2000

Assessing Nuclear and Mitochondrial DNA Sequence Variation within *Steinernema* (Rhabditida: Steinernematidae)

Allen L. Szalanski
University of Nebraska-Lincoln

David B. Taylor
University of Nebraska-Lincoln, dtaylor1@unl.edu

Peter G. Mullin
University of Nebraska-Lincoln

Journal of Nematology 32(2):229–233. 2000.

Assessing Nuclear and Mitochondrial DNA Sequence Variation Within *Steinernema* (Rhabditida: Steinernematidae)[1]

Allen L. Szalanski,[2] David B. Taylor,[3] and Peter G. Mullin[2]

Abstract: DNA sequence analysis was used to characterize the nuclear ribosomal DNA ITS1 region and a portion of the COII and 16S rDNA genes of the mitochondrial genome from *Steinernema* entomopathogenic nematodes. Nuclear ITS1 nucleotide divergence among seven *Steinernema* spp. ranged from 6 to 22%, and mtDNA divergence among five species ranged from 12 to 20%. No intraspecific variation was observed among three *S. feltiae* strains. Phylogenetic analysis of both nuclear and mitochondrial DNA sequences confirms the existing morphological relationships of several *Steinernema* species. Both the rDNA ITS1 and mtDNA sequences were useful for resolving relationships among *Steinernema* taxa.

Key words: DNA sequencing, entomopathogenic nematodes, genetic variability, mitochondrial DNA, nematode, ribosomal DNA ITS, *Steinernema*.

Entomopathogenic nematodes of the genus *Steinernema* Travossos, in conjunction with their symbiotic bacteria, *Xenorhabdus* spp., are pathogenic to many insects (Poinar, 1979) and are effective biological control agents for soil insect pests (Klein, 1990). *Steinernema* species have unique biological characteristics that make accurate identification important. However, morphological classification is difficult (Poinar, 1990). In addition, differentiation of released and native nematodes may be important for evaluating the impact and efficacy of augmentative releases for biological control.

DNA sequencing of the nuclear ribosomal DNA (rDNA) first internal transcribed spacer (ITS1) region and mitochondrial DNA (mtDNA) ND4 gene has proven useful for studying speciation, phylogenetic relationships, and molecular evolution in the Heterorhabditidae (Liu et al.,1997, 1999; Adams et al., 1998). However, published data that compare ITS1 and mtDNA sequence variation of *Steinernema* species are not available. The objective of this study was to assess the level of genetic variation among seven species of *Steinernema* and three strains of *S. feltiae* using DNA sequencing of the nuclear rDNA ITS1 region and from five *Steinernema* spp. using the mitochondrial cytochrome oxidase II (COII)-16S rDNA region.

Materials and Methods

Species, strains, and sources of nematodes used are presented in Table 1. Classification of nematode strains to species follows Poinar (1990) and Nguyen and Smart (1995). Nematodes were reared and harvested using methods similar to Taylor et al. (1998). Voucher specimens have been deposited in the nematode collection of the Department of Plant Pathology, University of Nebraska-Lincoln.

DNA was isolated from approximately 750 infective juveniles using a phenol/chloroform technique per Taylor et al. (1996), and the resulting DNA pellet was resuspended in 100 µl of 10 mM Tris:1 mM EDTA (pH 7.5). The ITS-1 region was amplified with the primers $rDNA_2$ (5′-TTGATTACGTCCCTGCCCTTT-3′, Vrain et al., 1992) and $rDNA_{1.58s}$ (5′-ACGAGCCGAGTGATCCACCG-3′, Cherry et al., 1997). The mtDNA primers, C2F3 (5′-GGTCAATGTTCAGAAATTTGTGG-3′) and LRNB1R (5′-ATAATTTTCCTTTCGTACT-3′), were described by Joyce et al. (1994). These primers amplify a 5′ portion of the mitochondrial CO II gene, tRNA-histidine, and the 16S RNA gene. The PCR protocol

Received for publication 7 February 2000.

[1] Journal Series No. 12600, Agricultural Research Division, University of Nebraska-Lincoln.

[2] Research Assistant Professor (corresponding author), and Graduate Research Assistant, Department of Plant Pathology, 406 Plant Science,University of Nebraska-Lincoln, Lincoln, NE 68583, and [3] Research Entomologist, USDA, ARS Midwest Livestock Insects Research Laboratory, Department of Entomology, University of Nebraska-Lincoln, Lincoln, NE 68583.

E-mail: aszalans@unlserve.unl.edu

The authors thank Jan Jackson for providing nematode stocks. This paper was edited by B. Hyman.

TABLE 1. Species and strains of *Steinernema* spp. used in this study.

Species	Strain	Origin	Source
S. arenarium (Artyukhovshy)			Jackson[a]
S. carpocapsae (Weiser)	Kapow	Kapow, Poland	Jackson[a]
S. feltiae (Filipjev)	UNK-35	South Dakota, USA	Jackson[a]
S. feltiae	SN	France	Jackson[a]
S. feltiae	Umea	Sweden	Pye[b]
S. glaseri (Steiner)			Jackson[a]
S. kushidai Mamiya			Jackson[a]
S. intermedium (Poinar)			Jackson[a]
S. riobrave Cabanillas, Poinar & Raulston		Texas, USA	Cabanillas[c]

[a] Jackson, J. J., Northern Grain Insects Research Laboratory, USDA–ARS, Brookings, SD.
[b] Pye, A., BioLogic Co., Willow Hill, PA.
[c] Cabanillas, H. E., Crop Insects Research Laboratory, Subtropical Agricultural Research Laboratory, USDA–ARS, Weslaco, TX.

for the nuclear DNA amplification reactions was 40 cycles of 94 °C for 45 seconds, 55 °C for 1 minute, and 72 °C for 2 minutes. The PCR amplification profile for the mtDNA amplification reactions consisted of 35 cycles of 94 °C for 1 minute, 48 °C for 1 minute, and 72 °C for 1.5 minutes. Samples were prepared for DNA sequencing following Szalanski et al. (2000) and sent to the DNA sequencing facility at Iowa State University (Ames, IA) for direct sequencing in both directions. Consensus sequences were derived from individual sequences in each direction using GAP (Genetics Computer Group, Madison, WI). Nucleotide sequences have been deposited in GenBank with accession numbers AF192982 to AF192995.

The DNADIST program of PHYLIP v3.57C (Felsenstein, 1993) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura, 1980) of sequence evolution. *Steinernema* DNA sequences were aligned using *Caenorhabditis elegans* rDNA (Ellis et al., 1986) and mtDNA sequences (Okimoo et al., 1992). Maximum likelihood and unweighted parsimony analyses on the alignments were conducted with PAUP* 4.0b2 (Swofford, 1999). Gaps were treated as missing characters for all analysis. The reliability of trees was tested with a bootstrap test (Felsenstein, 1985). Parsimony bootstrap analysis included 1,000 resamplings using the Branch and Bound algorithm of PAUP*. For maximum likelihood analysis, the single most-parsimonious tree generated in our parsimony search was used as the starting tree. Rates were assumed to follow a gamma distribution with shape parameter estimated via maximum likelihood based on the general-time-reversible model (GTR) (Yang, 1994). A total of 286 distinct data patterns were used under this model for the rDNA ITS1 dataset and 260 for the mtDNA data. The starting branch lengths were obtained using the Rogers-Swofford approximation method.

RESULTS AND DISCUSSION

DNA sequencing of the ITS1 PCR-amplified product revealed that it ranged in size from 498 to 527 bp among the seven *Steinernema* species. The mtDNA PCR-amplified product varied in size from 1,084 to 1,092 bp. The average base frequencies were A = 0.24, C = 0.21, G = 0.25, and T = 0.30 for the entire amplified rDNA region, and A = 0.34, C = 0.09, G = 0.14, and T = 0.43 for the amplified mtDNA locus. No nucleotide variation was observed among the three *S. feltiae* strains for both rDNA ITS1 and mtDNA sequences.

The rDNA and mtDNA sequence was aligned for all of the *Steinernema* species using *Caenorhabditis elegans* as the outgroup taxon. The aligned rDNA data matrix including the outgroup taxon resulted in a total of 708 characters, including gaps. Of these characters, 315 (44%) were variable and 120 (17%) were informative. The aligned mtDNA data matrix including the outgroup taxon resulted in a total of 1,107

characters, including gaps. Of these characters, 445 (40%) were variable and 239 (22%) were informative.

Pairwise Tajima-Nei genetic distances among the *Steinernema* taxa ranged between 6 and 22% for the rDNA ITS1 sequences and 12 to 20% for the mtDNA sequences (Table 2). Levels of rDNA ITS1 and mtDNA genetic variation among *Steinernema* species were comparable to those observed in *Heterorhabditis*. Adams et al. (1998) observed 4 to 16% divergence in the ITS1 region of seven *Heterorhabditis* species, while Liu et al. (1999) observed 14 to 23% sequence divergence in a portion of the mtDNA ND4 gene from five *Heterorhabditis* species.

The rDNA dataset had only one most parsimonious tree (Fig. 1A) (Length = 556, CI = 0.78, CI excluding uninformative sites = 0.61), as documented using the Exhaustive search algorithm of PAUP*, which examined all possible trees of this dataset. Bootstrap analysis of the aligned *Steinernema* species and *C. elegans* rDNA ITS1 sequences resulted in a consensus tree with two well-supported branches (Fig. 1A). *Steinernema arenarium* formed a distinct clade with *S. glaseri* relative to the other *Steinernema* taxa. *Steinernema feltiae* formed a distinct clade with *S. kushidai*. Regardless of whether the starting tree was the most parsimonious tree or was obtained via step-wise addition, the maximum likelihood search found only one tree. This maximum likelihood tree was similar to the most parsimonious tree with the exception of *S. carpocapsae, S. riobrave,* and *S. intermedium* forming a common clade (Fig. 1B).

Parsimony analysis of the mtDNA dataset found one most parsimonious tree (Fig. 1C) (length = 690, CI = 0.81, CI excluding uninformative sites = 0.65), as documented using the Exhaustive search algorithm of PAUP*. Bootstrap analysis resulted in a consensus tree with two well-supported branches (Fig. 1C). *Steinernema arenarium* formed a distinct clade with *S. glaseri,* and *S. carpocapsae* formed a clade with *S. riobrave*. The maximum likelihood tree (−Ln likelihood = 4126.74982, estimated value of the gamma shape parameter = 0.346809) was identical to the parsimony tree. When *S. kushidai* and *S. intermedium* were excluded from the rDNA ITS1 phylogenetic analysis, results of the parsimony and maximum likelihood analysis were identical to those of the mtDNA phylogenetic analysis.

A previous study by Hominick et al. (1997) revealed *S. glaseri* to be most similar morphologically to *S. arenarium,* and this relationship is supported by our rDNA ITS1 and mtDNA phylogenies. This relationship was not observed by a phylogenetic study using a 265-bp region of the rDNA 18S genome (Liu et al., 1997) and may be due to a lack of phylogenetically informative characters.

The rDNA ITS1 and mtDNA 16S sequences revealed similar levels of genetic divergence among the studies *Steinernema* taxa. To our knowledge, sequences of the mtDNA 16S-COII region have not been employed for phylogenetic analysis of Nemata. Joyce et al. (1994) found PCR-RFLP of the mtDNA 16S-COII marker to be useful for differentiating species of *Heterorhabditis*. The similarity of genetic variation in the rDNA ITS1 and mtDNA sequences is interesting

TABLE 2. Pairwise genetic divergence among seven *Steinernema*.

	1	2	3	4	5	6	7
1. *S. arenarium*	—	0.055	0.134	0.130	0.155	0.199	0.214
2. *S. glaseri*	0.118	—	0.139	0.143	0.155	0.219	0.218
3. *S. kushidai*	ND	ND	—	0.091	0.126	0.164	0.186
4. *S. feltiae*	0.163	0.166	0.007	—	0.114	0.159	0.186
5. *S. carpocapsae*	0.178	0.186	0.214	0.195	—	0.170	0.171
6. *S. riobrave*	0.145	0.166	ND	0.170	0.158	—	0.214
7. *S. intermedium*	ND	ND	ND	ND	ND	ND	—

ITS1 below the diagonal and mtDNA above the diagonal; ND = no data available.

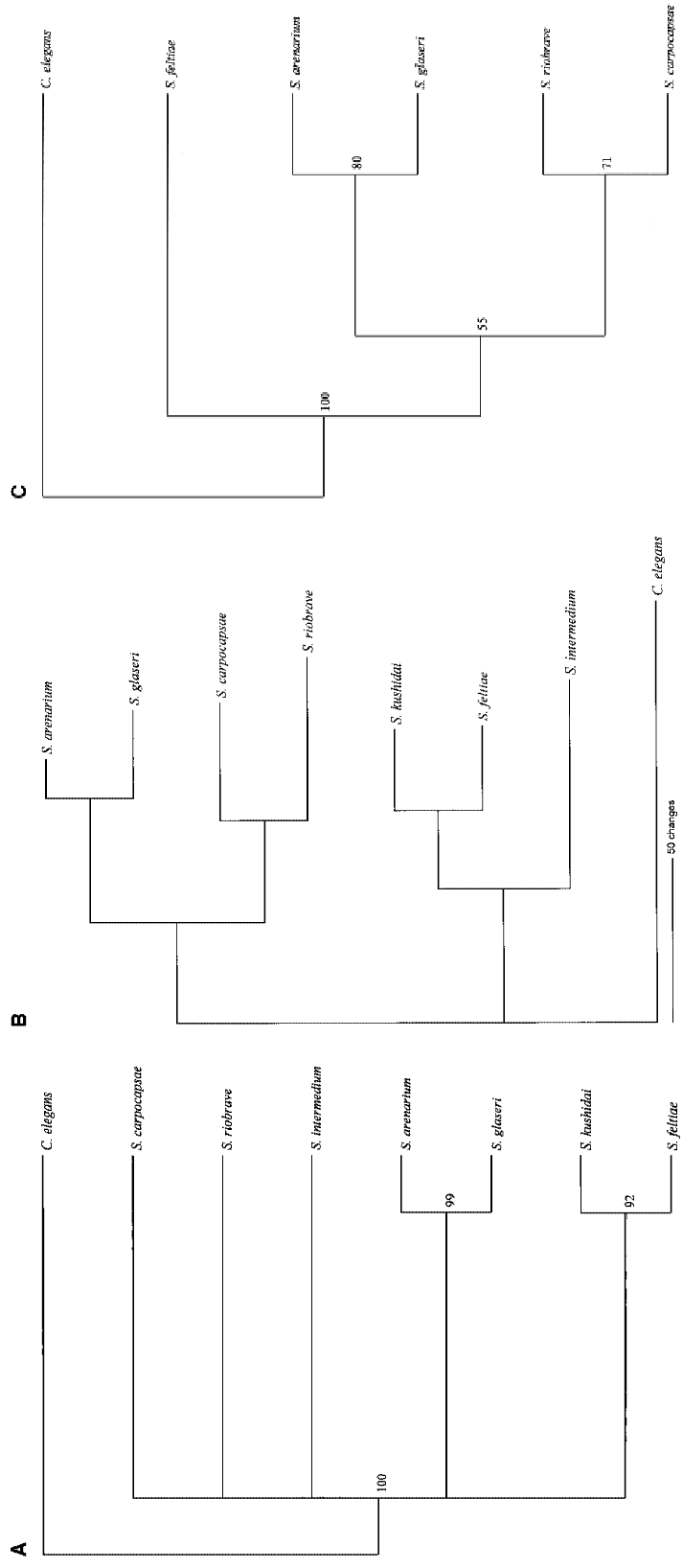

FIG. 1. A) Single most parsimonious tree during an Exhaustive search using PAUP* of rDNA ITS1 sequences with gapped characters excluded. Bootstrap values for 1,000 branch and bound replicates are listed above branches supported at ≥50%. B) Best maximum likelihood tree (–Ln likelihood = –3029.04710, estimated value of gamma shape parameter = 0.615257) based on rDNA ITS1 sequences. Branch lengths are proportional to the number of inferred changes. C) Single most parsimonious tree of mtDNA sequences with gapped characters excluded. Bootstrap values for 1,000 branch and bound replicates are listed above branches supported at ≥50%.

because the mtDNA COII region in Ascaridoids has been found to be saturated and weakly informative at this level of divergence (Nadler, 1995). However, another study by Liu et al. (1999) found the mtDNA ND4 region to be useful for revealing differences within and among species of *Heterorhabditis* and for resolving phylogenetic relationships. It is apparent that generalizations about the utility of mtDNA markers for determining nematode phylogenetic relationships cannot be made until additional taxa have been studied.

The combination of rDNA ITS1 and mtDNA 16S-CO II markers provides an informative dataset for future studies on phylogenetic relationships and molecular diagnostics of *Steinernema* at the species level. Also, based on the level of DNA sequence variation of the mtDNA 16S-COII region in *Steinernema,* this region may be useful for resolving phylogenetic relationships of other Nemata.

Literature Cited

Adams, B. J., A. M. Burnell, and T. O. Powers. 1998. A phylogenetic analysis of the genus *Heterorhabditis* (Nemata: Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. Journal of Nematology 30:22–39.

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

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

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

Felsenstein, J. 1993. PHYLIP: Phylogeny inference package, version 3.57c. Seattle: University of Washington.

Hominick, W. M., B. R. Briscoe, F. G. delPino, J. A. Heng, D. J. Hunt, E. Kozodoy, Z. Mracek, K. B. Nguyen, A. P. Reid, S. Spiridonov, P. Stock, D. Sturhan, C. Waturu, and M. Yoshida. 1997. Biosystematics of entomopathogenic nematodes: Current status, protocols, and definitions. Journal of Helminthology 71:271–298

Joyce, S. A., A. M. Burnell, and T. O. Powers. 1994. Characterization of *Heterorhabditis* isolates by PCR amplification of segments of mtDNA and rDNA genes. Journal of Nematology 26:260–270.

Klein, M. G. 1990. Efficacy against soil-inhabiting insect pests. Pp. 195–214 *in* R. Gaugler and H. K. Kaya, eds. Entomopathogenic nematodes in biological control. Boca Raton: CRC.

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

Liu, J., R. E. Berry, and M. S. Blouin. 1999. Molecular differentiation and phylogeny of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) based on ND4 gene sequences of mitochondrial DNA. Journal of Parasitology 85:709–715.

Liu, J., R. E. Berry, and A. F. Moldenke. 1997. Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rRNA gene sequences. Journal of Invertebrate Pathology 69:246–252.

Nadler, S. A. 1995. Advantages and disadvantages of molecular phylogenetics: A case study of Ascaroid nematodes. Journal of Nematology 27:423–432.

Nguyen, K. B., and G. C. Smart, Jr. 1995. Morphomerics of infective juveniles of *Steinernema* spp. and *Heterorhabditis bacteriophora* (Nemata: Rhabditida). Journal of Nematology 27:213–221.

Okimoto, R., J. L. Macfarlane, D. O. Clary, and D. R. Wolstenholme. 1992. The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum.* Genetics 130:471–498.

Poinar, G. O., Jr. 1979. Nematodes for biological control of insects. Boca Raton: CRC.

Poinar, G. O., Jr. 1990. Taxonomy and biology of Steinernematidae and Heterorhabditidae. Pp. 23–61 in R. Gaugler and H. K. Kaya, eds. Entomopathogenic nematodes in biological control. Boca Raton: CRC.

Swofford, D. L. 1999. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. MA: Sunderland.

Szalanski, A. L., D. S. Sikes, R. Bischof, and M. Fritz. 2000. Population genetics and phylogenetics of the endangered American burying beetle, *Nicrophorus americanus* (Coleoptera: Silphidae). Annals of the Entomological Society of America 93, in press.

Taylor, D. B., A. L. Szalanski, B. J. Adams, and R. D. Peterson II. 1998. Susceptibility of house fly, *Musca domestica* (Diptera: Muscidae), larvae to entomopathogenic nematodes (Rhabditida: Heterorhabditidae, Steinernematidae). Environmental Entomology 27:1514–1519.

Taylor, D. B., A. L. Szalanski, and R. D. Peterson II. 1996. Identification of screwworm species (Diptera: Calliphoridae) by polymerase chain reaction-restriction fragment length polymorphisms. Medical and Veterinary Entomology 10:63–70.

Vrain, T. C., D. A. Wakarchuk, A. C. Levesque, and R. I. Hamilton. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fundamental and Applied Nematology 15:563–573.

Yang, Z. 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: Approximate methods. Journal of Molecular Evolution 39:306–314.