

# Phylogenetic relationships of *Steinernema* Travassos, 1927 (Nematoda: Cephalobina: Steinernematidae) based on nuclear, mitochondrial and morphological data

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## Abstract

Entomopathogenic nematodes of the genus *Steinernema* are lethal parasites of insects that are used as biological control agents of several lepidopteran, dipteran and coleopteran pests. Phylogenetic relationships among 25 *Steinernema* species were estimated using nucleotide sequences from three genes and 22 morphological characters. Parsimony analysis of 28S (LSU) sequences yielded a well-resolved phylogenetic hypothesis with reliable bootstrap support for 13 clades. Parsimony analysis of mitochondrial DNA sequences (12S rDNA and *cox 1* genes) yielded phylogenetic trees with a lower consistency index than for LSU sequences, and with fewer reliably supported clades. Combined phylogenetic analysis of the 3-gene dataset by parsimony and Bayesian methods yielded well-resolved and highly similar trees. Bayesian posterior probabilities were high for most clades; bootstrap (parsimony) support was reliable for approximately half of the internal nodes. Parsimony analysis of the morphological dataset yielded a poorly resolved tree, whereas total evidence analysis (molecular plus morphological data) yielded a phylogenetic hypothesis consistent with, but less resolved than trees inferred from combined molecular data. Parsimony mapping of morphological characters on the 3-gene trees showed that most structural features of steinernematids are highly homoplastic. The distribution of nematode foraging strategies on these trees predicts that *S. hermaphroditum*, *S. diaprepesi* and *S. longicaudum* (US isolate) have cruise forager behaviours.

## Introduction

Nematodes of the genus *Steinernema* Travassos, 1927 (Cephalobina: Steinernematidae) are obligate parasites of insects harbouring mutualistic bacterial symbionts (*Xenorhabdus* spp.) that kill the insect host and digest its tissues, providing nutrients suitable for completing nematode growth and development within insect cadavers (Boemare et al., 1993; Forst & Neelson, 1996). Experimental studies of this mutualism indicate that strains of *Xenorhabdus* native to a particular species of *Steinernema* confer greater fitness than bacteria from congeners, suggesting that these bacterial-nematode interactions reflect a high level of

specialisation and coevolution (Sicard et al., 2004). *Steinernema* isolates and species show substantial (and in certain cases surprising) variation in behaviour, host range, infectivity, reproduction and environmental tolerances. For example, until recently all described species of *Steinernema* have been amphimictic, yet *S. hermaphroditum* Stock, Griffin & Chaenari, 2004 was found to reproduce as a self-fertile hermaphrodite (Griffin et al., 2001). This biological variation has stimulated interest in more fully characterising *Steinernema* genetic diversity because new strains and species may prove more useful than those currently used as biological control agents against agriculturally important pests (Simoes & Sosa, 1996; Brown & Gaugler, 1997; Campbell & Gaugler, 1997; de Doucet et al., 1999).

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Approximately 45 valid species of *Steinernema* have been described; however, many additional geographical isolates have yet to be fully characterised (Stock, 2005). Delimitation of *Steinernema* species has mainly been based on comparison of morphological or morphometric data and cross-breeding tests, with the biological species concept forming the conceptual framework for their delimitation (Poinar, 1990; Hominick et al., 1997; Kaya & Stock, 1997; Nguyen & Smart, 1997). Cross-breeding tests are labour- and time-intensive, and interpretation of morphological features for species identification of *Steinernema* requires substantial expertise to ensure accuracy. Diagnostic methods have been developed based on the polymerase chain reaction (PCR), including PCR-RFLP and Random Amplified Polymorphic DNA (RAPDs) that facilitate species identification by non-experts (Reid & Hominick, 1993; Joyce et al., 1994; Liu & Berry, 1995; Grenier et al., 1996; Stock et al., 1998). Molecular characters have also been used to examine if previously described species and new isolates of *Steinernema* are characterised by sequence differences (Szalanski et al., 2000; Spiridonov et al., 2004) or evidence of lineage independence (Nguyen et al., 2001; Stock et al., 2001; Spiridonov et al., 2004); the latter is required for species delimitation by evolutionary species concepts (Adams, 1998; Nadler, 2002). The application of nucleotide sequence data in conjunction with evolutionary (phylogenetic) species concepts represents a relatively new approach to finding and describing new species of nematodes (Adams, 1998; Baldwin et al., 2001; Nadler, 2002; Nadler et al., 2003), including steinernematids (Stock & Koppenhöfer, 2003; Stock et al., 2004).

Explicit evolutionary hypotheses for *Steinernema* spp. have mainly been based on molecular data from nuclear ribosomal DNA (rDNA), although mitochondrial DNA sequences have been used to investigate five species (Szalanski et al., 2000). The first *Steinernema* tree based on rDNA data (Reid, 1994) included 12 species and represented their relationships as a phenetic (similarity) dendrogram of genetic distances calculated from ribosomal DNA restriction sites. Subsequent molecular evolutionary investigations have been based on rDNA RFLP patterns (Reid et al., 1997), and combined analyses of morphological data and RAPD markers (Liu & Berry, 1996). Liu et al.

(1997) published the first nucleotide-based evolutionary hypothesis for steinernematids based on partial sequences of 18S (SSU) ribosomal DNA for eight *Steinernema* species. Subsequent studies have focused on less conserved genes, including 28S (LSU) rDNA (Stock et al., 2001), regions spanning the rDNA internal transcribed spacers (ITS-1, 5.8S, ITS-2; (Szalanski et al., 2000; Nguyen et al., 2001; Nguyen & Duncan, 2002; Spiridonov et al., 2004), and mitochondrial cytochrome oxidase subunit 2 and 16S rDNA sequences (Szalanski et al., 2000). Stock et al. (2001) presented phylogenetic hypotheses based on analysis of 28S (LSU) rDNA, independent analysis of morphological data and combined phylogenetic analysis of LSU sequences plus morphological characters. These phylogenetic trees were used to develop hypotheses for the evolution of certain complex traits, including foraging behaviour and morphological features (Stock et al., 2001; Campbell et al., 2003).

Stock et al. (2001) included substantial representation of described *Steinernema* species (21 of 25 described species available at that time) in their phylogeny. Spiridonov et al. (2004) included 84 *Steinernema* isolates representing 24 nominal species plus 16 putative undescribed species in a phylogenetic analysis of ITS sequences. A limitation of both these molecular hypotheses is that they were inferred using data from a single genetic locus (nuclear ribosomal DNA). To address the potential limitations of single-locus molecular hypotheses, phylogenetic relationships among 26 *Steinernema* taxa were estimated herein using sequences from 2 mitochondrial genes (12S rDNA and cytochrome *c* oxidase subunit 1), one nuclear gene (28S rDNA) and 22 morphological characters. The resulting phylogenetic hypotheses were used to examine patterns of evolutionary change for selected morphological and behavioural characters between species.

## Materials and methods

### *DNA amplification and sequencing*

Twenty-six *Steinernema* isolates representing 25 described species were used for sequencing, morphological study and phylogenetic analysis (Table 1). These nematode isolates were reared

Table 1. Specimen information and GenBank accession numbers for *Steinernema* species used in the phylogenetic analyses. Underlined accession numbers represent sequences published in Stock et al. (2001)

Species	Isolate name	Geographic origin	Voucher specimens	GenBank Numbers LSU, 12S, <i>cox 1</i>	Source
1. <i>S. abhasi Elawad</i> , Ahmad & Reid, 1997		Sultanate of Oman	UCDNC 3742–44	<u>AF331890</u> , AY944002, AY943976	B.R. Briscoe
2. <i>S. affine</i> (Bovien, 1937)	B1	England	UCDNC 3745–47	<u>AF331899</u> , AY944003, AY943977	B.R. Briscoe
3. <i>S. anatoliense</i> Hazir, Stock & Keskin, 2003	36–9	Kars, Turkey	UCDNC 3920–22	AY841761, AY944004, AY943978	S.P. Stock
4. <i>S. arenarium</i> (Artyokhovski, 1967)		Voronezh, Central Russia	UCDNC 3748–50	<u>AF331892</u> , AY944005, AY943979	H.K. Kaya
5. <i>S. bicornutum</i> Tallosi, Peters & Ehlers, 1995		Vojvodina, Yugoslavia	UCDNC 3751–53	AF331904, AY944006, AY943980	R.U. Ehlers
6. <i>S. carpocapsae</i> (Weiser, 1955)	All	Georgia, USA	UCDNC 3754–57	AF331900, AY944007, AY943981	H.K. Kaya
7. <i>S. ceratophorum</i> Jian, Reid & Hunt, 1997		Jining, China	UCDNC 3807–10	AF331888, AY944008, AY943982	B.R. Briscoe
8. <i>S. cubanum</i> Mrázek, Hernandez & Boemare, 1994		Pinar del Rio, Cuba	UCDNC 3758–60	<u>AF331889</u> , AY944009, AY943983	Z. Mrázek
9. <i>S. diaprepesi</i> Nguyen & Duncan, 2002		Florida, USA	UCDNC 3923–25	AY944001, AY944010, AY943984	L. Duncan
10. <i>S. feltiae</i> (Filipjev, 1934)	Bodega Bay	California, USA	UCDNC 3761–63	<u>AF331906</u> , AY944011, AY943985	S.P. Stock
11. <i>S. glaseri</i> (Steiner, 1929)	NC	North Carolina, USA	UCDNC 3764–66	<u>AF331908</u> , AY944012, AY943986	H.K. Kaya
12. <i>S. hermaphroditum</i> Stock, Griffin and Chaenari, 2004	T87	Ambon Island, Indonesia	UCDNC 3926–28	AY598358, AY944013, AY943987	S.P. Stock
13. <i>S. intermedium</i> (Poinar, 1985)	SC	South Carolina, USA	UCDNC 3767–69	AF331909, AY944014, AY943988	H.K. Kaya
14. <i>S. kari</i> Waturu, Hunt & Reid, 1997		Kirinyaga, Kenya	UCDNC 3770–72	AF331902, AY944015, AY943989	B.R. Briscoe
15. <i>S. kraussii</i> (Steiner, 1923)	Westphalia	Westphalia, Germany	UCDNC 3773–75	AF331896, AY944016, AY943990	Z. Mrázek
16. <i>S. kushidai</i> Mamiya, 1988		Shizuoka, Japan	UCDNC 3776–78	AF331897, AY944017, AY943991	H.K. Kaya
17. <i>S. longicaudum</i> Shen & Wang, 1992 (CH)	B2	Shandong, China	UCDNC 3782–84	AF331894, AY944018, AY943992	B.R. Briscoe
18. <i>S. longicaudum</i> Shen & Wang, 1992 (US)	CF1 VII	Lake Tahoe, California, USA	UCDNC 3779–81	<u>AF331901</u> , AY944019, AY943993	S.P. Stock
19. <i>S. monticolum</i> Stock, Choo & Kaya, 1997	Mt. Chiri	Gyeongnam, Korea	UCDNC 3785–87	AF331895, AY944020, AY943994	S.P. Stock
20. <i>S. oregonense</i> Liu & Berry, 1996	OS-10	Oregon, USA	UCDNC 3788–91	<u>AF331891</u> , AY944021, AY943995	J. Liu
21. <i>S. puertoricense</i> Román & Figueroa, 1994		Loiza, Puerto Rico	UCDNC 3792–94	AF331903, AY944022, AY943996	J. Román
22. <i>S. rarum</i> (de Doucet, 1986)	Sargento Cabral	Córdoba, Argentina	UCDNC 3795–97	AF331905, AY944023, AY943997	M.M. de Doucet
23. <i>S. riobrave</i> Cabanillas, Poinar & Raulston, 1994	TX	Texas, USA	UCDNC 3798–00	<u>AF331893</u> , AY944024, AY943998	H.K. Kaya
24. <i>S. scapterisci</i> Nguyen & Smart, 1992	Colón	Buenos Aires, Argentina	UCDNC 3801–3803	AF331898, AY944025, AY943999	S.P. Stock
25. <i>S. siamkayai</i> Stock, Somsook & Kaya, 1998	T9	Petchabun, Thailand	UCDNC 3804–07	<u>AF331907</u> , AY944026, AY944000	S.P. Stock
26. <i>S. websteri</i> Cutler & Stock, 2003	BJ	Beijing, China	UCDNC 3929–31	AY841762	S.P. Stock

*in vivo* using last instar *Galleria mellonella* (L.) larvae as hosts (Kaya & Stock, 1997), except for *S. scapterisci* Nguyen & Smart, 1992, which was reared in adult *Acheta domestica* (L.), and *S. kushidai* Mamiya, 1988, which was reared in larvae of *Cyclocephala hirta* Leconte. For most *Steinernema* isolates, nucleic acid preparations used for polymerase chain reaction (PCR) amplifications were extracted from one first-generation female. In some cases, (e.g. *S. scapterisci* and *S. abbasi* Elawad, Ahmad & Reid, 1997), pooled samples of several hundred infective juveniles from *in vivo* infections were washed three times with Ringer's solution and used for nucleic acid preparation. DNA was extracted from nematodes using various methods including two commercial kits (DNAzol, Molecular Research Center Inc.; ID Pure Genomic DNA Kit, ID Labs Biotechnology). Nematode DNA extracts obtained using kits were not quantified prior to use in PCR amplifications. Instead, the aliquot containing nucleic acids was concentrated by vacuum evaporation to 20  $\mu$ l and 1.5–3  $\mu$ l of this preparation was used in each PCR reaction (larger amounts typically for mtDNA amplifications). For bulk extraction of DNA from pooled infective juveniles, samples were incubated at 50°C in pH 8.0 TE buffer containing proteinase K (1  $\mu$ g/ $\mu$ l final concentration) and digested until only cuticle remained. Nucleic acids were extracted from the digestion supernatant using phenol-chloroform enrichment and ethanol/ammonium acetate precipitation (Ausubel et al., 1989). The resulting pellet was washed with 70% ethanol, resuspended in TE buffer (pH 8.0), treated with 50  $\mu$ g of RNase A (1 hr at 37°C) and DNA recovered following reprecipitation with ethanol. DNA from phenol-chloroform extracts was quantified by spectrophotometry, and 100–200 ng used per PCR reaction.

Regions of one nuclear gene (28S rDNA) and two mitochondrial genes (12S rDNA and cytochrome *c* oxidase subunit 1) were amplified by PCR. The nuclear large subunit rDNA gene region, corresponding to positions 3,745–4,700 in *Caenorhabditis elegans* and containing the D2 and D3 divergent domains, was amplified using forward primer #391 (5'-AGCGGAGGAAAAGAACTAA) and reverse primer #501 (5'-TCGGAAGGAACCAGCTACTA). Approximately 78% of the mitochondrial 12S rDNA corresponding to positions 1,027–1,568 in *C. elegans* was amplified

using forward primer #505 (5'-GTTCCAGAATAATCGGCTAGAC) and reverse primer #506 (5'-TCTACTTTACTACAACCTTACTCCCC). Approximately 45% of the mitochondrial *cox 1* gene, corresponding to positions 7,893–8,596 in *C. elegans*, was amplified using forward primer #507 (5'-AGTTCTAAT-CATAA(A/G)GATAT(C/T)G G) and reverse primer #588 (5'-TAAACTTCAGGGTGACCAA AAAATCA). Mitochondrial genes could not be amplified from *S. websteri* Cutler & Stock, 2003.

PCR reactions (25  $\mu$ l) consisted of 0.5  $\mu$ M of each primer, 200  $\mu$ M deoxynucleoside triphosphates, 0.5 unit Finnzymes DNAzyme EXT proof-reading polymerase (MJ Research) and MgCl<sub>2</sub> ranging from 1.5–3 mM as required to optimise amplification. PCR cycling conditions varied according to the locus amplified. For the LSU rDNA region, parameters included denaturation at 94°C for 3 min, followed by 33 cycles of 94°C for 30 sec, 52 C for 30 sec and 72°C for 1 min, followed by a post-amplification extension at 72°C for 7 min. For the 12S rDNA amplification, PCR parameters included denaturation at 94°C for 3 min, followed by 37 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec, followed by a post-amplification extension at 72°C for 7 min. For the *cox 1* region, PCR parameters included denaturation at 94°C for 3 min, followed by 36 cycles of 94°C for 1 min, 40 C for 1 min, and 72°C for 1 min, followed by a post-amplification extension at 72°C for 7 min.

PCR products were prepared for direct nucleotide sequencing using enzymatic treatment with exonuclease I and shrimp alkaline phosphatase (PCR product pre-sequencing kit, USB Corporation). PCR products were cloned when they could not be successfully sequenced directly (typically due to repeated sequence motifs). For cloning, PCR products were washed three times with TE buffer (pH 7.0) by spin filtration (Millipore Ultrafree-MC 30,000 NMWL, Millipore Corporation), ligated into pGEM-T vector (Promega) and cloned into JM109 *Escherichia coli*. Plasmid DNA was obtained using Qiaprep spin miniprep kits (Qiagen). Sequencing reactions were performed using dye-terminator sequencing chemistry, and reaction products were separated and detected using an ABI 3730 capillary DNA sequencer (PE Applied Biosystems). All sequences were completely double-stranded for verification using reactions primed from the PCR or vector primers, and two

or more internal sequencing primers as needed. Site polymorphisms in directly sequenced PCR products were recorded only when both alternative nucleotide peaks were present in all sequence reactions representing both DNA strands. If the heights of the alternative nucleotide peaks at polymorphic sites were not equal, the height of the minor peak was required to significantly exceed background terminations and comprise at least 25% of the major peak to be scored as a polymorphism. For cloned PCR products, 2 or more clones were sequenced per taxon; conflicts between different clones were recorded as polymorphisms. Contigs were assembled using Sequencher software (Version 3.0, Gene Codes). Sequences have been deposited in Genbank (accession numbers in Table 1). Nucleotide sequences were used to infer COI amino acids for *Steinernema* species using the *C. elegans* mtDNA code.

#### Morphological data

Specimens of first-generation males, first-generation females and third-stage infective juveniles (IJ) of each *Steinernema* isolate were used for assessments of morphological characters (Table 2) as detailed previously (Stock et al., 2001). A total of 22 morphological characters (14 qualitative and eight quantitative), including those typically used for diagnosis of species (Hominick et al., 1997), were evaluated. For cladistic analysis, continuous traits were coded as discrete states employing Simon's (Simon, 1983) homogenous subset coding method. One-way analysis of variance (ANOVA) was conducted for character values across the taxa using MSTAT-C v.2.0 (Freed et al., 1991). For all continuous characters, groups were defined that were significantly different at  $P < 0.05$ . This method confirmed the statistical distinctiveness of the groups within the sample, which is required for the application of subset gap coding. Finally, an *a posteriori* multiple comparisons test (Student-Newman-Keul's multiple range test) was performed to establish the subsets (character-states) within each character. Characters representing different measurements of adult and juvenile size (Table 2, characters 17-22) were not tested for potential correlation.

Morphological character-states were based on our observations and measurements, except for IJ lateral-field pattern (Mráček & Bednarek, 1991;

Tallosi et al., 1994; Elawad et al., 1997; Nguyen & Smart, 1997; Waturu et al., 1997; Stock et al., 1998) morphology of the gubernaculum cuneus (Hominick et al., 1997; Nguyen & Smart, 1997; Stock et al., 1998), and the presence of horn-like structures in juveniles (Tallosi et al., 1994; Nguyen & Adams, 2003). Of 22 morphological characters, six were binary and 16 were multi-state (Table 2); four missing values were coded as “?”

#### Phylogenetic analyses

*Caenorhabditis elegans* (Maupas, 1900) was used for rooting gene trees. A pragmatic reason for this choice was that *cox 1* genes could not be amplified in any of the three species (*Cervidellus alutus* (Siddiqi, 1993) [Cephalobidae], *Pseudacroboles variabilis* (Steiner, 1936) [Cephalobidae] and *Panagrellus redivivus* (L., 1767) [Panagrolaimidae]) used as outgroups in previous phylogenetic analyses of *Steinernema* LSU rDNA (Stock et al., 2001). From an evolutionary standpoint, phylogenetic analysis of 5.8 S sequence (Nguyen et al., 2001) also supports *C. elegans* as more closely related to *Steinernema* than other taxa that might be selected, such as plant-parasitic Tylenchida or vertebrate parasites including the Ascaridoidea. Using *C. elegans* as the outgroup is also consistent with a molecular phylogenetic analysis of LSU rDNA sequences that unexpectedly depicts *Steinernema* species as sharing more recent common ancestry with *Caenorhabditis* than with species from the Cephaloboidea, Panagrolaimoidea or Strongyloidoidea (see Nadler et al., submitted). From a practical standpoint, multiple alignments that included *C. elegans* had comparatively few alignment ambiguous sites in nuclear and mitochondrial rDNA. Cytochrome oxidase I amino acid sequences, translated from *cox 1* nucleotides using the *C. elegans* mtDNA code, required no indels to align *Steinernema* sequences with *C. elegans*. Nuclear and mitochondrial rDNA sequences were each aligned using ProAlign (Macintosh platform) Version 0.5 (Loytynoja & Milinkovitch, 2003). For each alignment, a ProAlign guide tree was constructed using corrected (for multiple hits) pairwise distances; this guide tree was used to estimate the hidden Markov model parameters ( $\delta$  and  $\epsilon$ ) for progressive multiple alignment. Program (Java) memory and bandwidth were increased as required to complete

Table 2. Morphological characters and character states for *Steinernema* species

Characters* Species	1	2	3	4	5	6	7	8	9	1	1	1	1	1	1	1	1	1	2	2	2	
										0	1	2	3	4	5	6	7	8	9	0	1	2
<i>S. abbasi</i>	0	1	1	0	2	1	0	2	2	1	0	1	1	0	0	2	1	2	2	2	1	2
<i>S. affine</i>	1	2	0	0	2	0	1	2	1	0	1	0	0	1	1	2	1	3	1	2	1	1
<i>S. anatoliense</i>	1	1	1	0	1	1	1	1	0	0	1	0	0	0	1	1	2	2	2	3	2	2
<i>S. arenarium</i>	0	1	0	0	0	0	2	0	0	1	2	0	0	0	0	1	1	2	0	1	1	1
<i>S. bicornutum</i>	0	1	0	0	0	1	0	0	1	0	0	0	1	0	0	2	1	1	1	2	1	1
<i>S. carpocapsae</i>	1	0	0	0	2	2	0	0	2	1	1	0	0	0	0	2	2	2	2	3	2	2
<i>S. ceratophorum</i>	0	1	0	0	2	1	1	2	1	0	2	0	1	0	0	1	2	2	1	2	1	1
<i>S. cubanum</i>	0	0	0	0	2	1	0	0	0	1	2	0	0	0	0	3	0	2	0	0	0	1
<i>S. diaprepesi</i>	0	1	0	0	1	1	0	1	3	1	0	0	0	0	0	1	0	2	0	2	1	1
<i>S. feltiae</i>	1	0	0	0	0	2	1	0	0	0	1	1	0	0	0	2	1	3	1	2	1	1
<i>S. glaseri</i>	0	2	0	0	0	0	3	0	0	1	2	0	0	0	0	1	1	1	0	1	1	1
<i>S. hermaphroditum</i>	0	0	2	0	1	1	2	0	0/1	2	0	0	0	0	1	2	2	2	1	2	1	1
<i>S. intermedium</i>	0	2	1	0	2	2	1	1	2	0	1	0	0	0	1	0	0	2	0	2	1	1
<i>S. kariii</i>	0	2	2	0	0	0	0	?	3	0	0	1	0	0	0	1	1	2	1	1	1	1
<i>S. kraussei</i>	1	2	0	0	2	2	1	0	2	0	1	0	0	0	1	2	1	0	1	2	1	1
<i>S. kushidai</i>	0	2	1	1	0	1	1	0	2	1	1	0	0	0	?	2	2	2	2	3	1	2
<i>S. longicaudum</i> (China)	1	2	0	0	2	1	1	0	1	1	2	0	0	0	0	0	0	2	0	1	1	0
<i>S. longicaudum</i> (USA)	1	2	0	0	2	1	1	0	1	1	2	0	0	0	0	1	0	2	0	1	1	0
<i>S. monticolum</i>	1	1	1	0	2	0	0	0	1	0	0	0	0	0	1	2	1	2	1	2	1	1
<i>S. oregonense</i>	0	1	2	0	0	0	0	0	3	0	2	0	0	0	?	2	1	2	1	2	1	1
<i>S. puertoricense</i>	0	2	0	0	0	0	3	0	3	0	2	1	0	0	0	1	0	2	0	1	1	0
<i>S. rarum</i>	1	2	1	0	2	2	0	0	1	1	2	0	0	0	0	3	2	3	2	3	2	2
<i>S. riobrave</i>	0	1	2	0	0	1	0	0	3	0	2	0	1	0	?	2	1	3	2	2	1	2
<i>S. scapterisci</i>	1	1	0	0	1	2	0	0	2	1	1	1	0	0	1	1	1	1	2	3	2	2
<i>S. siamkayai</i>	1	1	1	0	1	2	1	0	1	0	1	1	0	0	1	1	2	2	3	3	1	3
<i>S. websteri</i>	1	1	1	0	1	1	0	2	1	0	0	0	0	0	1	2	2	2	2	3	1	2

## \* Characters:

- Male tail mucro, (0) absent, (1) present;
- Spicule shape, (0) slightly curved, (1) moderately curved, (2) strongly curved;
- Spicule manubrium shape, (0) longer than wide, (1) square-rounded, (2) wider than long;
- Spicule lamina notch, (0) absent, (1) present;
- Spicule velum, (0) absent, (1) present, reduced, (2) present, well developed;
- Spicule rostrum, (0) absent, (1) present, slightly developed, (2) present, well developed;
- Spicule tip, (0) pointed, (1) blunt, not swollen, (2) blunt and swollen, (3) hook-shaped;
- Gubernaculum cuneus, (0) short, Y or V-shaped, (1) short, needle-shaped, (2) long, needle-shaped;
- Female tail shape, (0) blunt without mucro, (1) blunt with mucro, (2) conoid with pointy terminus, (3) conoid with mamillate terminus;
- Female post-anal swelling, (0) absent, (1) present;
- Vulval lips, (0) non-protruding, (1) protruding and symmetric, (2) protruding and asymmetric;
- Female double-flapped epiptygma, (0) absent, (1) present;
- IJ horn-like structures, (0) absent, (1) present;
- IJ tail spine, (0) absent, (1) present;
- IJ lateral field pattern, (0) 8 ridges, (1) < 8 ridges;
- Spicule length, (0) > 90  $\mu\text{m}$ , (1) 71–90  $\mu\text{m}$ , (2) 70–60  $\mu\text{m}$ , (3) < 60  $\mu\text{m}$ ;
- Male D%, (0) > 75, (1) 52–75, (2) < 52;
- Male SW ratio, (0) > 2.9, (1) 2.0–2.9, (2) 1.2–1.9, (3) < 1.2;
- IJ total body length, (0) > 1,000  $\mu\text{m}$ , (1) 650–1,000  $\mu\text{m}$ , (2) 450–650  $\mu\text{m}$ , (3) < 450  $\mu\text{m}$ ;
- IJ D%, (0) > 70, (1) 55–70, (2) 45–54, (3) < 45;
- IJ E%, (0) > 150, (1) 75–150, (2) < 75;
- IJ tail length, (0) > 90  $\mu\text{m}$ , (1) 61–90  $\mu\text{m}$ , (2) 40–60  $\mu\text{m}$ , (3) < 40  $\mu\text{m}$ .

the alignment. The average minimum posterior probability of sites was used as the criterion for detecting and removing unreliably aligned sequence, since this value is strongly correlated with correctness as determined by simulation studies (Loytynoja & Milinkovitch, 2003). To reduce the likelihood of excluding correctly aligned sites, the filter threshold was set to 60% minimum posterior probability, a value intermediate between the threshold of posterior probabilities for correctly versus incorrectly aligned sites in most simulation results (Loytynoja & Milinkovitch, 2003). For comparative purposes, this same ProAlign methodology was used to produce an alignment of ITS1-5.8S-ITS2 sequences of 10 *Steinernema* species and the *C. elegans* outgroup used by Spiridonov et al. (2004) in their phylogenetic analyses. These *Steinernema* species included *S. affine* (Bovien, 1937) (GenBank AY171289), *S. ceratophorum* Jian, Reid & Hunt, 1997 (AY230165), *S. cubanum* Mráček, Hernandez & Boemare, 1994 (AY230166), *S. intermedium* Poinar, 1985 (AY230172), *S. karii* Waturu, Hunt & Reid, 1997 (AY230173), *S. monticolum* Stock, Choo & Kaya, 1997 (AF122017), *S. oregonense* Liu & Berry, 1996 (AY230180), *S. riobrave* Cabanillas, Poinar & Raulston, 1994 (AY230182), *S. scapterisci* Nguyen & Smart, 1992 (AY230183) and *S. tami* Luc, Nguyen, Reid & Spiridonov, 2000 (AY171280). Phylogenetic trees were inferred separately for nuclear rDNA, mitochondrial DNA (12S rDNA plus *cox 1* sequences) and morphological characters. Datasets were also combined for phylogenetic analysis by unweighted maximum parsimony using PAUP\* 4.0b10 (Swofford, 1998) executed on a dual-processor Linux computer. Combined datasets included one for all molecular evidence (three genes) and total evidence (all sequence data plus morphological characters). Modeltest Version 3.06 (Posada & Crandall, 1998) was used to compare the fit of nucleotide substitution models for each gene using the Akaike information criterion, and the best-fit model for each gene was used for PAUP\* likelihood analyses. MrBayes v3.0b4 (Huelsenbeck & Ronquist, 2001) was used for a combined Bayesian analysis of all three genes, sampling trees over 100,000 generations (burnin determined empirically). In this analysis, a character partition corresponding to the three genes was invoked, and the parameters of the likelihood model were set to the GTR

substitution model with the invgamma model option (a proportion of sites are invariable, with the remaining sites modeled by a gamma distribution). The priors for the proportion of invariable sites were fixed separately for each gene using the pinvarpr command, with values as estimated for each gene according to the best-fit model. The best-fit model for the LSU dataset (GTR+G) included the parameters: base=(0.2513, 0.1882, 0.3130), Nst=6, Rmat=(0.7372, 3.1354, 1.6171, 0.2126, 6.5599), shape=0.3925 and pinvar=0. The best-fit model for the *cox 1* dataset (GTR+I+G) included the parameters: base=(0.2868, 0.0618, 0.1357), Nst=6, Rmat=(6651.9644, 113627.2031, 1210.4502, 49293.3359, 1124005.3750), shape=0.1808 and pinvar=0.4243. The best-fit model for the 12S dataset (K81uf+I+G) included the parameters: base=(0.3715, 0.0810, 0.1598), Nst=6, Rmat=(1.0000, 9.5504, 1.5998, 1.5998, 9.5504), shape=0.3869 and pinvar=0.3882.

Heuristic MP searches were conducted using 500 replicates of random taxon addition (RTA) with Tree-bisection-reconnection (TBR) branch swapping. Bootstrap MP searches were conducted using 1,000 pseudoreplicates, with 10 replicates of RTA and a search time limit of 60 sec per pseudoreplicate. Maximum likelihood trees were inferred using a neighbour-joining starting tree, with heuristic searching of tree space by TBR branch swapping. Bootstrap ML trees were produced using 100 pseudoreplicates of heuristic searches as indicated for individual ML trees, except each replicate was limited to 900 sec. Hypotheses for the evolution of characters were inferred by parsimony mapping on trees using MacClade version 4 (Maddison & Maddison, 2000). Datasets and treefiles from analyses have been deposited in TreeBASE (Sanderson et al., 1994).

## Results

For LSU rDNA sequences, using ProAlign to detect and remove unreliably aligned sites by their posterior probabilities excluded 230 of 910 potential characters. This LSU dataset of 680 remaining characters was used for all phylogenetic analyses and included 263 parsimony informative sites. For 12S rDNA sequences, the ProAlign 60% filter

excluded 83 of 474 alignment sites based on posterior probabilities. This 12S dataset of 391 characters was used for all phylogenetic analyses and included 115 parsimony informative sites. Based on the unambiguous amino acid alignment, the *cox 1* nucleotide dataset included 568 characters and 193 parsimony informative sites. The total evidence parsimony analysis was based on all three genes (filtered alignments for LSU and 12S rDNA datasets) and 22 morphological characters. This dataset included 1,660 characters, of which 574 were parsimony-informative. The total evidence dataset did not include *C. elegans* due to difficulties in determining homologous character states for many of the morphological characters selected and evaluated for *Steinernema* species. Consequently, the total evidence tree was rooted by

*S. siamkayai* Stock, Somsook & Kaya, 1998, *S. scapterisci*, *S. carpocapsae* (Weiser, 1955) and *S. monticolum*, as supported by previous analyses of LSU sequences (Stock et al., 2001) and the combined analysis of all molecular data herein. Inferred COI amino acid (AA) sequences (189 residues) included 13 sites with AA replacements (Table 3) among the *Steinernema* spp.

Parsimony analysis of the LSU dataset yielded five equally parsimonious trees of 678 steps (C.I. = 0.66); the strict consensus of these trees (Figure 1) was moderately well resolved. This tree included four species not represented in the published analysis of Stock et al. (2001): *S. anatoliense* Hazir, Stock & Keskin, 2003, *S. diaprepesi* Nguyen & Duncan, 2002, *S. hermaphroditum* Stock, Griffin & Chaenari, 2004 and *S. websteri* Cutler & Stock,

Table 3. Thirteen variable COI positions among *Steinernema* taxa. Amino acid numbers refer to position in complete *C. elegans* protein sequence. Unambiguously shared-derived amino acid replacements (by parsimony mapping), relative to the 3-gene trees or mtDNA trees are indicated and the apomorphic amino acids shown capital in bold.

Amino Acid No.	51	54	55	65	81	90	115	118	123	128	148	162	194
<i>S. abbasi</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Ser	Val	Thr
<i>S. affine</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. anatoliense</i>	<b>GLN</b>	Leu	<b>LEU</b>	Ileu	Ser	<b>MET</b>	Thr	Phe	<b>SER</b>	Thr	Ser	Ileu	<b>ALA</b>
<i>S. arenarium</i>	Lys	<b>VAL</b>	Phe	Val	Ser	Leu	Thr	Leu	Ala	Met	Asn	Ileu	Thr
<i>S. bicornutum</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Ser	Ileu	Thr
<i>S. carpocapsae</i>	<b>GLN</b>	Leu	<b>LEU</b>	Ileu	Ser	<b>MET</b>	Thr	Phe	<b>SER</b>	Thr	Ser	Ileu	<b>ALA</b>
<i>S. ceratophorum</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Phe	Ala	Met	Ser	Val	Thr
<i>S. cubanum</i>	Lys	<b>VAL</b>	Phe	Val	Ser	Leu	Thr	Leu	Ala	Met	Asn	Ileu	Thr
<i>S. diaprepesi</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. feltiae</i>	Lys	Leu	Phe	Val	Ser	Leu	Thr	Leu	Ala	Thr	Asn	Val	Thr
<i>S. glaseri</i>	Lys	<b>VAL</b>	Phe	Val	Ser	Leu	Thr	Leu	Ala	Met	Asn	Ileu	Thr
<i>S. hermaphroditum</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. intermedium</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. kariii</i>	Lys	Leu	Phe	Val	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. kraussei</i>	Lys	Leu	Phe	Ileu	Thr	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. kushidai</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. longicaudum</i> CH	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. longicaudum</i> US	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. monticolum</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Ileu	Thr
<i>S. oregonense</i>	Lys	Leu	Phe	Val	Ser	Leu	Thr	Met	Ala	Met	Asn	Val	Thr
<i>S. puertoricense</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Asn	Val	Thr
<i>S. rarum</i>	Lys	Leu	<b>LEU</b>	Ileu	Ser	Leu	Ala	Phe	Ala	Met	Asn	Val	Thr
<i>S. riobrave</i>	Lys	Leu	Phe	Ileu	Ser	Leu	Thr	Leu	Ala	Met	Ser	Val	Thr
<i>S. scapterisci</i>	<b>GLN</b>	Leu	<b>LEU</b>	Val	Ser	<b>MET</b>	Thr	Leu	<b>SER</b>	Thr	Ser	Ileu	Thr
<i>S. siamkayai</i>	<b>GLN</b>	Leu	<b>LEU</b>	Val	Ser	<b>MET</b>	Thr	Leu	<b>SER</b>	Thr	Ser	Ileu	Thr
<i>C. elegans</i>	Lys	Phe	Phe	Val	Thr	Leu	Thr	Leu	Ala	Met	Ser	Leu	Thr
3-gene tree(s) synapomorphy		X											
MtDNA tree synapomorphy	X		X			X			X				X

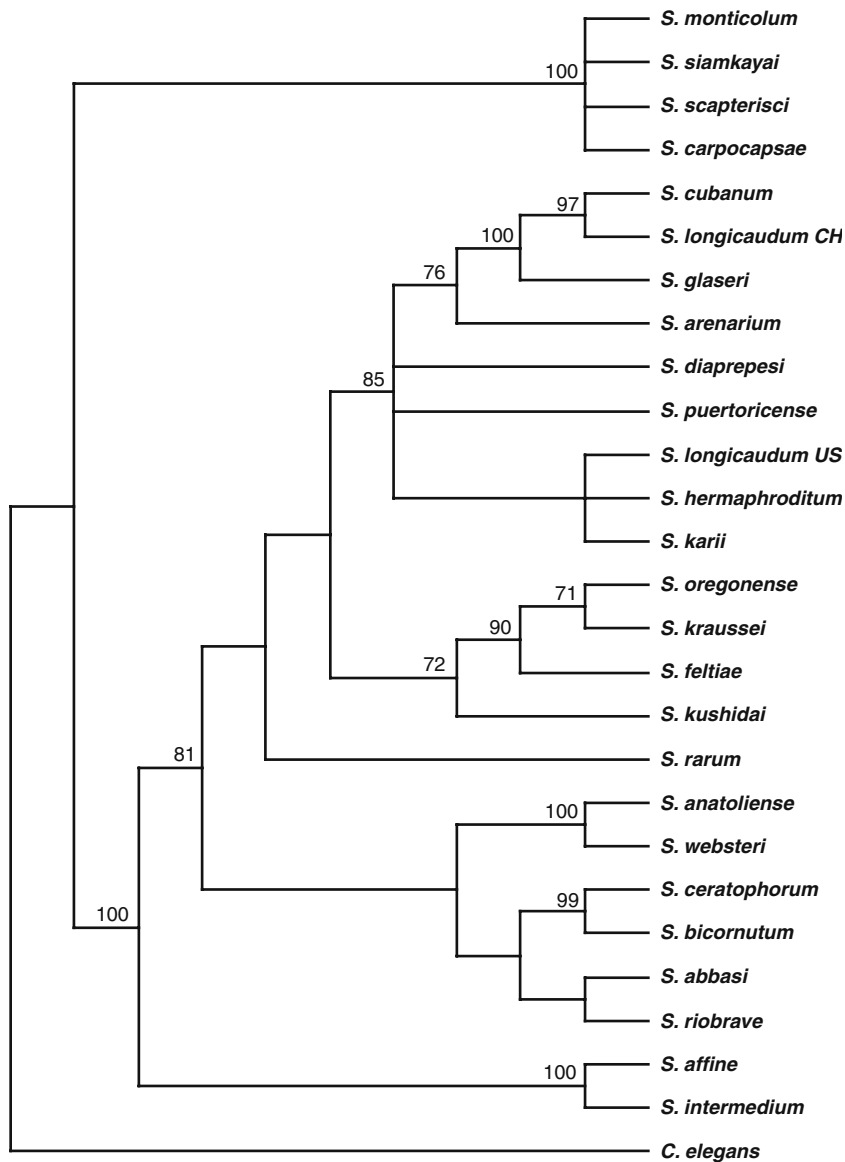


Figure 1. Strict consensus of five maximum parsimony trees inferred from LSU rDNA (680 characters, 263 parsimony informative). MP bootstrap clade frequency values  $\geq 70\%$  mapped above internal nodes

2003. Bootstrap MP analysis of these data revealed moderate ( $\geq 70\%$ ,  $< 90\%$ ) to high ( $\geq 90\%$ ) support for 13 of 19 clades in the strict consensus MP tree (Figure 1); there was no conflict between clades resolved in the LSU 50% majority-rule bootstrap consensus tree (not shown) and the strict consensus of most parsimonious trees. Strong MP bootstrap support ( $\geq 90\%$ ) was recovered for eight groups of taxa, including four sets of sister species.

Maximum likelihood analysis of the LSU dataset yielded one tree with a long branch

separating the clade of (*siamkayai*, *scapterisci*, *carpocapsae*, *monticolum*) from the clade including all other *Steinernema* species (Figure 2); each of these clades received 100% bootstrap support. Within the main (23-taxon) clade, branch lengths ranged from moderate to short. Moderate to high bootstrap support was recovered for nine clades within the ML tree (Figure 2), and seven of these were within the main clade. For these LSU rDNA data, all clades receiving high bootstrap support by ML also received  $\geq 90\%$

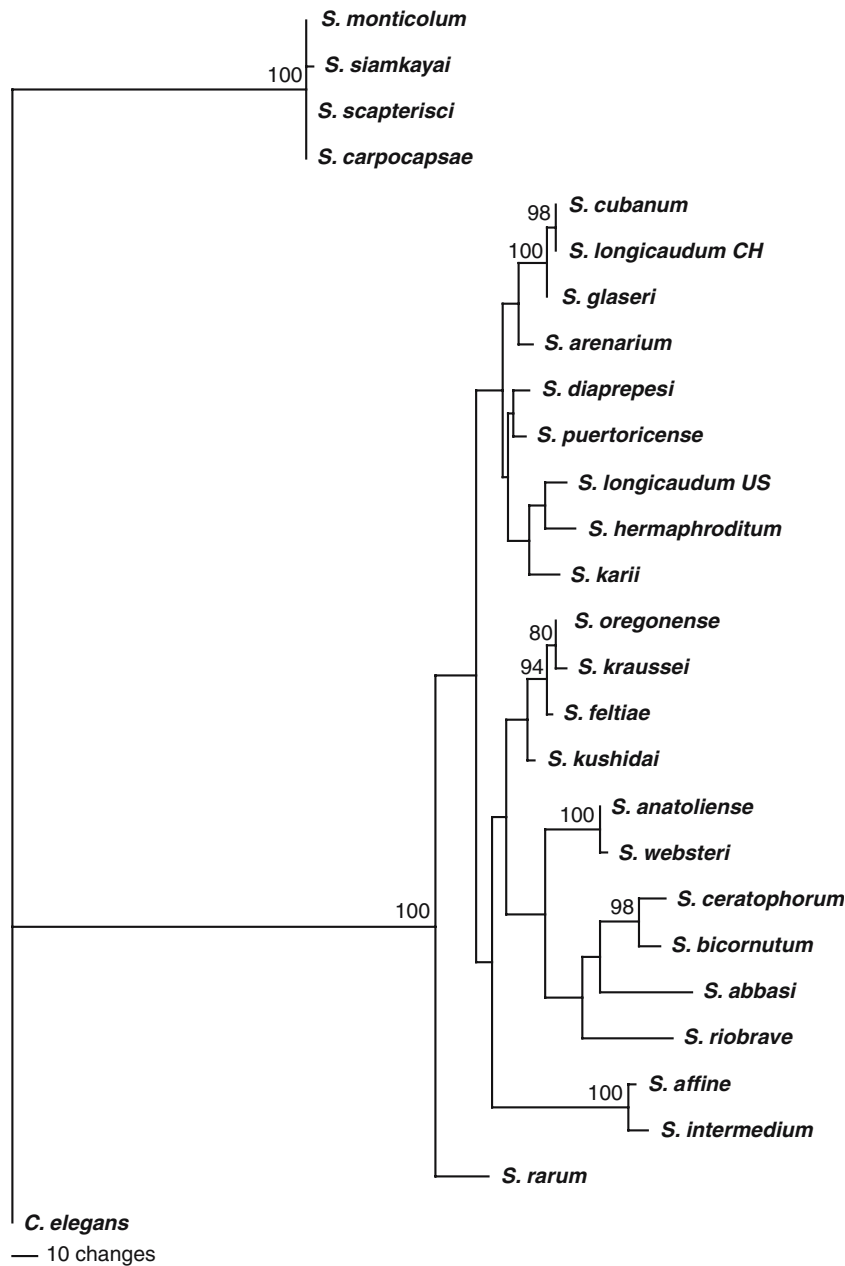


Figure 2. Maximum likelihood tree inferred from LSU rDNA with branch lengths scaled to the expected number of substitutions per site. ML bootstrap clade frequency values  $\geq 70\%$  mapped above internal nodes

support by MP bootstrap analysis (Figures 1, 2), and 10 of 13 clades receiving moderate to high bootstrap support by MP were characterised by similar ML bootstrap support. The topology of the LSU ML tree (Figure 2) showed substantial similarity to the MP strict consensus tree (Figure 1), particularly for clades receiving  $\geq 70\%$  support by MP bootstrap analysis. However,

some of the relationships among larger clades were different between these ML and MP hypotheses.

Parsimony analysis of the combined mtDNA (12S and *cox 1*) data (959 characters, 308 parsimony-informative) yielded two equally parsimonious trees of 1,581 steps (C.I. = 0.39); the strict consensus of these trees (Figure 3) was moderately

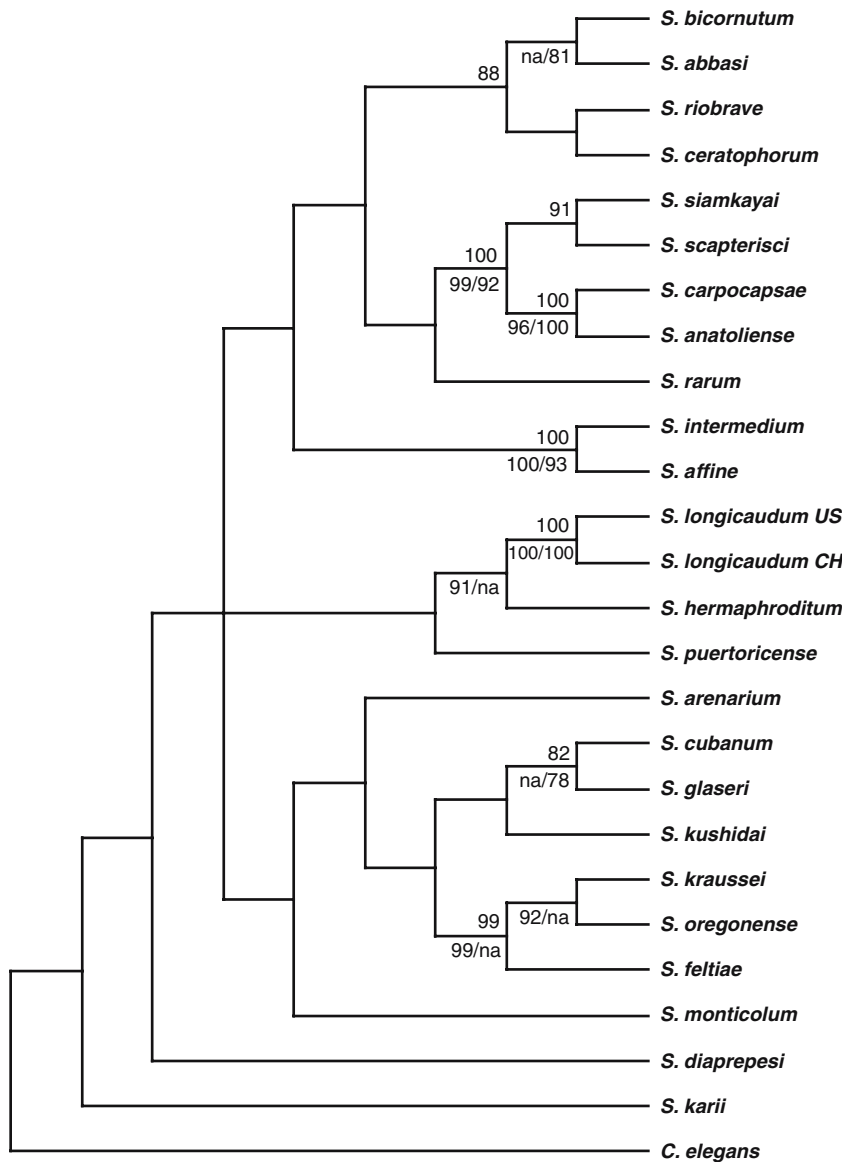


Figure 3. Strict consensus of two maximum parsimony trees for combined mtDNA (12S rDNA and *cox 1* genes). MP bootstrap clade frequency values  $\geq 70\%$  mapped above internal nodes. ML bootstrap values for analysis of individual genes shown below internal nodes (12S followed by *cox 1* frequency)

well resolved. Bootstrap MP analysis of these data revealed weaker clade support than for LSU data, with just eight clades having bootstrap values  $\geq 70\%$ . Some clades with  $< 70\%$  MP bootstrap support in the combined mtDNA data were reliably supported by ML bootstrap analysis of individual (12s or *cox 1*) genes (Figure 3). There was no conflict between clades resolved in the mtDNA bootstrap 50% majority-rule consensus tree (not shown) and the strict consensus of two

MP trees. There were two instances of topological discordance between the mtDNA and nuclear (LSU) MP gene trees (Figures 1, 3), for which the conflicting clades were each strongly supported by bootstrap resampling of these alternative loci. Specifically, in the mtDNA tree, *S. anatoliense* was sister to *S. carpocapsae* with 100% bootstrap support, whereas in the LSU tree, *S. anatoliense* was sister to *S. websteri* (species not successfully amplified for mtDNA) with 100% support, and

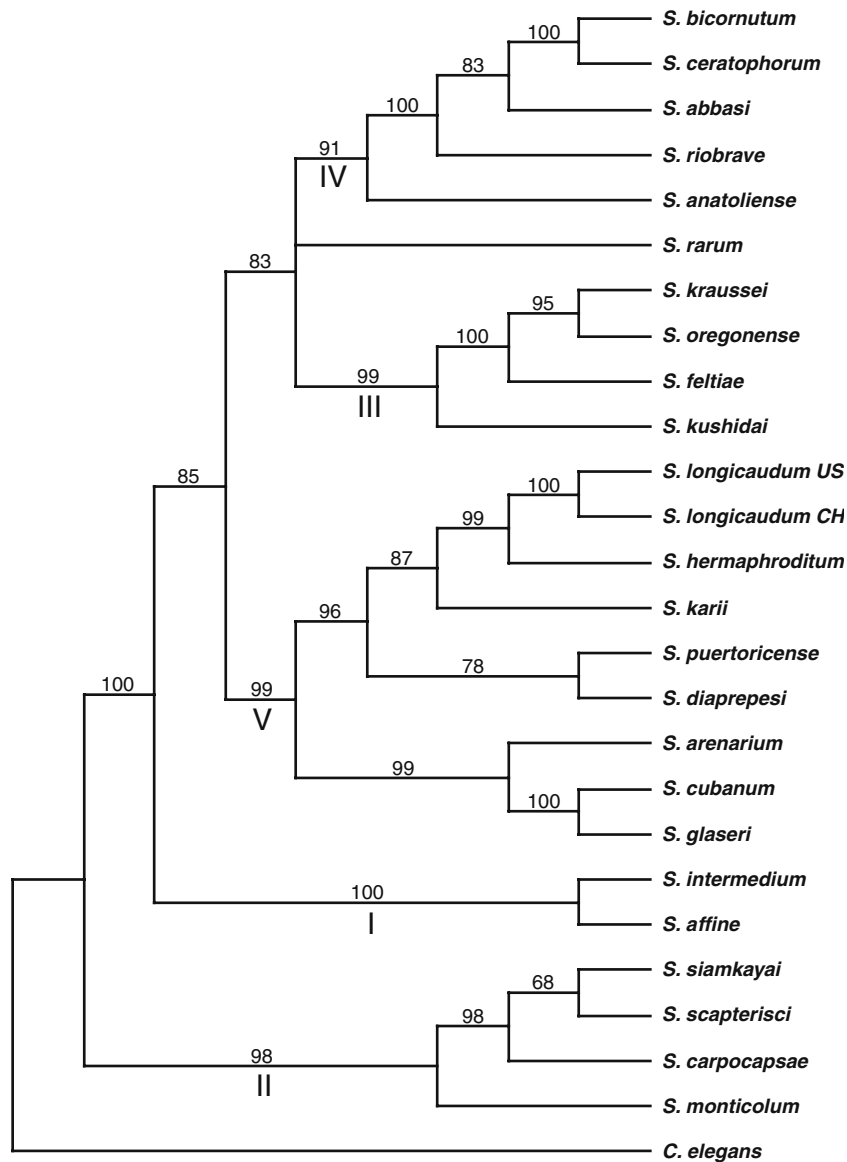


Figure 4. Bayesian tree inferred from combined analysis of all molecular data, partitioned by gene. MCMC posterior probabilities are shown above internal nodes. Roman numerals designate major clades discussed in the text

*S. carpocapsae* was part of a clade including *S. monticolum*, *S. siamkayai* and *S. scapterisci*. Similarly, in the LSU tree, the two *S. longicaudum* Shen & Wang, 1992 isolates (United States, China) were not closest relatives, with the Chinese *S. longicaudum* sister to *S. cubanum* with 97% bootstrap support. In contrast, these *S. longicaudum* isolates were sister taxa with 100% bootstrap support in the mtDNA tree. Other topological differences between LSU and mtDNA trees

involved clades that were weakly supported by one or both datasets.

Combined likelihood analysis of all molecular data using MrBayes yielded a highly resolved topology (Figure 4) with high posterior probabilities. Although these posterior probabilities are not directly comparable to bootstrap values, they are useful in assessing how well these data support this model-based analysis. Only the position of *S. rarum* (de Doucet, 1986) was poorly resolved

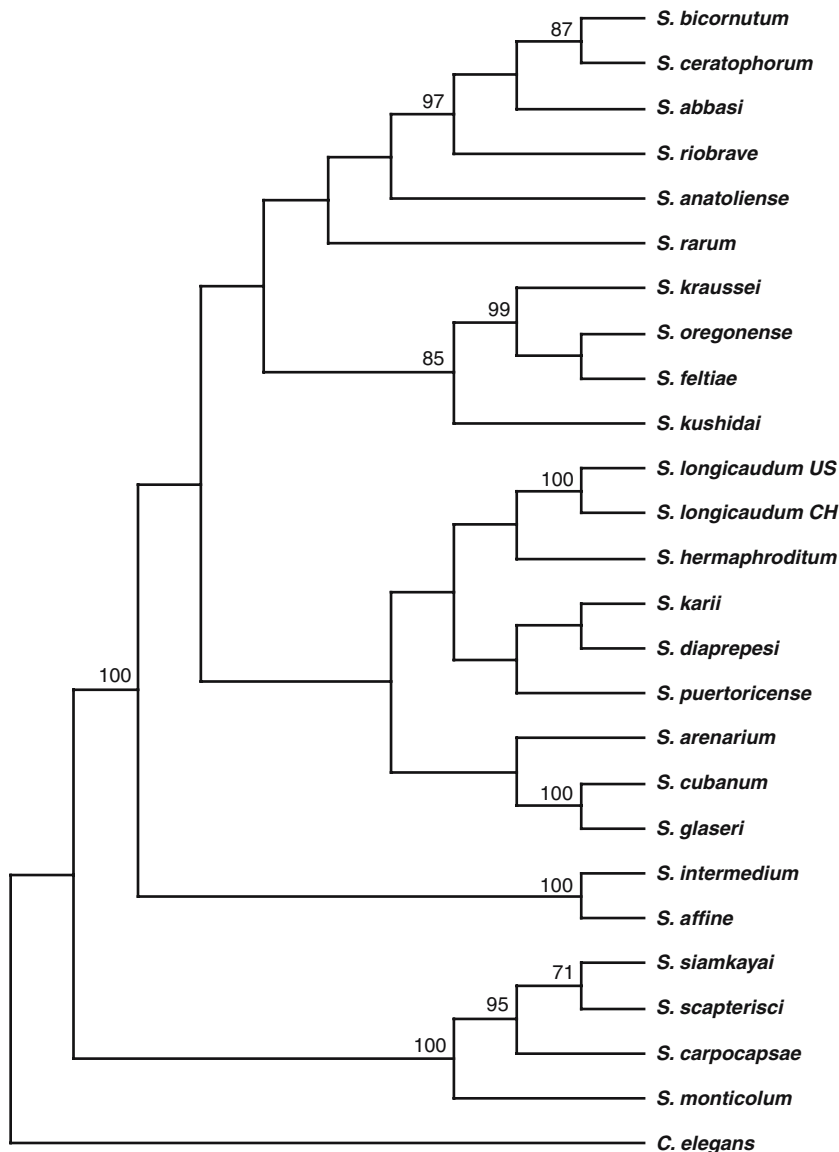


Figure 5. Most parsimonious tree inferred from combined molecular data (three genes, 1,639 characters, 565 parsimony informative). MP bootstrap clade frequency values  $\geq 70\%$  mapped above internal nodes

in the Bayesian tree, and relatively few nodes were weakly supported (i.e. *siamkayai* + *scapterisci*). Unlike results for individual LSU or mitochondrial datasets (Figures 1–3), deeper nodes in the Bayesian tree were moderately to strongly supported, as assessed by their posterior probabilities. For example, two large subclades, one including 10 species (posterior probability of 83%) and another nine species (posterior of 99%), were recovered by Bayesian analysis. Although the 9-species clade was recovered in both the LSU

and mtDNA gene trees, the 10-species clade was not. The Bayesian analysis reflected many of the strongly supported clades for individual molecular datasets, but also provided resolution for the few groups of taxa that conflicted between the LSU and mitochondrial trees. Combined MP analysis of all molecular data (1,639 characters, 565 parsimony-informative characters) yielded a single most parsimonious tree (Figure 5) of 2,404 steps (C.I. = 0.44). Bootstrap MP analysis of these data revealed moderate to high support for 11 of 23

Table 4. Consistency index range for morphological characters obtained by mapping on the 3-gene trees (Bayesian and parsimony)

Character	States	C. I. range
1. Male tail mucro	2	0.17
2. Male spicule shape	3	0.22
3. Male spicule manubrium	3	0.20
4. Male spicule lamina notch	2	1.0
5. Male spicule velum	3	0.18
6. Male spicule rostrum	3	0.22
7. Male spicule tip	4	0.27
8. Male gubernaculum cuneus	3	0.33
9. Female tail	4	0.25–0.27
10. Female postanal swelling	2	0.22
11. Female vulval lips	3	0.20–0.22
12. Female epiptygma	2	0.20
13. IJ horn-like structures	2	1.0
14. IJ tail spine	2	1.0
15. IJ lateral field pattern	2	0.20
16. Male spicule length	4	0.33
17. Male D%	3	0.18
18. Male SW ratio	4	0.38
19. IJ total body length	4	0.33
20. IJ D%	4	0.38
21. IJ E%	3	0.40
22. IJ tail length	4	0.43

clades found in the most parsimonious tree; only one clade in the bootstrap 50% majority-rule consensus tree (not shown) conflicted with the topology of the MP tree ((*kraussei*, *oregonense*, *feltiae*)), but this sister-species relationship was poorly supported by bootstrap (56%). The topology of the MP tree (Figure 5) was quite similar to the Bayesian tree (Figure 4), with the exception of differences in some taxa that were poorly supported by MP bootstrap resampling (e.g. *karii*, *diaprepesi*, *puertoricense*). Some clades in the combined molecular trees conflicted with clades inferred for analysis of individual datasets (LSU, mtDNA). However, the combined molecular tree did not suffer a loss of resolution for species that had conflicting positions in LSU versus mtDNA trees, and relationships for these species conformed to one of the alternative hypotheses produced by the two datasets (e.g. monophyly of the *longicaudum* isolates reflects the topology inferred by mtDNA).

Parsimony analysis of the morphological dataset (20 parsimony-informative characters) yielded

676 equally parsimonious trees of 123 steps (C.I. = 0.37); the strict consensus of these trees was very poorly resolved (tree not shown). The topology of this strict consensus tree showed little similarity to results from total evidence or combined molecular datasets. Bootstrap MP analysis yielded only one clade (*longicaudum* US, *longicaudum* CH) receiving  $\geq 70\%$  support. Parsimony mapping of morphological characters on the 3-gene trees (Bayesian and MP) revealed high homoplasy for all characters (Table 4) except two that were autapomorphic and one (horn-like structures of infective juveniles) that was synapomorphic for the clade of *S. bicornutum* Tallosi, Peters & Ehlers, 1995, *S. ceratophorum*, *S. abbasi* Elawad, Ahmad & Reid, 1997 and *S. riobrave*.

Parsimony analysis of the total evidence dataset recovered three trees of 2,333 steps (C.I. = 0.43). The strict consensus of these trees (Figure 6) showed moderate resolution at the tree tips, but poor resolution along the backbone of the tree. There was moderate to high bootstrap support for nine of the 14 monophyletic groups resolved in the strict consensus MP tree (Figure 6). All clades recovered with  $\geq 70\%$  bootstrap support did not conflict with the topology of the Bayesian analysis of all three genes (Figure 4).

## Discussion

Evolutionary hypotheses for steinernematids have been based on a variety of evidence (Poinar, 1993), although formal analyses by phylogenetic methods have been based mainly on genetic data. Although several different types of molecular markers have been used in these studies (e.g. RAPDS, RFLPs), nuclear ribosomal DNA sequences have been the primary source of molecular data for inferring *Steinernema* phylogeny. Sequences analysed include 18S (SSU) rDNA (Liu et al., 1997), internal transcribed spacer (ITS) regions (Szalanski et al., 2000; Nguyen et al., 2001; Nguyen & Duncan, 2002; Spiridonov et al., 2004), 5.8S rDNA (Nguyen et al., 2001) and 28S (LSU) rDNA (Stock et al., 2001, 2004). Each of these rDNA regions has different strengths and weaknesses for inferring *Steinernema* phylogeny. All ribosomal DNA sequences are compromised to a greater or lesser degree due to the ambiguity of inferring positional

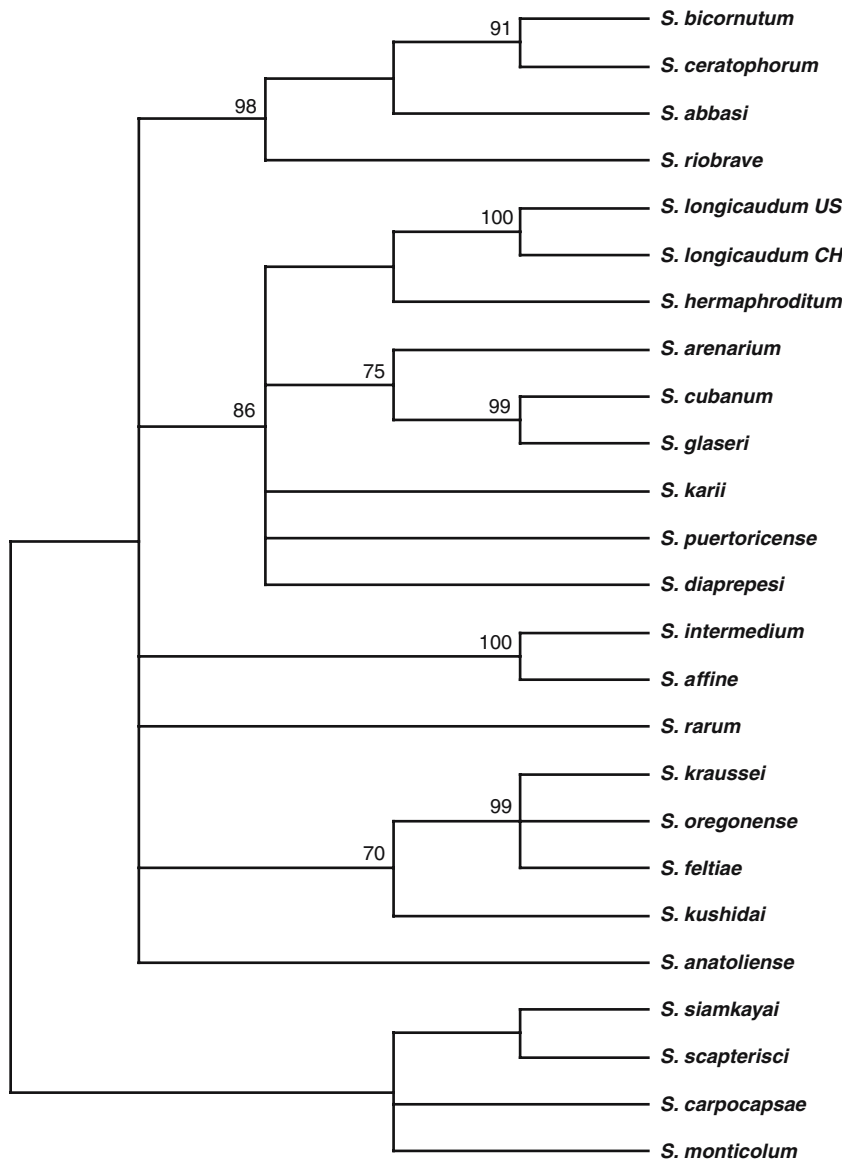


Figure 6. Strict consensus of three maximum parsimony trees inferred from total evidence dataset (1,660 total characters, 574 parsimony informative). MP bootstrap clade frequency values  $\geq 70\%$  mapped above internal nodes

homology of characters. Accurate positional homology determinations are critical for construction of characters used to estimate nematode molecular phylogenies (Nadler, 1995), and the relative reliability of *Steinernema* clades is known to be sensitive to alignment ambiguity of rDNA sequences (Nguyen et al., 2001; Stock et al., 2001). In addition, studies of nematode SSU rDNA have shown that topologies of phylogenetic trees are significantly correlated with alignment parameter choice (gap opening and extension penalties) when

all alignment characters are included without consideration of alignment ambiguity (A. Smythe, pers. comm.).

Among *Steinernema* species, SSU sequences are highly conserved and include the fewest alignment ambiguous sites; unfortunately, SSU sequences of steinernematids contain few phylogenetically informative characters and yield poorly supported trees (Stock et al., 2001). Ribosomal ITS regions are highly variable among *Steinernema* species, providing more potentially informative characters

and resolution among closely related species than SSU or LSU rDNA (Nguyen et al., 2001; Stock et al., 2001; Spiridonov et al., 2004). However, *Steinernema* ITS sequences are also quite variable in length (> 100 bp difference between some species) and nucleotide composition, making inferences of positional homology dubious across extensive regions of sequence (Nguyen et al., 2001; Spiridonov et al., 2004). For example, Nguyen et al. (2001) reported that < 50% of the ITS positions in their comparison of 10 species were free from significant alignment ambiguity. Herein, the ProAlign program was used to compare ITS1-5.8S-ITS2 sequences from 10 *Steinernema* species (two from each of the five major clades) in the analysis of Spiridonov et al. (2004) and the *C. elegans* outgroup. Only 12% of these characters had posterior probabilities meeting the 60% threshold, and when only the 10 *Steinernema* species were aligned (no outgroup included), 40% of the characters met this threshold. These results support the findings of Nguyen et al. (2001), who first reported that *Steinernema* ITS sequences are too variable to reliably infer relationships among diverse *Steinernema* species, and that aligning these ITS sequences to more distantly related outgroup species was not feasible. In addition to alignment difficulties, Spiridonov et al. (2004) have reported within-isolate ITS variation (including length variation) that can cause practical problems for directly sequencing DNA and theoretical difficulties for analysis (e.g. establishing orthology). Nuclear LSU sequences have proved more phylogenetically informative than SSU sequences and, although not free of alignment difficulties, LSU sequences have fewer ambiguously aligned positions than ITS rDNA. For example, in this analysis using the ProAlign 60% filter, 75% of the LSU characters could be aligned among *Steinernema* species and the *C. elegans* outgroup.

Independent of issues regarding alignment ambiguity and phylogenetic information content, a shortcoming of previously published studies is that trees inferred from nuclear ribosomal DNA represent hypotheses based on data from a single genetic locus. Although a gene tree from one locus can represent the evolutionary history of organisms (rather than a gene history), topologies inferred from a single locus may be misleading due to a variety of causes, including lineage sorting artifacts, and rate variation and compositional

bias among lineages that may affect the accuracy of tree reconstruction. Whether such problems compromise any individual gene tree can be difficult to establish, and this uncertainty has led investigators to seek evidence of clade congruence from gene trees representing independent loci. Repeated phylogenetic patterns from independent loci most likely reflect the underlying common cause of organismal evolutionary history. Comparisons of *Steinernema* gene trees for LSU rDNA and mitochondrial DNA sequences meet these criteria because nematode mitochondrial DNA, with its maternal inheritance (Anderson et al., 1995), is transmitted independently of nuclear genes. A study of nuclear rDNA (ITS-2 sequences) and mitochondrial gene sequences for five *Steinernema* species demonstrated that multilocus approaches show promise for this genus (Szalanski et al., 2000). The current study is the first phylogenetic hypothesis employing sequences from multiple loci for a substantial sample of described *Steinernema* species.

In the present study, DNA sequences from the nuclear (28S LSU) and mitochondrial (12S rDNA and *cox 1*) genomes were used to infer phylogenetic relationships in both separate and combined analyses. These 2 rDNA genes have higher rates of substitution than the SSU rDNA, but lower rates than ITS genes. A previous study of *Steinernema* phylogeny based on this LSU region (which includes the D2 and D3 domains) used a manual ('by eye') approach to remove sites that were judged potentially ambiguous (27% of total sites) with respect to positional homology (Stock et al., 2001). In the current study, the ProAlign program was used to assess alignment ambiguity according to the posterior probability of sites. An advantage of using this program is that it removes potential investigator bias from decisions concerning alignment ambiguity and exclusion of characters. This posterior probability method excluded 25% of the sites in the LSU alignment and 18% of the 12S sites. No *cox 1* sites were considered ambiguous with respect to alignment because the inferred protein sequences required no indels.

Differences of opinion exist concerning whether it is always warranted to combine independent datasets (e.g. different genes) for phylogenetic analysis. Advocates of the "total evidence" approach argue that phylogenies inferred from analysis of all available data maximises explanatory

power (Eernisse & Kluge, 1993; Kluge, 1998). Alternatively, recognising that different datasets (e.g. different genes) can have different histories, advocates of the ‘congruence’ approach advocate evaluating trees from independent datasets separately, and combining datasets only if they lack incongruence as determined by statistical tests. Herein, we consider the combined analyses (combined molecular, molecular plus morphology) as representing the best working hypotheses for *Steinernema* evolutionary history, recognising that this approach has certain caveats. For example, trees inferred from nuclear (LSU) and mitochondrial (12S rDNA + *cox 1*) datasets showed some clades that were both strongly supported and conflicting. Furthermore, the partition homogeneity test revealed that these two molecular datasets are incongruent ( $P=0.001$ ). Notably, nuclear rDNA does not support the monophyly of the *S. longicaudum* isolates (Figures 1, 2), a result reported previously (Stock et al., 2001), whereas mtDNA does (Figure 3). Notably, these two isolates do not cross-breed in the laboratory (S.P. Stock, pers. comm.), consistent with the hypothesis that they represent separate species. Another example of incongruence between trees from these datasets is that LSU rDNA includes a supported clade of (*monticolum*, *siamkayai*, *scapterisci*, *carpocapsae*), whereas the mtDNA hypothesis includes a clade with three of these species (*siamkayai*, *scapterisci*, *carpocapsae*) plus *S. anatoliense*. Most of the other topological differences between trees inferred from LSU rDNA and mtDNA appear to have relatively little character support as assessed by bootstrap resampling. On the other hand, certain clades are congruent between MP analyses of these nuclear and mitochondrial datasets including: ((*kraussei*, *oregonense*), *feltiae*); (*intermedium*, *affine*); and (*bicornutum*, *abbasi*, *riobrave*, *ceratophorum*). These clades should be considered particularly well corroborated. Beyond these comparisons of relatively closely related species, there is little bootstrap support for more taxonomically comprehensive (deeper) nodes in trees inferred from either the LSU rDNA or mtDNA datasets. Previous analyses of *Steinernema* LSU datasets (with exclusion of alignment ambiguous sites) were also relatively poorly resolved at deeper nodes (Stock et al., 2001). Comparison of consistency indices for these LSU and mitochondrial datasets (0.66 versus 0.39, respectively) suggests that these mtDNA sequence characters are

considerably more homoplastic than the LSU characters. This result reflects previous studies indicating that nematode mtDNA tends to be of less phylogenetic utility than nuclear genes (Blouin et al., 1998; Nadler & Hudspeth, 2000), although not in every case (Liu et al., 1999).

Combined phylogenetic analysis of all three genes in a Bayesian framework yielded a well-resolved hypothesis with high posterior probabilities. This result is in contrast to the node support inferred for individual LSU rDNA and mtDNA datasets, which had good resolution, but lacked strong bootstrap support for many clades. Some of this variation in support may be due to differences in the number of characters analysed, but another major factor concerns differences between inference methods. The posterior probabilities in Bayesian analysis and bootstrap clade frequencies are not equivalent measures of confidence (Alfaro et al., 2003). A clade receiving a high bootstrap percentage is expected to be found in other analyses of new datasets generated by the same fundamental process (Felsenstein, 1985), i.e. bootstrap resampling measures repeatability (Berry & Gascuel, 1996). In contrast, Bayesian MCMC posterior probability sampling is useful for assessing how well data support results of a fully probabilistic model of character evolution, i.e. Bayesian posterior probabilities are results conditioned on the observed data and models. In this case, the Bayesian model involved partitioning the combined molecular dataset into component genes (LSU, 12S, *cox 1*), each with different priors on elements of the substitution model (gamma distributed rate variation across sites, with a fixed proportion of invariable sites, as estimated separately for each dataset using Modeltest). Given these models and the data, probabilities of most clades appear high, with a few exceptions. Maximum parsimony analysis of these same data yielded a single resolved tree with much topological similarity to the Bayesian result. But in contrast to the Bayesian posterior probability values, MP bootstrap support for many clades were lower (11 clades had values  $\geq 70\%$ ). Differences in topology between the MP and Bayesian trees involved relationships of species that did not have  $\geq 70\%$  bootstrap MP support. Differences in clade ‘support’ between Bayesian and MP trees (for the same dataset) illustrates how different analyses employing different models of sequence

evolution impact on interpretation of results. These Bayesian MCMC posterior probabilities (BPP) derive from models of (gene specific) substitution encompassing all sequence data (1,639 characters), whereas unweighted MP analysis is based on only those sequence characters that are parsimony informative (565 in the 3-gene dataset). The greatest impact of this difference between inference methods involves interpretation of support for relationships among major clades of *Steinernema* species. Similarly, the total evidence (molecular plus morphological data) MP strict consensus hypotheses is significantly less resolved than the 3-gene MP tree or 3-gene Bayesian tree, yet clades in the total evidence tree are all also represented in the 3-gene trees (Figures 4–6).

Previous studies of rDNA have indicated that the diversity of *Steinernema* species is represented by several different clades (Stock et al., 2001; Spiridonov et al., 2004). For example, in a phylogenetic study of 22 isolates (21 described species) based on LSU rDNA, Stock et al. (2001) found that these species were represented by five main clades. Likewise, Spiridonov et al. (2004) analysed ITS1-5.8S-ITS2 rDNA sequences from 88 *Steinernema* isolates (24 described species) and recovered clades that were primarily consistent with results inferred using LSU rDNA. Spiridonov et al. (2004) formally enumerated these clades and described their membership based on ITS trees. Direct comparison of the results of Spiridonov et al. (2004) with the current study is feasible only for the 20 described species in common to both; the ITS1-5.8S-ITS2 study did not include *anatoliense*, *kushidai*, *hermaphroditum* or *puertoricense* Román & Figueroa, 1994, whereas the current 3-gene study does not include *neocurtillae* Nguyen & Smart, 1992, *pakistanense* Shahina, Anis, Reid, Rowe & Maqbool, 2001, *tami* or *weiseri* Mracek, Sturhan & Reid, 2003. There is not an exact correspondence between the composition of the five clades enumerated in the study of Spiridonov et al. (2004) and those inferred from analysis of the 3-gene dataset. Retaining the clade terminology of Spiridonov et al. (2004) with minimal modifications (see Figure 4), Clade I (*affine*, *intermedium*) has the same composition in the 3-gene trees, and is strongly supported by BPP and bootstrap resampling. Clade II results are incongruent due to a different placement of *S. monticolum* in the ITS analysis of Spiridonov et al. (2004). In the

3-gene analyses, there is strong BPP and bootstrap support for Clade II being composed of (*siamkayai*, *scapterisci*, *carpocapsae*, *monticolum*); the first three of these species were part of Clade II as defined by Spiridonov et al. (2004). Clade III results are again incongruent due to the alternative placement of *S. monticolum* in the ITS analysis. In the 3-gene analyses, there is strong BPP and moderate bootstrap support for defining clade III as including (*kraussei*, *oregonense*, *feltiae*, *kushidai*). There is no incongruence between Clade IV results for the 3-gene and ITS trees, but the different sampling in the 3-gene dataset indicates that *S. anatoliense* should be included, i.e. Clade IV represented as (*anatoliense*, *bicornutum*, *ceratophorum*, *abbasi*, *riobrave*). This clade receives strong BPP support, and the subclade (*bicornutum*, *ceratophorum*, *abbasi*, *riobrave*) receives strong bootstrap support. Likewise, there is no incongruence between Clade V results for the 3-gene and ITS trees, but the different sampling in the 3-gene dataset shows that *S. hermaphroditum* and *S. puertoricense* belong to this clade, i.e. Clade V represented as (*longicaudum*, *hermaphroditum*, *karii*, *puertoricense*, *diaprepesi*, *arenarium*, *cubanum*, *glaseri*). Clade V is strongly supported by BPP, but unsupported by MP bootstrap resampling of the combined molecular dataset. Phylogenetic relationships between Clades I–V were not reliably resolved by analyses of ITS rDNA (Spiridonov et al., 2004) and were weakly supported by analysis of LSU rDNA (Figures 1, 2). In contrast, for the 3-gene analyses (MP and Bayesian), relationships between these clades were resolved and identical, with strong BPP support for each node. These analyses depicted Clades III and IV as sister groups, with Clade V sister to III + IV. The largest (non-trivial) monophyletic group was represented by Clades I, III, IV and V, and this clade also received 100% bootstrap support. In both Bayesian and MP analyses, clade II was sister to all other *Steinernema* species. Although the composition and relationships of these clades appear well supported by BPP, it is highly likely that this phylogenetic hypothesis undersamples steinernematids relative to their true diversity (Hominick et al., 1996; Stock, 2005). Such incomplete phylogenies should be interpreted cautiously because adding unsampled lineages to phylogenetic analyses may substantially alter interpretation of relationships and tree topology. For

example, Nguyen & Duncan (2002) suggested that *S. diaprepesi* was most closely related to *S. glaseri* (Steiner, 1929), based on their phylogenetic analysis of 11 species whereas the 3-gene analyses of 25 species suggests that *S. diaprepesi* is most closely related to *S. puertoricense* (Bayesian analysis) or *S. karii* (MP analysis).

Cytochrome oxidase subunit I amino acid replacements occurred at a moderate frequency among *Steinernema* spp., occurring at 7% of the positions examined. Six of these sites included replacements that were unambiguously synapomorphic when mapped by parsimony relative to molecular phylogenetic hypotheses (Table 3). Five of these putative shared-derived AA replacements reflected clades in the mtDNA parsimony tree (and were homoplastic relative to the 3-gene trees), whereas one replacement was synapomorphic on the 3-gene trees. Three putative AA replacement synapomorphies defined a clade consisting of (*scapterisci*, *siamkayai*, *carpocapsae*, *anatoliense*).

Previous studies of morphological character evolution in steinernematids relative to phylogenetic hypotheses have revealed high levels of homoplasy (Stock et al., 2001; Spiridonov et al., 2004). Stock et al. (2001) reported that only one character, the presence of horn-like structures in infective juveniles, was synapomorphic for species, with most other morphological characters having consistency indices of <0.50. Parsimony mapping of these same 22 morphological characters on the 3-gene hypotheses (Bayesian and MP trees) revealed lower consistency indices for 14 of these characters when compared to results reported previously by Stock et al. (2001). This result is not unexpected for highly homoplastic features because as more species are added, additional instance of homoplasy are likely to be detected. Given the 3-gene trees, only the horn-like structures of infective juveniles appear to be a shared-derived character, mapping to the clade including *S. bicornutum*, *S. ceratophorum*, *S. abbasi* and *S. riobrave*. Spiridonov et al. (2004) also concluded that horn-like structures were a synapomorphy for Clade IV species. Other structural features coded and mapped by Spiridonov et al. (2004), including spermatozoon morphology, bacterial vesicle features and spicule colour were not characterised during our morphological study. Two of these traits (spicule colour and bacterial vesicle structure) appear too variable to be coded

for species. Bacterial vesicle size and shape varies depending on culture conditions and level of bacterial colonisation; similarly, spicule colour varies among individuals within populations (Stock et al., unpublished). Foraging strategy categories have also been mapped on molecular phylogenetic trees to develop hypotheses for their evolution (Campbell et al., 2003); these workers suggested that the ambush strategy had evolved once, that cruise foragers had evolved three times and that an intermediate foraging strategy was plesiomorphic. Detailed experimental studies characterising foraging strategies are lacking for species newly represented in our molecular trees; however, given the potential predictive value of phylogenies, it can be suggested that *S. hermaphroditum*, *S. diaprepesi* and *S. longicaudum* (US) are cruise foragers, as are the six other characterised members of this strongly supported clade.

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### References

- Adams, B.J. (1998) Species concepts and the evolutionary paradigm in modern nematology. *Journal of Nematology*, **30**, 1–21.
- Alfaro, M.E., Zoller, S. & Lutzoni, F. (2003) Bayes or Bootstrap? A simulation study comparing the performance of Bayesian Markov chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. *Molecular Biology and Evolution*, **20**, 255–266.
- Anderson, T.J.C., Komuniecki, R., Komuniecki, P.R. & Jaenike, J. (1995) Are mitochondria inherited paternally in *Ascaris*? *International Journal for Parasitology*, **25**, 1001–1004.
- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (1989) *Short protocols in molecular biology*. New York, New York: Greene Publishing Associates and Wiley-Interscience, 387 pp.
- Baldwin, J.G., De Ley, I.T., Mundo-Ocampo, M., De Ley, P., Nadler, S.A. & Gebre, M. (2001) *Acromoldavicus mojaviensis* n sp (Nematoda: Cephalobidae) from the Mojave Desert, California. *Nematology*, **3**, 343–353.
- Berry, V. & Gascuel, O. (1996) On the interpretation of bootstrap trees: appropriate threshold of clade selection and induced gain. *Molecular Biology and Evolution*, **13**, 999–1011.

- Blouin, M.S., Yowell, C.A., Courtney, C.H. & Dame, J.B. (1998) Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Molecular Biology and Evolution*, **15**, 1719–1727.
- Boemare, N.E., Akhurst, R.J. & Mourant, R.G. (1993) DNA relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus*, new genus. *International Journal of Systematic Bacteriology*, **43**, 249–255.
- Brown, I.M. & Gaugler, R. (1997) Temperature and humidity influence emergence and survival of entomopathogenic nematodes. *Nematologica*, **43**, 363–375.
- Campbell, J.F. & Gaugler, R.R. (1997) Inter-specific variation in entomopathogenic nematode foraging strategy: dichotomy or variation along a continuum? *Fundamental and Applied Nematology*, **20**, 393–398.
- Campbell, J.F., Lewis, E.E., Stock, S.P., Nadler, S.A. & Kaya, H.K. (2003) Evolution of host search strategies in entomopathogenic nematodes. *Journal of Nematology*, **35**, 142–145.
- de Doucet, M., Bertolotti, M.A., Giayetto, A.L. & Miranda, M.B. (1999) Host range, specificity, and virulence of *Steinernema feltiae*, *Steinernema rarum*, and *Heterorhabditis bacteriophora* (Steinernematidae and Heterorhabditidae) from Argentina. *Journal of Invertebrate Pathology*, **73**, 237–242.
- Eernisse, D.J. & Kluge, A.G. (1993) Taxonomic congruence versus total evidence, and amniote phylogeny inferred from fossils, molecules, and morphology. *Molecular Biology and Evolution*, **10**, 1170–1195.
- Elawad, S., Ahmad, W. & Reid, A.P. (1997) *Steinernema abbasi* sp. n. (Nematoda: Steinernematidae) from the Sultanate of Oman. *Fundamental and applied Nematology*, **20**, 435–442.
- Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783–791.
- Forst, S. & Nealson, K. (1996) Molecular biology of the symbiotic-pathogenic bacteria *Xenorhabdus* spp. and *Photorhabdus* spp. *Microbiological Reviews*, **60**, 21–43.
- Freed, R., Eisensmith, S.P., Goetz, S., Reicosky, R., Smail, V.W. & Wolberg, P. (1991) *User's guide to MSTAT-C: A software program for the design, management, and analysis of agronomic research experiments*. East Lansing, Michigan: Michigan State University.
- Grenier, E., Bonifassi, E., Abad, P. & Laumond, C. (1996) Use of species-specific satellite DNAs as diagnostic probes in the identification of Steinernematidae and Heterorhabditidae entomopathogenic nematodes. *Parasitology*, **113**, 483–489.
- Griffin, C.T., O'Callaghan, K.M. & Dix, I. (2001) A self-fertile species of *Steinernema* from Indonesia: further evidence of convergent evolution amongst entomopathogenic nematodes? *Parasitology*, **122**, 181–186.
- Hominick, W.M., Briscoe, B.R., Del Pino, F.G., Heng, J., Hunt, D.J., Kozodoy, E., Mracek, Z., Nguyen, K.B., Reid, A.P., Spiridonov, S., Stock, P., Sturhan, D., Waturu, C. & Yoshida, M. (1997) Biosystematics of entomopathogenic nematodes: current status, protocols and definitions. *Journal of Helminthology*, **71**, 271–298.
- Hominick, W.M., Reid, A.P., Bohan, D.A. & Briscoe, B.R. (1996) Entomopathogenic nematodes: Biodiversity, geographical distribution and the convention on biological diversity. *Biocontrol Science and Technology*, **6**, 317–331.
- Huelsenbeck, J.P. & Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, **17**, 754–755.
- Joyce, S.A., Griffin, C.T. & Burnell, A.M. (1994) The use of isoelectric focusing and polyacrylamide gel electrophoresis of soluble proteins in the taxonomy of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae). *Nematologica*, **40**, 601–612.
- Kaya, H.K. & Stock, S.P. (1997) Techniques in insect nematology. In: Lacey, L.A. (Ed.) *Manual of techniques in insect pathology*. San Diego: Academic Press, pp. 281–324.
- Kluge, A.G. (1998) Total evidence or taxonomic congruence: cladistics or consensus classification. *Cladistics*, **14**, 151–158.
- Liu, J. & Berry, R.E. (1995) Determination of PCR conditions of RAPD analysis in entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae). *Journal of Invertebrate Pathology*, **65**, 79–81.
- Liu, J. & Berry, R.E. (1996) Phylogenetic analysis of the genus *Steinernema* by morphological characters and randomly amplified polymorphic DNA fragments. *Fundamental and Applied Nematology*, **19**, 463–469.
- Liu, J., Berry, R.E. & Moldenke, A.F. (1997) Phylogenetic relationships of entomopathogenic nematodes (Heterorhabditidae and Steinernematidae) inferred from partial 18S rRNA gene sequences. *Journal of Invertebrate Pathology*, **69**, 246–252.
- Liu, J., Berry, R.E. & Blouin, M.S. (1999) Molecular differentiation and phylogeny of entomopathogenic nematodes (Rhabditida: Heterorhabditidae) based on ND4 gene sequences of mitochondria DNA. *Journal of Parasitology*, **85**, 709–715.
- Loytynoja, A. & Milinkovitch, M.C. (2003) A hidden Markov model for progressive multiple alignment. *Bioinformatics*, **19**, 1505–1513.
- Maddison, D.R. & Maddison, W.P. (2000) *MacClade 4: Analysis of phylogeny and character evolution*. Sunderland, Massachusetts: Sinauer Associates Inc.
- Mráček, Z. & Bednarek, A. (1991) The morphology of the lateral field of infective juveniles of entomopathogenic nematodes of the family Steinernematidae (Rhabditida). *Nematologica*, **37**, 63–71.
- Nadler, S.A. (1995) Advantages and disadvantages of molecular phylogenetics: a case study of ascaridoid nematodes. *Journal of Nematology*, **27**, 423–432.
- Nadler, S.A. (2002) Species delimitation and nematode biodiversity: phylogenies rule. *Nematology*, **4**, 615–625.
- Nadler, S.A., Carreno, R.A., Adams, B.J., Kinde, H., Baldwin, J.G. & Mundo-Ocampo, M. (2003) Molecular phylogenetics and diagnosis of soil and clinical isolates of *Halicephalobus gingivalis* (Nematoda: Cephalobina: Panagrolaimoidea), an opportunistic pathogen of horses. *International Journal for Parasitology*, **33**, 1115–1125.
- Nadler, S.A. & Hudspeth, D.S.S. (2000) Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. *Journal of Parasitology*, **86**, 380–393.
- Nguyen, K.B. & Adams, B.J. (2003) SEM and systematic studies of *Steinernema abbasi* Elawad et al., 1997, and *S. riobrave* Cabanillas et al., 1994 (Rhabditida: Steinernematidae). *Zootaxa*, **179**, 1–10.
- Nguyen, K.B. & Duncan, L.W. (2002) *Steinernema diaprepesi* n. sp. (Rhabditida: Steinernematidae), a parasite of the citrus

- root weevil *Diaprepes abbreviatus* (L) (Coleoptera: Curculionidae). *Journal of Nematology*, **34**, 159–170.
- Nguyen, K.B., Maruniak, J. & Adams, B.J. (2001) Diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. *Journal of Nematology*, **33**, 73–82.
- Nguyen, K.B. & Smart, G.C. Jr (1997) Scanning electron microscope studies of spicules and gubernacula of *Steinernema* spp (Nemata: Steinernematidae). *Nematologica*, **43**, 465–480.
- Poinar, G.O. Jr (1990) Taxonomy and biology of Steinernematidae and Heterorhabditidae. In: Gaugler, R. & Kaya, H.K.K. (Eds) Entomopathogenic nematodes in biological control. Boca Raton, Florida: CRC Press, pp. 23–61.
- Poinar, G.O. Jr (1993) Origins and phylogenetic relationships of the entomophilic rhabditids *Heterorhabditis* and *Steinernema*. *Fundamental and Applied Nematology*, **16**, 333–338.
- Posada, D. & Crandall, K.A. (1998) MODELTEST: Testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.
- Reid, A.P. (1994) Molecular taxonomy of *Steinernema*. In: Burnell, A.M., Ehlers, R.-U. & Mason, J.P. (Eds) Genetics of entomopathogenic nematode-bacterium complexes. Luxembourg City: European Commission Luxembourg, pp. 49–58.
- Reid, A.P. & Hominick, W.M. (1993) Cloning of the rDNA repeat unit from a British entomopathogenic nematode (Steinernematidae) and its potentials for species identification. *Parasitology*, **107**, 529–536.
- Reid, A.P., Hominick, W.M. & Briscoe, B.R. (1997) Molecular taxonomy and phylogeny of entomopathogenic nematode species (Rhabditida: Steinernematidae) by RFLP analysis of the ITS region of the ribosomal DNA repeat unit. *Systematic Parasitology*, **37**, 187–193.
- Sanderson, M.J., Donoghue, M.J., Piel, W. & Eriksson, T. (1994) TreeBASE: A prototype database of phylogenetic analyses and an interactive tool for browsing the phylogeny of life. *American Journal of Botany*, **81**, 183.
- Sicard, M., Ferdy, J.B., Pages, S., Le Brun, N., Godelle, B., Boemare, N. & Moulia, C. (2004) When mutualists are pathogens: an experimental study of the symbioses between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria). *Journal of Evolutionary Biology*, **17**, 985–993.
- Simoës, N. & Sosa, J.S. (1996) Pathogenicity and host specificity of entomopathogenic nematodes. *Biocontrol Science and Technology*, **6**, 403–411.
- Simon, C. (1983) A new coding procedure for morphometric data with an example from periodical cicada wing veins. In: Felsenstein, J. (Ed.) Numerical Taxonomy. Berlin: Springer-Verlag, pp. 378–383.
- Spiridonov, S.E., Reid, A.P., Podrucka, K., Subbotin, S.A. & Moens, M. (2004) Phylogenetic relationships within the genus *Steinernema* (Nematoda: Rhabditida) as inferred from analyses of sequences of the ITS1–5.8S-ITS2 region of rDNA and morphological features. *Nematology*, **6**, 547–566.
- Stock P. (2005) Insect pathogenic nematodes: from lab curiosities to model organisms. *Journal of Invertebrate Pathology*, (in press).
- Stock, P. & Koppenhöfer, A.M. (2003) *Steinernema scarabaei* n. sp. (Rhabditida: Steinernematidae), a natural pathogen of scarab beetle larvae (Coleoptera: Scarabaeidae) from New Jersey. *Nematology*, **5**, 191–204.
- Stock, S.P., Campbell, J.F. & Nadler, S.A. (2001) Phylogeny of *Steinernema* travassos, 1927 (Cephalobina: Steinernematidae) inferred from ribosomal DNA sequences and morphological characters. *Journal of Parasitology*, **87**, 877–889.
- Stock, S.P., Griffin, C.T. & Chaerani, R. (2004) Morphological and molecular characterisation of *Steinernema hermaphroditum* n. sp. (Nematoda: Steinernematidae), an entomopathogenic nematode from Indonesia, and its phylogenetic relationships with other members of the genus. *Nematology*, **6**, 401–412.
- Stock, S.P., Somsook, V. & Reid, A.P. (1998) *Steinernema siamkayai* n. sp. (Rhabditida: Steinernematidae), an entomopathogenic nematode from Thailand. *Systematic Parasitology*, **41**, 105–113.
- Swofford, D.L. (1998) *PAUP\**. *Phylogenetic Analysis Using Parsimony (\*and Other Methods)*. Version 4. Sinauer Associates, Inc: Sunderland, Massachusetts.
- Szalanski, A.L., Taylor, D.B. & Mullin, P.G. (2000) Assessing nuclear and mitochondrial DNA sequence variation within *Steinernema* (Rhabditida: Steinernematidae). *Journal of Nematology*, **32**, 229–233.
- Tallosi, B., Peters, A. & Ehlers, R.-U. (1994) *Steinernema bicornutum* (Rhabditida: Steinernematidae) from Vojvodina, Yugoslavia. *Russian Journal of Nematology*, **3**, 71–80.
- Waturu, C.N., Hunt, D.J. & Reid, A.P. (1997) *Steinernema karii* sp. n. (Nematoda: Steinernematidae), a new entomopathogenic nematode from Kenya. *International Journal of Nematology*, **7**, 68–75.