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Diagnostic and Phylogenetic Utility of the rDNA Internal Transcribed Spacer Sequences of *Steinernema*[1]

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Abstract: The ITS regions of 10 species of *Steinernema* were PCR amplified and directly sequenced. Restriction mapping of these sequences revealed diagnostic variation such that the number of cuts and the length of the resulting fragments can be used to diagnose *Steinernema* species. Nevertheless, identical fragment sizes produced by non-homologous restriction sites also were identified. Pronounced variation in sequence length and nucleotide composition resulted in optimized alignments containing extensive regions of dubious homology. Significant shifts in nucleotide base composition exist among taxa and appear to mirror evolutionary history. These shifts do not have an observable influence on phylogenetic reconstruction and are probably due to descent as opposed to convergence. Alignment instability and the presence of alignment-ambiguous regions had the greatest effect on phylogeny reconstruction. Our results support the taxonomic utility of the ITS region to diagnose nematode species of the genus *Steinernema,* and all sampled taxa show evidence (in the form of numerous autapomorphic characters) of lineage independence. However, the ITS region appears to be phylogenetically informative only for closely related sister species. High variability among more distantly related taxa preclude its use for confidently resolving relationships among all members of the genus.

Key words: Entomopathogenic nematodes, evolution, ITS region, molecular systematics, nematode, phylogeny, ribosomal DNA, species delimitation, species diagnosis, systematics, taxonomy.

The genus *Steinernema* currently contains 24 species, and their taxonomic identification is often complicated. Although some taxonomic keys (Hominick et al., 1997; Nguyen and Smart, 1992, 1996; Poinar, 1990) have been constructed to help identify species, the overlap of morphometrics and similar morphological structures of some species make identification difficult. The polymerase chain reaction (PCR) in combination with RFLP (Hominick et al., 1997; Joyce et al., 1994; Nasmith et al., 1996; Reid and Hominick, 1992) has been used to confirm the identification of *Steinernema* species and to estimate their phylogenetic relationships.

DNA sequences of internal transcribed spacer (ITS) regions yield more detailed information about variation within and among nematode species than PCR-RFLP approaches. These spacer sequences have been used successfully to diagnose species and populations of nematodes (e.g., Blok et al., 1997; Cherry et al. 1997; Ferris et al., 1993; Ibrahim et al., 1997; Powers et al., 1997; Szalanski et al., 1997). Analyses of ITS rDNA sequences also have been used to reconstruct phylogenetic relationships and delimit nematode species (Adams, 1998; Adams et al., 1998; Kaplan et al., 2000; Nadler et al., 2000). Sequence variation of the ITS1 region among seven species of *Steinernema* was recently explored by Szalanski et al. (2000).

We distinguish the diagnosis and identification of species as being distinct from the activity of species delimitation and phylogeny reconstruction. The latter activities produce hypotheses that carry meaning in an evolutionary context, require hypothesis testing, and emanate from homologous, heritable characters. Alternatively, species diagnosis consists of determining whether entities are merely ''the same'' or ''different.'' Therefore, we explored the potential of this molecular marker to diagnose or identify steinernematid species as well as reconstruct their evolutionary history and delimit species. For our investigation, ITS regions of ribosomal DNA from 10 *Steinernema* species were amplified using PCR and sequenced. Our intent was to determine the taxonomic and phylogenetic utility of the entire ITS region for systematic work in the genus *Steinernema.*

Materials and Methods

The nematode species used in this paper were *Steinernema bicornutum* Tallosi, Peters & Ehlers 1995 (''Yugoslavia'' isolate), *S. carpocapsae* (Weiser, 1955) Wouts, Mracek, Gerdin & Bedding 1982 (''Breton'' isolate), *S. feltiae* (Filipjev, 1934) Wouts, Mracek, Gerdin & Bedding 1982 (''SN'' isolate), *S. glaseri* (Steiner, 1929) Wouts, Mracek, Gerdin & Bedding 1982 (''NJ'' isolate), *S. intermedium* (Poinar 1985) Mamiya 1988 (''SC'' isolate), *S. monticolum* Stock, Choo & Kaya 1997 (''Korea'' isolate), *S. neocurtillae* Nguyen & Smart 1992 (''La Crosse'' isolate), *S. oregonense* Liu & Berry 1996 (''Oregon'' isolate), *S. scapterisci* Nguyen & Smart 1990 (''Uruguay'' isolate), and a new species of *Steinernema.*

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All are maintained in the nematode evolution laboratory of the Entomology and Nematology Department, University of Florida, Gainesville, Florida. For some analyses we included previously published sequences of the ITS1 region for *S. arenarium, S. carpocapsae, S. feltiae, S. glaseri, S. kushidai, S. intermedium, and S. riobrave* (GenBank accession numbers AF192982-192989; Szalanski et al., 2000).

Extraction of DNA: DNA of each nematode species was extracted from a single female (or juvenile) using the method reported by Hominick et al. (1997). The nematode was crushed in 20 µl (10 µl for juvenile) of lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin, and 60 µg/ml proteinase K) on a sterilized hanging-drop glass slide and transferred to a sterilized 0.5-ml microcentrifuge tube on ice. The tube was frozen at –80 °C for 10 minutes, incubated at 65 °C for 1 hour, and followed by a 95 °C incubation for 10 minutes to completely lyse the cells, digest the proteins, and inactivate proteinase K, respectively. Subsequently, the tube was cooled on ice and centrifuged at 12,000 rpm for 2 minutes. The supernatant containing the DNA was collected and kept at 4 °C for future use.

PCR amplification: The ITS region of the ribosomal DNA was PCR in a 100-µl reaction with a DNA polymerase kit from AMRESCO, Inc. The following were used to assemble the reaction: 10 µl of 10× PCR buffer (100 mM Tris-HCl (pH 8.8), 15 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100), 2 µl of dNTP mixture (10 mM each), 2 µl of 10 µM forward primer, 2 µl of 5 pM reverse primer, 0.5 µl of Thermalase Thr™ (2 U/µl), 74 µl of distilled water, and 10 µl of DNA extract. Mineral oil (100 µl) was placed on top of the solution in the tube to minimize evaporation. The primers used in this study were reported by Vrain et al. (1992): 18S: 5′-TTGATTACGTCCCTGCCCTTT-3′ (forward) and 26S: 5′-TTTCACTCGCCGTTACTAAGG-3′ (reverse) corresponding to nucleotide position 2503–2523 and 3774–3794, respectively, of the sequence of the rDNA tandem unit from *Caenorhabditis elegans* (GenBank accession number X03680). All PCR reactions were conducted in a PTC-100 Thermocycler (MJ Research, Inc., Waltham, MA) with the cycling profile suggested by Hominick et al. (1997): 1 cycle at 94 °C for 2 minutes followed by 40 cycles at 94 °C for 30 seconds, 45 °C for 60 seconds, and 72 °C for 90 seconds. The last step was 72 °C for 5 minutes.

Sequencing: PCR products were purified with a QIAquick PCR purification kit (QIAGEN Inc., Santa Clarita, CA). Purified DNA was sequenced directly using an ABI PRISM ™ Dye Terminator Cycling Sequencing Ready Reaction Kit (Perkin-Elmer Corp., Foster City, CA). For each sequencing reaction the following reagents were added to a 0.5-ml tube: 4 µl Terminator Ready Reaction Mix, 1.5 µl primer (3.2 pM), 2.5 µl template DNA from the PCR reaction, 2 µl water, and 10 µl of mineral oil to avoid evaporation. The primers used in this step were the 18S and 26S described earlier and two internal primers that were synthesized for this study: KN58, 5′-GTATGTTTGGTTGAAGGTC-3′ and KNRV, 5′-CACGCTCATACAACTGCTC-3′. The KN58 primer was designed with the Prime program from the Genetic Computer Group (GCG) Package, Madison, Wisconsin; the KNRV primer was selected from the alignment of 10 sequences obtained by sequencing PCR products of 10 species with the two PCR primers. The two internal primers were used to obtain complete sequence of both strands of the PCR product. The sequencing reactions were conducted in a PTC-100 thermal cycler, and the following cycling profile was used: 25 cycles at 96 °C for 30 seconds, 50 °C for 15 seconds, and 60 °C for 4 minutes. The sequencing product was ethanol-precipitated to remove primers and unincorporated nucleotides. For this, 1 µl 3 M sodium acetate, pH 4.6, and 25 µl 95% ethanol were added to the 10-µl sequencing reaction, incubated on ice for 10 minutes, and centrifuged in a microcentrifuge for 15 minutes. The pellet was washed with 70% ethanol and dried in a vacuum desiccator. The DNA was sequenced at the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center on an Applied Biosystems Model 373A DNA Sequencer or 377 DNA Sequencer. The sequences flanked by the 18S and 26S primers were assembled using Auto-Assembler Version 2 (PerkinElmer, Wellesley, MA). At least two sequencing reactions were performed per strand.

Alignment of multiple sequences: To establish a root for an analysis of only steinernematid taxa, sequences of the 5.8S subunit were aligned to other, more distantly related nematodes. Outgroup taxa included *Heterodera avenae* (U12389), *H. glycines* (AF216579), *Nacobbus aberrans* (U71375), *Meloidogyne arenaria* (AF077083), *Aphelenchus avenae* (AF119048), *Ascaris suum* (AJ001506), *Toxocara canis* (AJ001498), *Heterakis gallinarum* (AJ001510), and *Caenorhabditis elegans* (X03680). The 5.8S sequences were aligned using the default parameters of ClustalX (Thompson et al., 1997) and edited manually.

An alignment of the ingroup taxa for the entire sequenced product was carried out using a modified elision method (Maddison et al., 1999; Wheeler et al., 1995). The aligned sequences included the entire amplified and sequenced PCR product (172 bases of the 18S flanking region, the entire ITS1-5.8S-ITS2, and 78 bases of the 28S flanking region). Using MALIGN (Wheeler and Gladstein, 1994), eight separate multiple-sequence alignments were created with gap insertion penalties that varied sequentially from 3 to 10 and gap extension penalties that varied from 1 to 8. The eight alignments were then concatenated into a single matrix. A second independent alignment was created

with gap parameters estimated according to Wheeler (1990), from which regions of dubious homology were removed. These regions were identified as having two or more character states within columns that could be shifted two or more columns (either to the left or right) without unambiguously improving the homology statement by increasing the number of taxa that share the same character state. In addition, to this alignment (optimized, edited) the sequences of the ITS1 region of Szalanski et al. (2000) were added using the Profile Alignment command of ClustalX (Thompson et al., 1997). All alignments, including assumption sets specifying rooting assumptions and deleted characters, are available from TreeBASE submission number S595 (http://www.herbaria.harvard.edu/treebase/).

Mapping: GCG Map and Mapsort programs (GCG package) were used to map the sites and sizes of fragments based on potential cuts by 217 available restriction enzymes.

Similarity: PAUP* 4.0b8 (Swofford, 2001) was used to create a table of pairwise comparisons among the 10 aligned, unedited sequences. The Bestfit program (GCG package) also was used to compare *Steinernema* sequences with those of the same species previously reported in the GenBank: *S. carpocapsae* (GenBank, Accession numbers AF036947 and AF036604).

Phylogenetic analysis: Maximum parsimony analyses were performed for the 5.8s matrix, the concatenated ITS matrix, and the optimized ITS alignment. In addition, the optimized ITS alignment underwent independent analyses with the alignment-ambiguous regions removed, gaps treated as a fifth base, and gaps treated as missing data. Maximum likelihood analyses were conducted on the 5.8S matrix and the optimized ITS alignment. Neighbor-joining trees based on LogDet distances were performed only on the optimized ITS alignment.

Maximum parsimony trees were constructed using PAUP* 4.0b8 (Swofford, 2001) with a branch and bound search strategy. All characters were treated as equally weighted. Model choice and parameters for maximum likelihood analyses were selected based on likelihood ratio tests (Modeltest version 3.0; Posada and Crandall, 1998). The 5.8S data set assumed a Kimura (1980) model of sequence evolution with Tl/Tv ratio of 1.9896. Base frequencies were assumed to be equal, and rate heterogeneity implied zero invariable sites and a γ distribution shape parameter of 0.8591. The maximum likelihood analysis of the optimized ITS alignment assumed the sequence substitution model of Tamura and Nei (1993) (substitution rate matrix: Ra = 1.0, Rb = 1.6456, Rc = 1.0, Rd = 1.0, Re = 2.2714, Rf = 1.0; base frequencies: freqA = 0.2673, freqC = 0.1582, freqG = 0.2061, freqT = 0.3683; proportion of invariable sites = 0.3954; γ shape parameter = 2.1477). We did not perform a maximum likelihood analysis of the concatenated ITS matrix or the optimized ITS matrix with alignment-ambiguous regions deleted because the edited alignments violate the interdependence-of-sites assumptions of the maximum likelihood models. Maximum likelihood trees were estimated using a heuristic search algorithm (starting trees generated by random sequence addition [30 replicates], with tree-bisection-reconnection branch-swapping). Branch support for the maximum likelihood tree was estimated via bootstrapping (100 replicates) using the same treesearch procedure. Branch support for parsimony analyses was estimated by bootstrapping (via branch and bound search, 1,000 replicates) and Bremer support (AutoDecay 4.0.2) (Bremer, 1988, 1994; Eriksson, 1998; Källersjö et al., 1992). Trees based on the 5.8S data were rooted with taxa representing members of the Tylenchida (as per Blaxter et al., 1998). Trees based on the analyses of only Steinernematids were rooted according to the relationships established by the 5.8S tree.

Results and Discussion

ITS sequence characterization: The PCR reactions successfully amplified part of the ribosomal DNA of 10 species of *Steinernema.* No evidence of intraindividual sequence polymorphism was found. The length of the PCR-amplified DNA of these species varied from 916 to 1,061 base pairs (as determined by sequencing) (Table 1) and correspond to nucleotide positions 2524–3773 of *C. elegans.* The nucleotide composition of the sequences is presented in Table 2. Average base composition among Steinernematid taxa was 0.26 (A), 0.18 (C), 0.23 (G), and 0.33 (T). The sequences were AT rich (mean = 0.59), and an among-taxon test revealed significant departure from base frequency homogeneity using a X^2 test ($P < 0.001$).

Multiple alignments: The alignment of the 10 sequences of *Steinernema* shows that the 18S (partial), 5.8S, and 28S (partial) regions are less variable among taxa than the ITS1 and ITS2 regions, which were both highly variable. Several regions of the optimized ITS alignment contained multiple insertion and deletion events among all taxa. Six blocks of 118, 61, 5, 92, 183, and 187 characters were considered of dubious homology and were removed prior to some of the parsimony analyses. Removal of the alignment-ambiguous regions dramatically reduced the number of indels and eliminated the need for re-coding multiple, adjacent indels as unique events.

The lengths of the amplified region for the sampled species of *Steinernema* were often diagnostic: *S. monticolum,* 916 bp (Accession number AF122017); *S. scapterisci,* 959 bp (AF122020); *S. neocurtillae,* 968 bp (AF122018); *S. oregonense,* 973 bp (AF122019); *S. feltiae,* 1,016 bp (AF121050); *Steinernema* sp., 1,020 bp (AF122021); and *S. intermedium,* 1,061 bp (AF122016). However, the sequence length of the three species *S.*

TABLE 1. Number of cuts (boldfaced numbers) for each restriction enzyme and fragment arrangement (in descending order) of ITS sequences of 10 *Steinernema* spp. cut by 22 identifying enzymes.

Enzyme	*bicornutum* (1,016 bp)	*carpocapsae* (987 bp)	*feltiae* (980 bp)	*glaseri* (988 bp)	*intermedium* (1,061 bp)	*monticolum* (916 bp)	*neocurtillae* (968 bp)	*oregonense* (973 bp)	*scapterisci* (959 bp)	sp. (1,022 bp)
AluI	**2;** 716, 177, 123	**5;** 429, 177, 150, 120, 66, 45	**3;** 512, 258, 177, 33	**5;** 355, 293, 139, 108, 61, 32	**3;** 475, 226, 181, 179	**1;** 742, 177	**1;** 791, 177	**4;** 509, 177, 149, 105, 33	**5;** 269, 215, 177, 163, 71, 64	**8;** 216, 177, 175, 162, 116, 88, 55, 22, 11
ApoI	**1;** 547, 469	**1;** 545, 442	**4;** 318, 213, 165, 144, 130	**1;** 545, 443	**3;** 491, 404, 101, 65	**3;** 530, 197, 98, 94	**1;** 523, 445	**3;** 533, 245, 148, 47	**1;** 513, 446	**1;** 568, 454
Bcef	**2;** 735, 156, 125	**1;** 731, 256	**4;** 571, 147, 125, 88, 49	**3;** 627, 149, 125, 87	**1;** 763, 298	**3;** 522, 138, 134, 125	**2;** 710, 133, 125	**4;** 572, 148, 125, 78, 50	**1;** 704, 255	**3;** 638, 149, 125, 110
BfaI	**4;** 450, 332, 160, 66, 8	**5;** 612, 180, 102, 70, 15, 8	**2;** 626, 346, 8	**2;** 745, 235, 8	**2;** 918, 135, 8	**3;** 479, 332, 97, 11	**3;** 616, 219, 125, 8	**3;** 337, 334, 294, 8	**4;** 580, 179, 122, 70, 8	**2;** 669, 345, 8
DdeI	**3;** 676, 201, 111, 28	**4;** 642, 190, 101, 28, 26	**3;** 647, 271, 34, 28	**2;** 926, 34, 28	**3;** 726, 284, 28, 23	**4;** 697, 141, 32, 28, 21	**2;** 801, 139, 28	**3;** 758, 153, 34, 28	**3;** 513, 230, 188, 28	**4;** 590, 197, 173, 34, 28
FokI	**0**	**1;** 542, 445	**0**	**0**	**1;** 974, 87	**0**	**1;** 881, 87	**0**	**0**	**1;** 708, 314
HhaI	**1;** 543, 473	**2;** 541, 240, 206	**1;** 880, 100	**4;** 792, 116, 68, 10, 2	**2;** 493, 307, 261	**0**	**0**	**2;** 762, 107, 104	**2;** 533, 221, 205	**1;** 954, 68
Hin4I	**0**	**0**	**2;** 853, 71, 56	**3;** 587, 274, 71, 56	**2;** 746, 244, 71	**2;** 645, 200, 74	**1;** 897, 71	**2;** 846, 71, 56	**0**	**2;** 895, 71, 56
HincII	**2;** 620, 249, 147	**0**	**0**	**1;** 605, 383	**0**	**2;** 746, 158, 15	**2;** 826, 118, 24	**0**	**1;** 815, 144	**2;** 825, 121, 76
HindIII	**0**	**1;** 767, 220	**0**	**1;** 817, 171	**0**	**0**	**0**	**0**	**0**	**1;** 858, 164
HinfI	**4;** 378, 377, 168, 62, 31	**3;** 372, 268, 241, 106	**3;** 611, 194, 166, 9	**0**	**0**	**4;** 422, 202, 143, 113, 39	**1;** 620, 348	**4;** 613, 194, 147, 10, 9	**2;** 616, 237, 106	**2;** 682, 244, 96
MaeIII	**4;** 322, 243, 183, 131, 127	**3;** 564, 154, 138, 131	**5;** 353, 269, 170, 131, 40, 17	**3;** 374, 315, 168, 131	**2;** 734, 194, 133	**4;** 307, 284, 161, 131, 36	**4;** 376, 405, 156, 84, 47	**7;** 270, 187, 171, 148, 131, 30, 19, 17	**2;** 675, 153, 131	**5;** 438, 182, 156, 131, 68, 47

TABLE 1. *Continued*

Enzyme	*bicornutum* (1,016 bp)	*carpocapsae* (987 bp)	*feltiae* (980 bp)	*glaseri* (988 bp)	*intermedium* (1,061 bp)	*monticolum* (916 bp)	*neocurtillae* (968 bp)	*oregonense* (973 bp)	*scapterisci* (959 bp)	sp. (1,022 bp)
MseI	**19;** 151, 101, 100, 100, 79, 76, 66, 64, 60, 45, 44, 30, 21, 20, 15, 13, 10, 9, 7, 5	**9;** 324, 134, 102, 100, 93, 85, 60, 43, 32, 14	**8;** 252, 189, 168, 144, 100, 43, 41, 29	**3;** 550, 295, 100, 43	**13;**173, 167, 166, 104, 102, 101, 80, 51, 43, 29, 17, 15, 8, 5	**7;** 224, 187, 169, 100, 93, 86, 46, 14	**22;** 202, 100, 92, 60, 57, 56, 50, 43, 43, 43, 35, 29, 29, 23, 21, 16, 15, 13, 13, 11, 8, 5, 4	**7;** 334, 243, 168, 100, 43, 41, 29, 15	**11;** 378, 136, 100, 92, 73, 68, 43, 28, 13, 12, 9, 7	**8;** 348, 180, 145, 100, 79, 51, 43, 42, 34
MwoI	**4;** 335, 231, 200, 156, 94	**5;** 429, 200, 112, 53, 41	**3;** 461, 425, 53, 41	**6;** 225, 224, 205, 177, 63, 53, 41	**6;** 264, 249, 215, 187, 53, 50, 43	**3;** 411, 262, 152, 94	**1;** 501, 467	**4;** 417, 257, 205, 53, 41	**6;** 397, 199, 157, 73, 53, 41, 39	**4;** 476, 247, 205, 53, 41
PvuII	**1;** 716, 300	**0**	**0**	**2;** 494, 293, 201	**0**	**0**	**0**	**0**	**1;** 619, 340	**1;** 729, 293
RsaI	**2;** 806, 208, 2	**3;** 363, 316, 306, 2	**5;** 431, 236, 148, 117, 46, 2	**5;** 652, 130, 129, 63, 12, 2	**2;** 688, 371, 2	**5;** 656, 137, 67, 47, 10, 2	**3;** 767, 180, 19, 2	**4;** 423, 240, 189, 119, 2	**3;** 642, 183, 132, 2	**3;** 693, 190, 137, 2
SaII	**0**	**0**	**0**	**1;** 603, 385	**0**	**1;** 759, 160	**0**	**0**	**0**	**0**
Sau3A1	**4;** 532, 164, 162, 142, 16	**3;** 521, 235, 164, 67	**4;** 518, 239, 87, 77, 59	**3;** 522, 263, 164, 39	**5;** 500, 168, 166, 157, 54, 16	**5;** 468, 164, 107, 90, 67, 23	**3;** 524, 280, 87, 77	**7;** 231, 203, 165, 120, 87, 77, 59, 31	**3;** 525, 252, 164, 18	**2;** 533, 325, 164
Sau96I	**0**	**0**	**1;** 827, 153	**0**	**1;** 671, 390	**0**	**0**	**0**	**0**	**0**
SimI	**1;** 604, 412	**1;** 602, 385	**2;** 382, 341, 257	**2;** 386, 369, 233	**1;** 627, 434	**1;** 587, 332	**1;** 580, 388	**1;** 590, 383	**1;** 570, 389	**2;** 397, 392, 233
TaqI	**8;** 383, 119, 107, 103, 92, 68, 66, 40, 38	**5;** 419, 309, 83, 72, 66, 38	**9;** 275, 155, 119, 112, 102, 66, 49, 40, 38, 24	**9;** 254, 148, 148, 119, 85, 84, 66, 38, 37, 9	**6;** 404, 308, 119, 85, 68, 39, 38	**9;** 366, 119, 87, 72, 66, 64, 49, 40, 38, 18	**5;** 359, 346, 119, 66, 40, 38	**10;** 241, 138, 125, 119, 102, 66, 52, 50, 38, 26, 16	**5;** 347, 223, 159, 126, 66, 38	**13;** 244, 205, 138, 119, 84, 66, 38, 38, 28, 22, 14, 12, 9, 5
Tth111II	**1;** 698, 318	**3;** 497, 254, 164, 72	**2;** 576, 355, 49	**3;** 433, 359, 147, 49	**6;** 292, 229, 186, 133, 127, 49, 45	**3;** 304, 302, 261, 52	**2;** 558, 361, 49	**3;** 356, 354, 214, 49	**2;** 706, 197, 56	**1;** 800, 222

TABLE 2. Base frequencies among representative *Steinernema.*

Taxon	A	C	G	T	AT%	# sites
S. bicornutum	0.26476	0.17126	0.22638	0.33760	0.60236	1,016
S. feltiae	0.25714	0.18367	0.23061	0.32857	0.58571	980
S. sp.	0.24853	0.21233	0.24951	0.28963	0.53816	1,022
S. glaseri	0.23583	0.21761	0.27126	0.27530	0.51113	988
S. oregonense	0.25385	0.19322	0.23535	0.31757	0.57142	973
S. intermedium	0.25636	0.16682	0.21960	0.35721	0.61357	1,061
S. carpocapsae	0.24417	0.17427	0.23202	0.34954	0.59371	987
S. scapterisci	0.25235	0.16580	0.22836	0.35349	0.60584	959
S. monticolum	0.27402	0.17358	0.23908	0.31332	0.58734	916
S. neocurtillae	0.27686	0.14979	0.19731	0.37603	0.65289	968
Mean	0.25623	0.18095	0.23293	0.32989	0.58621	987.00

carpocapsae, 986 bp (AF121049); *S. bicornutum,* 987 bp (AF121048); and *S. glaseri,* 988 bp (AF122015) are very similar (Tables 1 and 2). We note differences between our sequences and those of Szalanski et al. (2000) for *S. glaseri, S. feltiae,* and *S. intermedium.* At the nucleotide position that is five bases from the terminal 5′ end of their sequences is a cytosine (C); in our sequences there is a thymidine (T). The differences occur within the 5′ primer sequence of Szalanski et al. (2000).

Mapping: Morphological identification of *Steinernema* is often complicated, and ITS PCR-RFLP has been proposed as a useful tool for diagnosing or identifying described species of *Steinernema* (Hominick et al., 1997). Table 1 presents restriction enzymes yielding distinct profiles that can be used for diagnostic purposes among the 10 species included in this study. Where the number of restriction fragments are identical, fragment lengths can be used for further discrimination. For example, the enzyme *Alu*I cleaves both *S. feltiae* and *S. intermedium* at three sites; however, the resulting fragments are different lengths (512, 258, 177, and 33 bps for *S. feltiae* and 475, 226, 181, and 179 bps for *S. intermedium*). The data in Table 1 corroborate the ITS PCR-RFLP results of Reid (1994) and Hominick et al. (1997) as useful diagnostic tools for Steinernematid taxonomy, and isolates that produce unique RFLP profiles can be considered candidates for further investigation as new species. However, ITS PCR-RFLP will be of limited use for phylogenetic analyses or delimiting new species because, without *a priori* knowledge of cleavage site homology, interpretation of fragment patterns can be complicated or misleading. For example, *Mse*I produces multiple bands of identical size within a single taxon (two 100- bp bands in *S. bicornutum,* three 43- bp bands in *S. neocurtillae*), complicating an accurate interpretation of the number of fragments visualized on a standard agarose gel. Additionally, *Mwo*I produces a 200-bp fragment in *S. bicornutum* and *S. carpocapsae* and a 205-bp fragment in *S. glaseri* and *S. oregonense;* however, in both cases fragments of identical size are produced by non-homologous cleavage sites. Thus, without restriction site maps, fragment patterns cannot reliably produce the homologous characters required to infer phylogenetic relationships or delimit species. We are skeptical that PCR-RFLP of this locus is a cost-effective or efficient taxonomic phylogenetic tool for *Steinernema,* given the additional information gained from two to four sequencing reactions.

Similarity ratio: Uncorrected pairwise comparisons of the sequences among 10 *Steinernema* species are presented as similarity ratios in Table 3. For the closely related species *S. feltiae* and *S. oregonense,* this ratio is 94.3%; for *S. carpocapsae* and *S. scapterisci,* the ratio is 91.4%; and for *S. glaseri* and *Steinernema* sp. the ratio is 90.4%. Maximum likelihood corrected distances are presented in Table 4.

Intuitively, the similarity in DNA sequences appears to agree with morphological observation. Morphologically, *S. oregonense* is similar to *S. feltiae* except the E% (distance from anterior end/tail length × 100) is larger (Hominick et al., 1997) and the mucron on its male tail terminus is absent. *Steinernema scapterisci* has been characterized as closely related to *S. carpocapsae* because the body lengths of the two species are similar (Nguyen and Smart, 1990). *Steinernema glaseri* and *S. intermedium,* two species that appear to be distantly related by morphological traits (Nguyen and Smart, 1996), present a DNA similarity ratio of only 74.1% and are the least similar of the 10 species of *Steinernema* studied. Statistical testing

TABLE 3. Pairwise similarity among 10 species of *Steinernema.*

Species[a]	BIC	FEL	SP.	GLA	ORE	INT	CAR	SCA	MON	NEO
S. bicornutum	—	0.18894	0.23231	0.23845	0.18828	0.23633	0.23432	0.23973	0.23174	0.23026
S. feltiae	181	—	0.21164	0.20779	0.05723	0.22813	0.21844	0.23503	0.17332	0.21577
S. sp.	220	200	—	0.09657	0.21627	0.26393	0.22835	0.24062	0.21808	0.22807
S. glaseri	222	192	93	—	0.21304	0.25934	0.24439	0.25934	0.22953	0.23378
S. oregonense	180	55	202	196	—	0.21458	0.22247	0.23378	0.17257	0.21768
S. intermedium	229	219	251	243	206	—	0.27413	0.28287	0.26554	0.26172
S. carpocapsae	213	199	211	218	200	250	—	0.08613	0.16816	0.17457
S. scapterisci	216	212	218	229	209	256	82	—	0.18110	0.18074
S. monticolum	203	152	193	199	151	235	150	161	—	0.17095
S. neocurtillae	210	197	208	209	197	240	162	167	153	—

[a] BIC = *Steinernema bicornutum,* FEL = *S. feltiae,* SP. = *S.* sp., GLA = *S. glaseri,* ORE = *S. oregonense,* INT = *S. intermedium,* CAR = *S. carpocapsae,* SCA = *S. scapterisci,* MON = *S. monticolum,* NEO = *S. neocurtillae.* Values in the lower diagonal are total character differences; those in the upper diagonal are mean character differences. Missing data (gaps) were distributed proportionally to unambiguous changes.

TABLE 4. Maximum-likelihood distance matrix.

Species[a]	BIC	FEL	SP.	GLA	ORE	INT	CAR	SCA	MON	NEO
S. bicornutum	—									
S. feltiae	0.28135	—								
S. sp.	0.38261	0.32724	—							
S. glaseri	0.40231	0.31784	0.11151	—						
S. oregonense	0.27679	0.06204	0.33404	0.32607	—					
S. intermedium	0.40547	0.38273	0.49054	0.47851	0.34410	—				
S. carpocapsae	0.39864	0.35396	0.36765	0.41406	0.35834	0.52408	—			
S. scapterisci	0.42250	0.39877	0.40333	0.45726	0.39011	0.55681	0.09874	—		
S. monticolum	0.39642	0.25245	0.34569	0.37772	0.24819	0.50573	0.23579	0.26282	—	
S. neocurtillae	0.39713	0.35122	0.37653	0.39274	0.35281	0.50708	0.24741	0.26229	0.24497	—

[a] GLA = *Steinernema glaseri*, SP. = *Steinernema* sp., CAR = *S. carpocapsae*, SCA = *S. scapterisci*, FEL = *S. feltiae*, ORE = *S. oregonense*, MON = *S. monticolum*, NEO = *S. neocurtillae*, BIC = *S. bicornutum*, INT = *S. intermedium*. Model of sequence substitution is Tamura and Nei (1993) with γ distributed rate heterogeneity and an estimate of the number of invariant sites.

of apparent trends between morphological and molecular features are currently under way.

The similarity ratio of the ITS1 sequence of *S. carpocapsae* strain ''Breton'' in this study and that of *S. carpocapsae* strain ''All'' reported by Adams et al. (1998) is 99.6% similar, differing only by two bases. The 18S portion of *S. carpocapsae* strain ''Breton'' is identical to that of the *S. carpocapsae* sequence reported by Blaxter et al. (1998).

Phylogenetic analysis: Because of the high degree of ITS sequence dissimilarity among Steinernematid taxa, aligning these taxa to other more distantly related taxa with confidence was not feasible. The analysis of the 5.8S data set produced trees that strongly supported *Steinernema* as a monophyletic group and tenuously, but consistently, placed *S. intermedium* as the sister taxon to the rest of the Steinernematids (Fig. 1). For this reason, we treated *S. intermedium* as the outgroup taxon for resolving the relationships among the rest of the *Steinernema* species using the entire ITS sequence array. Thus, for the practical purpose of character polarization and resolution among the remaining *Steinernema, S. intermedium* is treated as if it were the direct ancestor of the remaining taxa. This assumption is most certainly violated.

Estimates of phylogenetic relationships among the sampled *Steinernema* taxa are presented in Figure 2. Figures 2A and 2B depict the two most parsimonious solutions to the edited, optimized data set. The concatenated data matrix produced a single tree that is congruent with Figure 2B. The maximum likelihood, maximum parsimony (unedited, optimized alignment), and LogDet (via neighbor-joining) solution is depicted in Figure 2C. Figure 2D is a consensus of the three alternative solutions and depicts Bremer support, as well as parsimony and maximum likelihood bootstrap support where the alternative trees are concordant.

Log-determinant transformed distances (LogDet) can account for apparent non-independence of nucleotide composition among taxa, a condition that can confound conventional tree-building methods (Lockhart et al., 1994). The congruence between the maximum likelihood, maximum parsimony (without deleted ambiguous characters), and the LogDet neighbor-joining tree suggests that base composition bias (Table 2), although significant, does not affect the maximum likelihood or parsimony analyses. Instead, the concordance

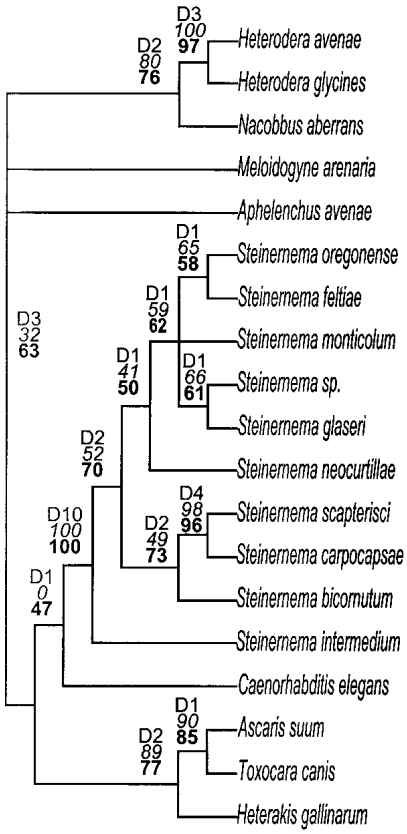

FIG. 1. Relationships among 10 species of *Steinernema* and representative outgroup taxa based on parsimony analysis of the 5.8S subunit gene. Tree is a combinable components consensus of the two most parsimonious solutions. Branch support indices appear to the left of each estimated node. Bremer support indices are preceded by the letter ''D''. Bootstrap values for maximum parsimony (1,000 replicates) are italicized. Bootstrap values for maximum likelihood (100 replicates) are in bold. Though weakly supported, the best phylogenetic estimates based on the 5.8S data depict *S. intermedium* as the sister taxon to the rest of the Steinernematids.

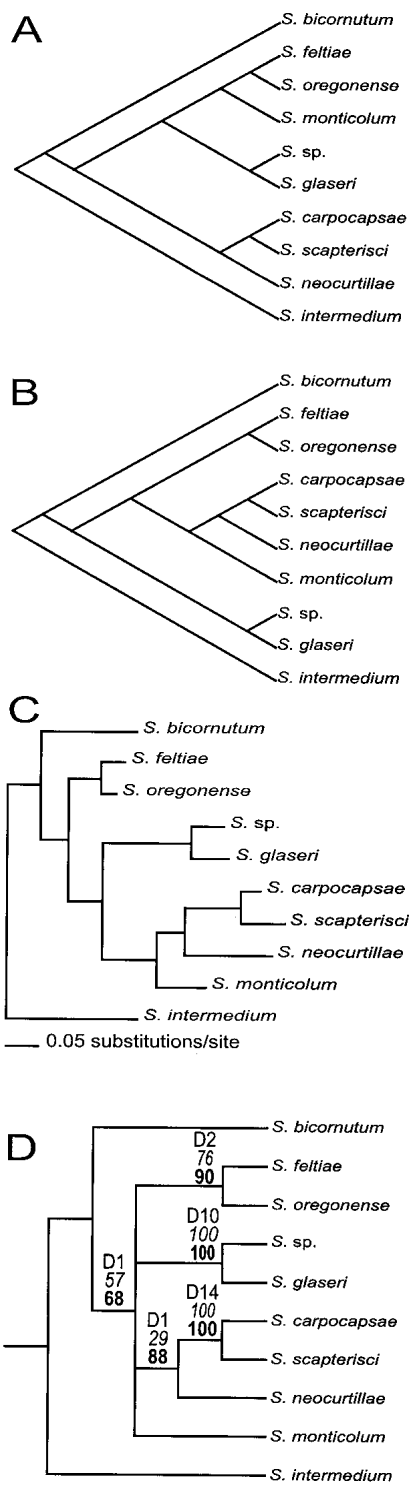

FIG. 2. Hypotheses of steinernematid evolution based on ITS data produced by different tree-building methods, and a summary of support. A-B) The two most parsimonious trees produced by the optimized ITS data set. Fig. 2B is also the single most parsimonious solution to the concatenated ITS matrix. C) Maximum likelihood, LogDet, and maximum parsimony tree produced from an optimized matrix retaining alignment-ambiguous regions. D) Combinable components consensus tree of likelihood and parsimony solutions to the ITS data set, with support indices. The letter ''D'' precedes Bremer support indices. Bootstrap support indices for maximum parsimony (1,000 replicates) are italicized, and maximum likelihood (100 replicates) indices are in bold.

between shifts in base composition and phylogeny appear to be the result of shared evolutionary history rather than convergence. Thus, the topological discordance among the different analyses is solely a function of the multiple-sequence alignment (homology statements)—specifically, whether characters of dubious homology are included or removed.

The maximum parsimony tree produced by the modified elision (concatenated) matrix is concordant with one of the two most parsimonious solutions produced by the matrix that had alignment-ambiguous regions deleted. This finding is consistent with the idea that unstable (dubious) regions of the alignment contain the majority of the phylogenetic noise in the data set, and that this noise is overwhelmed by the signal content of the more stable regions. We conclude that the discrepancy between maximum parsimony trees and those produced by LogDet distances or maximum likelihood is not a product of the optimality criterion but is instead due to the high noise level in the unstable regions of the alignment.

Relationships between the sister taxa *S. feltiae* and *S. oregonense, S. glaseri* and *Steinernema* sp., and *S. carpocapsae* and *S. scapterisci* are well supported, but deeper nodes are not. In the maximum parsimony solutions, the lineages of *S. monticolum, S. neocurtillae,* and *S. glaseri* + *Steinernema* sp. are not unambiguously resolved. Bootstrap support for their positions in Figure 2A are less than 50% (31%, 29%, and 41%, respectively). Maximum likelihood bootstrap support for *S. neocurtillae* as sister to *S. carpocapsae* + *S. scapterisci* is greater than par-

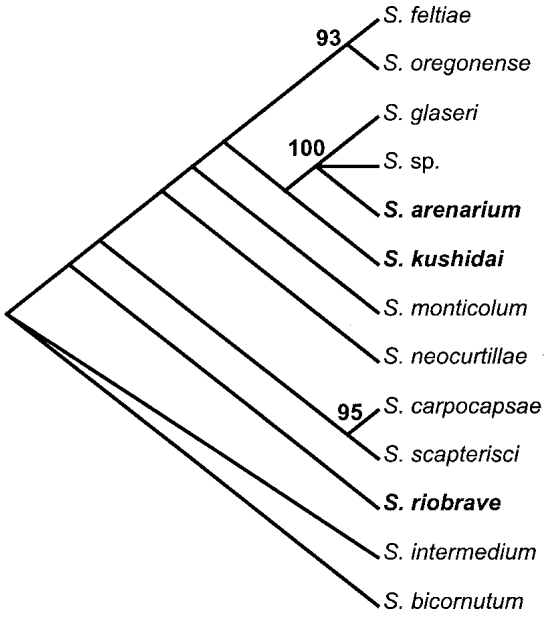

FIG. 3. Maximum parsimony hypothesis of steinernematid evolution based on the ITS1 region only. Bootstrap values are indicated at nodes with >50% support. Taxa representing sequences not generated in the present study appear in bold. Inclusion or exclusion of gapped characters had no effect on tree topology or relative branch support.

simony bootstrap (88% vs. 29%, respectively). The greatest discrepancy in topological support is for a clade consisting of *S. carpocapsae, S. scapterisci, S. neocurtillae,* and *S. monticolum,* where maximum likelihood bootstrap support is 100%, yet parsimony bootstrap support is less than 29%. Rooted with *S. intermedium,* there is only weak support for *S. bicornutum* as sister taxon to the rest of the sampled steinernematids.

The ITS1 sequences of Szalanski et al. (2000) included three taxa we were unable to sample—*S. arenarium, S. kushidai,* and *S. riobrave* (Fig. 3). A strict consensus of the two most parsimonious trees depicts *S. arenarium* forming a well-supported clade with *S. glaseri* and *S.* sp. *Steinernema kushidai* appears as sister taxon to the clade containing *S. glaseri, S.* sp., and *S. arenarium. Steinernema riobrave* appears as the sister taxon to the rest of the Steinernematids to the exclusion of *S. intermedium* and *S. bicornutum.* However, without 5.8S sequence data we cannot tell if this relationship is a function of its being constrained to the ingroup. The relationship of these additional taxa to those we sampled is consistent with the ITS1 tree topology of Szalanski et al. (2000).

Although our survey of steinernematid taxa is incomplete, the representative ITS sequences provide evidence (in the form of numerous autapomorphic characters) that all the presumably integrated lineages we sampled are evolving independently of one another and, thus, appear to be species. However, a consideration of only the ITS1 region failed to reveal autapomorphies that could serve to delimit as species *S. glaseri, S. arenarium,* and *S.* sp.

In conclusion, PCR amplification of the ITS regions followed by DNA sequencing of the PCR product provides reliable data for diagnosing *Steinernema* species. However, the use of the ITS region for species delimitation and phylogenetic reconstruction should be restricted to only those nucleotide positions for which character polarization and homology statements are robust. Efforts to delimit species using ITS data must extend taxon sampling to include the other species in the genus and incorporate an experimental design that adequately addresses the nature of variability within and among individuals and populations of *Steinernema.* Unlike Szalanski et al. (2000), we fail to see evidence that the ITS1 region, or even the entire array, will be an adequate source of characters to confidently resolve relationships among the Steinernematidae. The ITS region is useful for resolving relationships among closely related Steinernematid taxa, but it is too variable to reliably infer relationships among all species in the genus. Conversely, the 5.8S region is too conserved to resolve all Steinernematid relationships. As with species delimitation, sound phylogenetic inference will require more extensive taxon sampling but with additional sources of phylogenetically informative characters.

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