., and Gordon, T. R. 1996. Relationships among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. Molecular Plant-Microbe Interactions 9:125–138.

Armbruster, G. F. J., van Moorsel, C. H. M., and Gittenberger, E. 2000. Conserved sequence patterns in the non-coding ribosomal ITS-1 of distantly related snail taxa. Journal of Molluscan Studies 66:570–573.

Bellocq, J. G., Ferte, H., Depaquit, J., Justine, J., Tillier, A., and Durette-Desset, M. 2001. Phylogeny of the Trichostrongylina (Nematoda) inferred from 28S rDNA sequences. Molecular Phylogenetics and Evolution 19 (3): 430–442.

Body, G., Van der Schoot, J., and Vosman, B. 2000. Heterogeneity of internal transcribed spacer 1 (ITS1) in Tulipa (Liliaceae). Plant Systematics and Evolution 225:29–41.

Bulman, S. R., and Marshall, J. W. 1997. Differentiation of Australasian potato cyst nematode (PCN) populations using the polymerase chain reaction (PCR). New Zealand Journal of Crop and Horticultural Science 25:123–129.

Campbell, C., Wojciechowski, M. F., Baldwin, B. G., Alice, L. A., and Donoghue, M. J. 1997. Persist nuclear ribosomal DNA sequence polymorphism in the Amelanchier Agamic complex (Rosaceae). Molecular Biology and Evolution 14 (1): 81–90.

Carranza, S., Giribet, G., Ribera, C., Baugna, R., and Riutort, M. 1996. Evidence that two types of 18S rDNA coexist in the genome of *Dugesia* (Schmidtea) *mediterranea* (Platyhelminthes, Turbellaria, Tricladida). Molecular Biology and Evolution 13:824–832.

Castagnone-Sereno, P. 2006. Genetic variability and adaptive evolution in parthenogenetic root-knot nematodes. Heredity 96:282–289.

Cherry, T., Szalanski, A. L., Todd, T. C., and Powers, T. O. 1997. The internal transcribed Spacer region of *Belonolaimus* (Nemata; Belonolaimidae). Journal of Nematology 29 (1): 23–29.

Chilton, N. B., Huby-Chilton, F., and Gasser, R. B. 2003. First complete large subunit ribosomal RNA sequence and secondary structure for parasitic nematode: Phylogenetic and diagnostic implications. Molecular and Cellular Probes 17:33–39.

De Luca, F., Reyes, A., Grunder, J., Kunz, P., Agostinelli, A., De Giorgi, C., and Lamberti, F. 2004. Characterization and sequence variation in the rDNA region of six nematode species of the Genus *Longidorus* (Nematoda). Journal of Nematology 36 (2): 147–152.

Duncan, L. W., Inserra, R. N., Thomas, W. K., Dunn, D., Mustika, I., Frisse, L. M., Mendes, M. L., Morris, K., and Kaplan, D. T. 1999. Molecular and morphological analysis of isolates of *Pratylenchus coffeae* and closely related species. Nematropica 29:61–80.

Ellis, R. E., Sulston, J. E., and Coulson, A. R. 1986. The rDNA of *C. elegans*: Sequence and structure. Nucleic Acids Research 14:2345–2364.

Fassuliotis, G. 1974. Host range of the columbia lance nematode *Hoplolaimus columbus*. Plant Disease Reporter 58:1000–1002.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Fortuner, R. 1991. The Hoplolaiminae. Chapter 15:669–719 *in* William R. Nickle. Manual of agricultural nematology. New York: Marcel Dekker, Inc.

Hall, T. A. 1999. Bioedit. A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95–98.

Handoo, Z. A., and Golden, A. M. 1992. A key and diagnostic compendium to the species of the genus Hoplolaimus Daday, 1905. Journal of Nematology 24 (1): 45–53.

Heethoff, M., Maraun, M., and Scheu, S. 2000. Genetische Variabilität der ribosomalen ITS 1-Sequenzen bei der parthenogenetischen Hornmibe Platynothrus (C.L. Koch, 1839) (Acari: Oribatia). Berichte des naturwissenschaftlich-medizinischen Vereins Innsbruck 87:339–354.

Henn, R. A., and Dunn, R. A. 1989. Reproduction of *Hoplolaimus galeatus* and growth of seven St. Augustine grass *(Stenotaphrum secundatum)* cultivars. Nematropica 19 (1): 81–87.

Hijri, M., Hosny, M., van Tuinen, D., and Dulieu, H. 1999. Intraspecific ITS polymorphism in *Scutellospora castanea* (Glomales, Zygomycota) is structured within multinucleate spores. Fungal Genetics and Biology 26:141–151.

Hillis, D. M., and Dixon, M. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. The Quarterly Review of Biology 66 (4): 411–446.

Hugall, A., Stanton, J., and Mortiz, C. 1999. Reticulate Evolution and the origins of Ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. Molecular Biology and Evolution 16 (2): 157–164.

Hyman, B. C. 1990. Molecular diagnosis of *Meloidogyne* species. Journal of Nematology 22:24–30.

Ibrahim, S. K., Perry, R. N., Burrows, P. R., and Hooper 1994. D.J. 1994. Differentiation of species and populations of *Ditylenchus angustus* using a Fragment of Ribosomal DNA. 1994. Journal of Nematology 26 (4): 412–421.

Insua, A., López-piñón, J., Freire, R., and Méndez, J. 2003. Sequence analysis of the ribosomal DNA internal transcribed spacer region in some scallop species (Mollusca: Bivalvia: Pectinidae). Genome 46:595–604.

Kaplan, D. T., Thomas, W. K., Frisse, L. M., Sarah, J. L., Stanton, J. M., Speijer, P. R., Marin, D. H., and Opperman, C. H. 2000. Phylogenetic Analysis of Geographically Diverse *Radopholus similis* via rDNA Sequence Reveals a Monomorphic Motif. Journal of Nematology 32 (2): 134–142.

Kearney, M. R. 2003. Why is sex so unpopular in the Australian desert?. Trends in Ecology and Evolution 18 (2): 605–607.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative study of nucleotide sequences. Journal of Molecular Evolution 16:111–120.

Ko, K., and Jung, H. 2002. Three nonorthologous ITS1 types are present in a polypore fungus *Trichaptum abietinum*. Molecular Phylogenetics and Evolution 23:112–122.

Koenning, S. R., Kikpatrick, T. L., Starr, J. L., Wrather, J. A., Walker, N. R., and Mueller, J. D. 2004. Plant-parasitic nematodes attacking cotton in United States; old and emerging production challenges. Plant Disease 88 (2): 100–113.

Lewis, S. A., Smith, F. H., and Powell, W. M. 1976. Host-Parasite relationships of *Hoplolaimus columbus* on cotton and soybean. Journal of Nematology 8 (2): 142–145.

Little, T. J., and Hebert, P. D. 1997. Clonal diversity in high arctic ostracodes. Journal of Evolutionary Biology 10:233–252.

Maraun, M., Heethoff, M., Scheu, S., Norton, R. A., Weigmann, G., and Thomas, R. H. 2003. Radiation in sexual and parthenogenetic oribatid mites (Oribatida, Acari) as indicated by genetic divergence of closely related species. Experimental and Applied Acarology 29:265–277.

Noe, J. P. 1993. Damage functions and population changes of *Hoplolaimus columbus* on cotton and soybean. Journal of Nematology 25:440–445.

Nyczepir, A. P., and Lewis, S. A. 1979. Relative tolerance of selected soybean cultivars to *Hoplolaimus columbus* and possible effects of soil temperature. Journal of Nematology 11:27–31.

Odorico, D. M., and Miller, D. J. 1997. Variation in the ribosomal internal transcribed spacers and 5.8S rDNA among five species of *Acropora* (Cnidaria: Scleractinia): Patterns of variation consistent with reticulate evolution. Molecular Biology and Evolution 14 (5): 465–473.

Olivier, C., van de Pas, S., Lepp, P. W., Yoder, K., and Relman, D. A. 2001. Sequence variability in the first internal transcribed spacer region within and among *Cyclospora* species is consistent with polyparasitism. International Journal of Parasitology 31:1475–1487.

Poinar, G. O., and Ricci, C. 1992. Bdelloid rotifers in Dominican amber: evidence for parthenogentic continuity. Experientia 48:408–410.

Posada, D., and Crandall, K. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818.

Powers, T. O., Todd, T. C., Burnell, A. M., Murray, C. B., Fleming, C. C., Szalanski, A. L., Adams, B. A., and Harris, T. S. 1997. The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. Journal of Nematology 29 (4): 441–450.

Robbins, R. T., Riggs, R. D., and Von Steen, D. 1987. Results of annual phytoparasitic nematode surveys of Arkansas soybean fields, 1978–1986. Annals of Applied Nematology 1:50–55.

Robbins, R. T., Riggs, R. D., and Von Steen, D. 1989. Phytoparasitic nematode survey of Arkansas cotton fields, 1986–1988. Supplement to the Journal of Nematology 21:619–623.

Roehrdanz, R. L., Szalanski, A. L., and Levine, E. 2003. Mitochondrial DNA and ITS1 differentiation in geographical populations of North corn rootworm, *Diabrotica barberi* (Coleoptera: Chrysomelidae): Identification of distinct genetic populations. Annals of the Entomological Society of America 96 (6): 901–913.

Subbotin, S. A., Halford, P. D., Warry, A., and Perry, R. N. 2000. Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasiting solanaceous plants. Nematology 2 (6): 591–604.

Subbotin, S. A., Vierstraete, A., Ley, P. D., Rowe, J., Waeyenberge, L., Moens, M., and Vanfletern, J. R. 2001. Phylogenetic relationships within the cyst-forming nematodes (Nematoda, Heteroderidae) based on analysis of sequences from the ITS regions of Ribosomal DNA. Molecular Phylogenetics and Evolution 21:1–16.

Szalanski, A. L., Sui, D., Harris, T. S., and Powers, T. O. 1997. Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. Journal of Nematology 29 (3): 255–267.

Swofford, D. L. 2001. PAUP. Phylogenetic analysis using parsimony (* and other method), ver. 4.0b10. Sunderland, MA; Sinauer.

Tajima, F., and Nei, M. 1984. Estimation of evolutionary distance between nucleotide sequences. Molecular Biology and Evolution 1:269–285.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22:4673–4680.

Trudgill, D. L. 1997. Parthenogenetic root-knot nematodes (*Meloidogyne* spp.) how can these biotrophic endoparasites have such an enormous host ranges? Plant Pathology 46:26–32.

Van Herwerden, L., Blair, D., and Agatsuma, T. 1998. Intra- and inter-specific variation in nuclear ribosomal internal transcribed spacer 1 of the *Schistosoma japonicum* species complex. Parasitology 116:311–317.

Vogler, A. P., and DeSalle, R. 1994. Evolution and phylogenetic information content of ITS1 region in the Tiger Beetle Cicindela dorsalis. Molecular Biology and Evolution 1 (3): 393–405.

Von der Schulenburg, J. H. G., Hancock, J. M., Pagnamenta, A., Sloggett, J. J., Majerus, M. E. N., and Hurst, G. D. 2001. Extreme length and length variation in first ribosomal internal transcribed spacer of Ladybird Beetles (Coleoptera; Coccinellidae). Molecular Biology and Evolution 18 (4): 648–660.

Weaver, P. L., Sun, C., and Chang, T. H. 1997. Dbp3p, a putative RNA helicase in *Saccharomyces cerevisiae*, is required for efficient pre-rRNA processing predominantly at site A(3). Molecular and Cellular Biology 17:1354–1365.

Ye, W., Szalanski, A., and Robbins, R. T. 2004. Phylogenetic relationship and genetic variation in *Longidorus* and *Xiphinema* speceis (Nematoda; Logidoridae) using ITS1 sequences of nuclear ribosomal DNA. Journal of Nematology 36 (1): 14–19.

Zheng, J., Subbotin, S. A., Waeyenberge, L., and Moens, M. 2000. Molecular characterization of Chinese *Heterodera glyceines* and *H. avenae* populations based on RFLPs and sequences of rDNA-ITS regions. Russian journal of Nematology 8 (2): 109–113.