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Steinernema diaprepesi n. sp. (Rhabditida: Steinernematidae), a Parasite of the Citrus Root Weevil *Diaprepes abbreviatus* (L) (Coleoptera: Curculionidae)[1]

Khuong B. Nguyen and Larry W. Duncan[2]

Abstract: A nematode collected from *Diaprepes abbreviatus* is identified and described as a new species, *Steinernema diaprepesi* n. sp. The new species is closely related to *S. feltiae, S. glaseri,* and *S. oregonense* and can be distinguished from these species by the following characteristics: *Males:* Spicule averaging 79 (71–90) µm and spicule shape; D% (distance from anterior end to excretory pore/esophagus length × 100) about 80; the ratio SW (spicule length/anal body width) about 1.8. *Females:* Vulva with short, double-flapped epiptygma; tail terminus usually with 5 papillae-like structures. *Infective juveniles:* Body averaging 1,002 (880–1,133) µm, EP (distance from anterior end to excretory pore) = 74 (66–83) µm; tail length = 83 (65–91) µm, and E% (EP/tail length × 100) = 89.6 (78–114). Lateral field pattern variable, the formula for the arrangement of ridges from head to tail is: 2, 6, 7, 8, 4, 2. The portion with eight ridges is the longest. This new species can be differentiated further from three closest species (*S. feltiae, S. glaseri,* and *S. oregonense*) by characteristic sequences of their ITS regions, including sequence lengths, ratios of similarity, composition, and differences in base characters in sequence alignment.

Key words: citrus root weevil, *Diaprepes abbreviatus,* DNA, entomopathogenic nematodes, nematodes, new species, *Steinernema diaprepesi,* taxonomy.

Entomopathogenic nematodes have been used in Florida citrus groves for more than a decade in an attempt to manage subterranean larvae of a weevil pest, *Diaprepes abbreviatus* L. Schroeder (1992) used *Steinernema carpocapsae* (Weiser, 1955) Wouts, Mracek, Gerdin and Bedding, 1982 and *Heterorhabditis bacteriophora* Poinar, 1976 to control citrus root weevil. He reported that weevil emergence was reduced by 70% compared with an untreated control. Formulated products containing *S. riobrave* Cabanillas, Poinar and Raulston, 1994, and *H. indica* Poinar, Karunakar and David, 1992 are currently used for this purpose. Field trials using commercially formulated *S. riobrave* and *H. indica* reported efficacy against *D. abbreviatus* ranging from >85% suppression to no measurable effect (Adair, 1994; Bullock et al., 1999; Duncan et al., 1996; Duncan and McCoy, 1996; McCoy et al., 2000; McCoy et al., 2001; Stansly et al., 1997). Variation in soils (Duncan et al., 2001), application rates (McCoy et al., 2000), quality of products, and application methods may contribute to unpredictability in the response to application of these nematodes. It was also demonstrated that persistence of *S. riobrave* and *H. bacteriophora* Poinar, 1976 in citrus groves is relatively low (Duncan et al., 1996; Duncan and McCoy, 1996). Efficacy is measurable only during the first week after application (McCoy et al., 2000); thus, nematodes are used periodically as inundative rather than classical biological control agents. Entomopathogenic nematodes adapted to persist at higher population density in the citrus rhizosphere might provide greater and less variable efficacy against weevil pests.

A nematode was collected from larvae of *D. abbreviatus* that were buried in cages beneath citrus trees in an experiment conducted in Polk County, Florida. During a 2-year survey, the nematode was found to be the major identifiable cause of mortality to the weevil larvae in soil (Duncan and Graham, unpubl.). In plots treated twice annually with *S. riobrave,* indigenous populations of the new nematode killed an average of 4 times more *D. abbreviatus* than did *S. riobrave,* except during treatment months. The nematode is a member of the genus *Steinernema* but is different morphologically from other species of the genus. The nematode is most similar in general morphology to *S. feltiae* (Filipjev, 1934) Wouts, Mracek, Gerdin & Bedding, 1982; *S. glaseri* (Steiner, 1929) Wouts, Mracek, Gerdin & Bedding, 1982; and *S. oregonense* Liu & Berry, 1996, but morphological characters, morphometrics, genetic characteristics, and cross hybridization suggest that this nematode is a new species. The new species is described herein as *Steinernema diaprepesi* n. sp., named after the host insect from which the nematode was collected.

Materials and Methods

Nematodes collected from the field by baiting with caged buried larvae of *D. abbreviatus* were maintained in the laboratory on last instar *Galleria mellonella* L larvae (Dutky et al., 1964). For taxonomic studies, 10 *G. mellonella* were exposed to 5,000 infective juveniles (IJ) in a petri dish (100 × 15 mm) lined with two moistened filter papers. First- and second-generation adult nematodes were obtained by dissecting infected insects 2 to 4 days and 5 to 7 days, respectively, after the insects died. Third-stage IJ were obtained when they emerged from the cadavers after 7 to 10 days. All observations and measurements were performed within a week after col-

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[1] Florida Agricultural Experiment Station Journal series No. R-07913.
[2] Entomology and Nematology Department, and Citrus Research and Education Center, respectively, Institute of Food and Agricultural Science, University of Florida, Gainesville, FL 32611-0620.
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E-mail: kbn@gnv.ifas.ufl.edu
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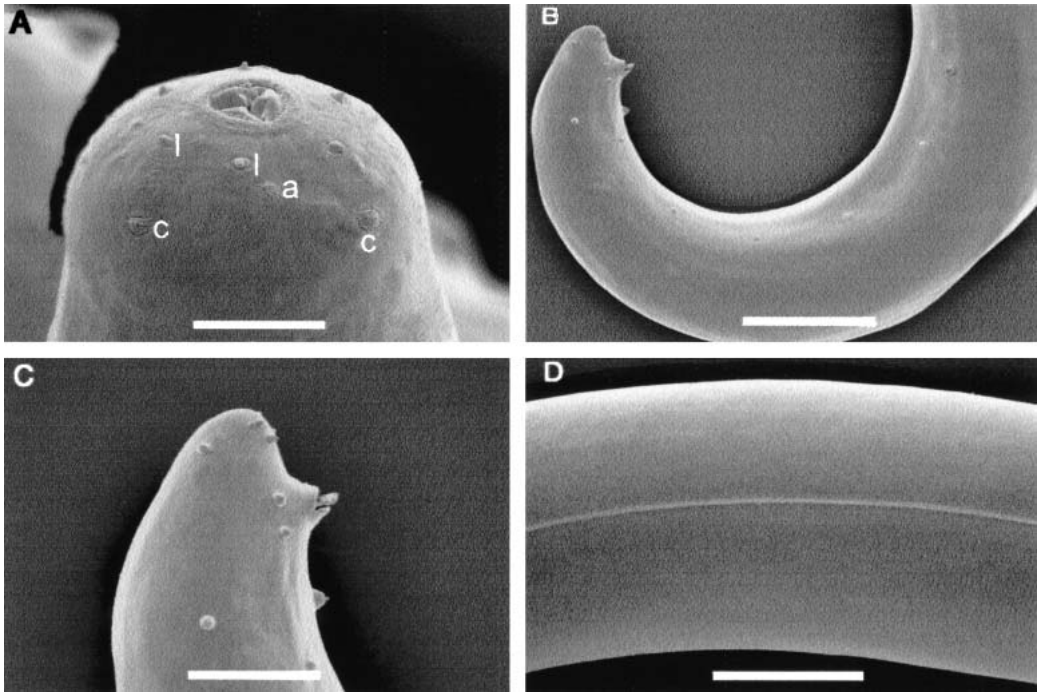

FIG. 1. *Steinernema diaprepesi* n. sp., SEM of first-generation males. A) Slightly swollen head, showing anterior end of stoma with a perioral ring, six labial papillae (l) and two of four cephalic papillae (c). B,C) Posterior region of males showing number and distribution of genital papillae (p). D) Lateral field at mid-body with a single ridge. Scale bars: A = 10 µm, B = 66.7 µm, C,D = 30 µm.

lection. For light microscope observation, at least 20 males and females and 50 IJ were examined live. Additional specimens of different stages were killed in warm water (40 °C) or fixed in TAF (Courtney et al., 1955) or in lactophenol (Franklin and Goodey, 1949). These nematodes were used further when more observation was needed to confirm the morphology or variation of some structures. Nematodes fixed in TAF were processed to glycerin by the Seinhorst method (Seinhorst, 1959). Type specimens were mounted in glycerin. Coverglass supports were used in all cases to avoid flattening of specimens. Nematode males, females, and IJ collected from *D. abbreviatus* (10 larvae were inoculated with 5,000 IJ) also were used in this study.

Scanning electron microscopy: Adults and IJ were fixed in 3% glutaraldehyde buffered with 0.1 M sodium cacodylate at pH 7.2 for 24 hours at 8 °C (Nguyen and Smart, 1995a). They were post-fixed with 2% osmium tetroxide solution for 12 hours at 25 °C, dehydrated in a graded ethanol series, critical-point-dried with liquid CO_2, mounted on SEM stubs, and coated with gold. Spicules and gubernacula were prepared as suggested by Nguyen and Smart (1995a).

Cross hybridization test: Reproductive compatibility of *S. feltiae* (strain SN), *S. oregonense* (strain Oregon), *S. glaseri* (strain NJ), and *S. diaprepesi* n. sp. (strain Polk) was tested via modification of the first method reported by Nguyen and Smart (1990), using *G. mellonella* hemolymph. A drop of hemolymph of *G. mellonella* was placed in a sterile petri dish (35 × 10 mm), and one IJ of *S. diaprepesi* n. sp. and one IJ of *S. glaseri* were added. For control, the crosses between IJ of the same species were conducted. All treatments were replicated 30 times. All petri dishes were kept in closed plastic bags containing a piece of moistened facial tissue to keep

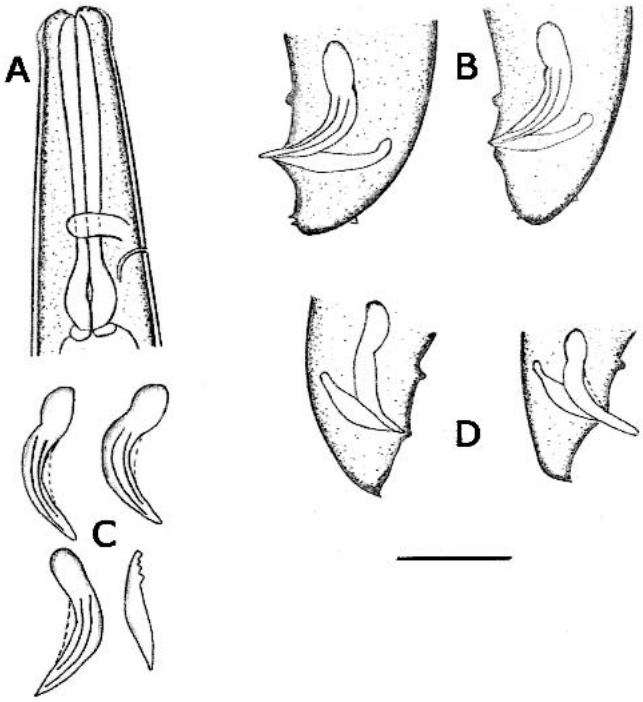

FIG. 2. *Steinernema diaprepesi* n. sp., first- and second-generation males. A) Anterior region. B) Posterior region. C) Variation in spicule shape and a gubernaculum. D) Tail of some second-generation males showing mucron. Scale bar: A–D = 33 µm.

the hemolymph from drying. Cross hybridization between *S. diaprepesi* n. sp. and *S. feltiae* and *S. oregonense* were conducted in the same way.

Extraction of DNA: DNA of *S. feltiae, S. oregonense, S. glaseri,* and *S. diaprepesi* n. sp. was extracted from a single female using the modified method reported by Joyce et al. (1994).

PCR amplification: The ITS region of the ribosomal DNA was amplified by the polymerase chain reaction (PCR) in a 100-µl reaction with a DNA polymerase kit (AMRESCO, INC., Solon, OH). All tubes were kept on ice, and the following PCR mix was added to each tube: 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

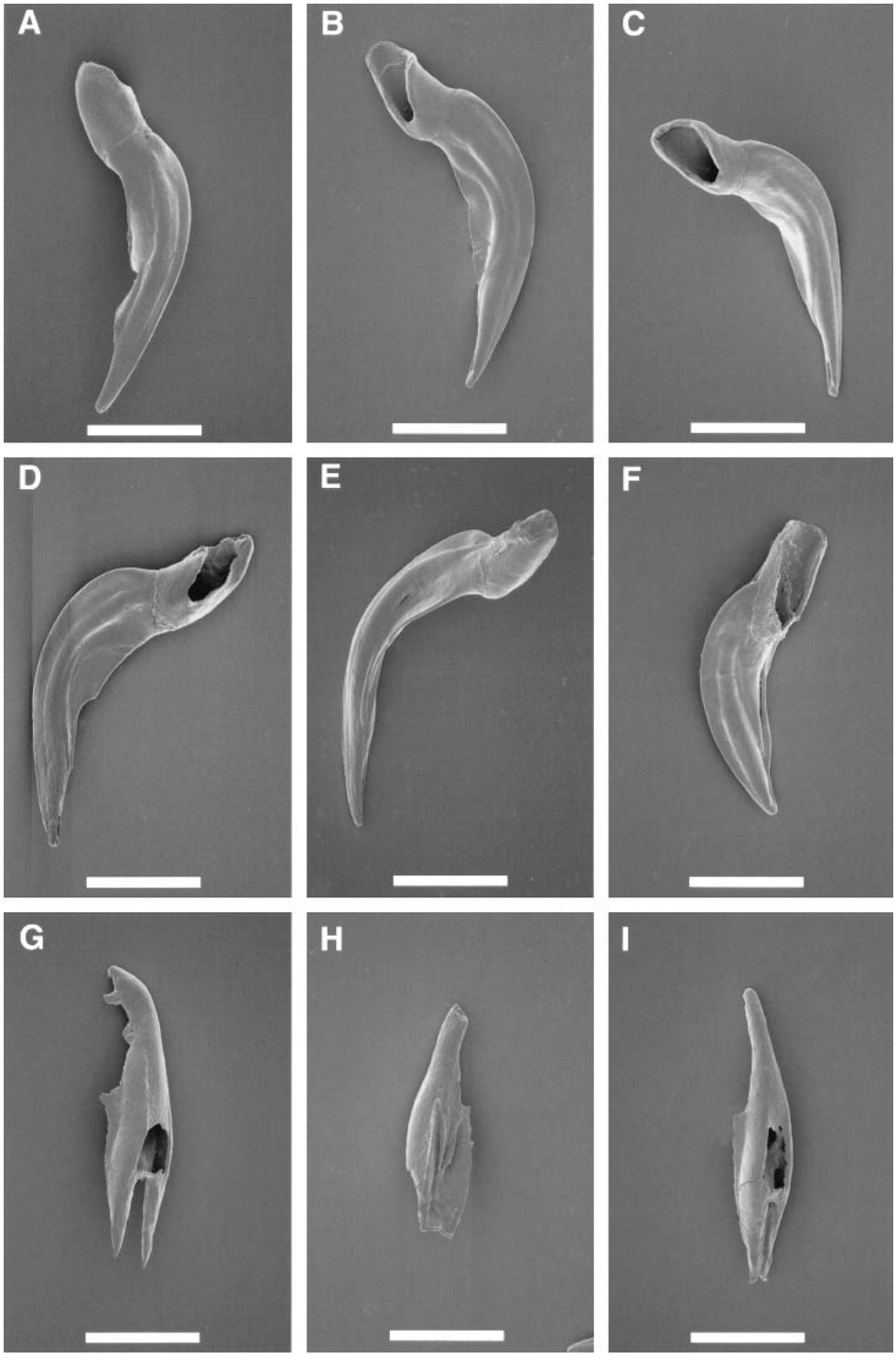

FIG. 3. *Steinernema diaprepesi* n. sp., spicules and gubernacula of first-generation males. A–D) Common types of spicules. E,F) Uncommon types of spicule. G–I) Gubernacula in different views. Scale bars: all scale bars = 20 µm.

dNTP mixture (10 mM each), 2 µl of 5 pM forward primer, 2 µl of 5 pM reverse primer, 0.5 µl of Thermalase Tbr (AMRESCO, INC., Solon, OH) (2 U/µl), 74 µl of distilled water, and 10 µl of DNA. 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 Hominick et al. (1997): AB28: 5′-ATATGCTTAAGTTCAGCGGGT-3′ (forward) and TW81: 5′-GTTTCCGTAGGTGAACCTGC-3′ (reverse). All PCR reactions were run in a PTC-100 Thermocycler (MJ Research, Inc., Waltham, MA) with the cycling profile: 1 cycle of 94 °C for 2 minutes followed by 40 cycles of 94 °C for 30 seconds, 45 °C for 60 seconds, 72 °C for 90 seconds. The last step was 72 °C for 15 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 (PerkinElmer, Inc., Foster City, CA). For each 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 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 AB28 and TW81 described earlier and two internal primers suggested by Nguyen et al. (2001): KN58 = 5′-GTATGTTTGGTTGAAGGTC-3′ and KNRV = 5′-CACGCTCATACAACTGCTC-3′. The sequences flanked by the AB28 and TW81 primers were assembled using Auto-Assembler Version 2 (PerkinElmer, Inc., Foster City, CA).

DNA analysis

Multiple alignment: Sequences of studied species were aligned using the default parameters of Clustal W (Thompson et al., 1994). The alignment would be used to compare the similarity and difference of nucleotides of the four sequences.

Ratios of similarity: This ratio was obtained using default parameters of the Olddistances program (Wiscon-

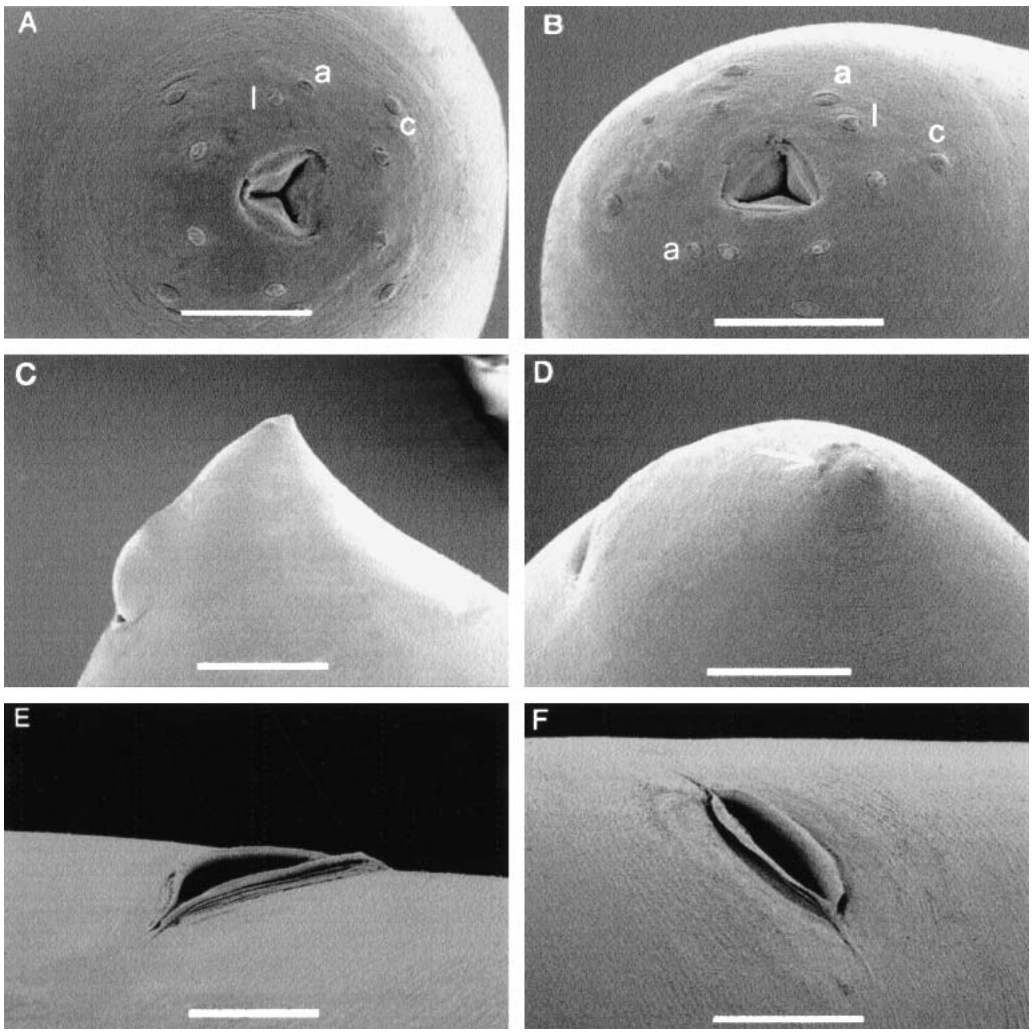

FIG. 4. *Steinernema diaprepesi* n. sp., first-generation females. A,B) Anterior end showing stoma, 6 labial papillae (1), 2 amphids (a), and 4 cephalic papillae (c). C,D) Tails showing anus, ventral postanal swelling (C), and tail terminus with papillae-like structures (D, arrow). E,F) Low double-flapped epiptygma. Scale bars: A,B = 10 µm, C = 20 µm, D = 15 µm, E = 10 µm, F = 15 pm.

sin package, GCG) to create a table of pairwise similarities within the four aligned sequences. The similarity value is the number of matches between each sequence pair divided by the sequence length. If the sequences of two nematodes are 100% identical, the distance is 1.0.

Composition: Composition of the sequences of *S. diaprepesi* n. sp., *S. feltiae, S. glaseri,* and *S. oregonense* were obtained using the Composition program (Wisconsin package, GCG). Data from this program were used to compare the percentage of nucleotides in four sequences of the studied nematode species.

Phylogenetic relationships: Maximum parsimony trees were obtained using PAUP, 4.0b8 (Swofford, 2001). The nematode species used in this process are: *S. carpocapsae* (NCBI Genbank, Accession #AF121049), *S. bicornutum* (AF121048), *S. ceratophorum* (AF440765), *S. diaprepesi* n. sp. (AF440764), *S. feltiae* (AF121050), *S. glaseri* (AF122015), *S. intermedium* (AF122016), *S. monticolum* (AF122017), *S. neocurtillae* (AF122018), *S. oregonense* (AF122019), and *S. scapterisci* (AF122020). All data were assumed to be unordered, all characters have equal weight, gaps are treated as missing data, and tree evaluation was made using a heuristic search with 1,000 replicates. The species *S. intermedium* was treated as the out group taxon (Nguyen et al., 2001) to resolve the relationships among other species. Branch support was estimated by boot-trap analysis (100 replicates) using heuristic search (the alignment-ambiguous regions were excluded before running the program). The aligned data matrix is available from the corresponding author.

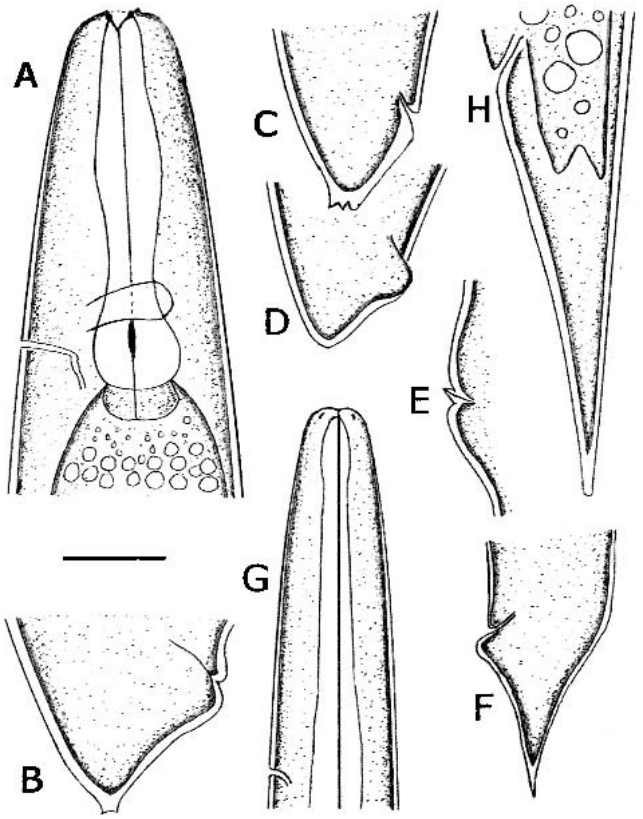

FIG. 5. *Steinernema diaprepesi* n. sp., first- and second-generation females and infective juvenile. A) Anterior region of a first-generation female. B–D) Variation in tail shapes of first-generation females. E) Epiptygma of a second-generation female. F) Posterior region of a second-generation female. G,H) Anterior and posterior regions of an infective juvenile, note that the hyaline portion is longer than half the tail length. Scale bar: A–F = 33 µm, G,H = 20 µm.

SYSTEMATICS

Steinernema diaprepesi n. sp.
(Fig. 1–6)

Holotype (male, first generation): Length 1,669 µm; width 127 µm; stoma width 16.6 µm; stoma length 7.6 µm; distances from anterior end to excretory pore (EP) 106 µm; to nerve ring 97 µm; to end of esophagus (ES) 132 µm; testis reflexion 533 µm; tail length 21 µm; spicule length 77 µm; spicule width 16.6 µm; gubernaculum length 53 µm; gubernaculum width 10.6 µm; SW (spicule length/anal body width) 2.02; GS (gubernaculum length/spicule length) 0.7; D = EP/ES × 100 = 80.

Males, first generation: Measurements of 20 males are given in Table 1. Body curved ventrally posteriorly (Fig. 1B), C-shaped when heat-killed. Lateral field present in mid-body with one narrow ridge (Fig. 1D). Head rounded, usually slightly swollen (Fig. 1A). Anterior end with a perioral disc around stoma; six labial papillae, two amphids (usually covered with exudate), and four cephalic papillae (Fig. 1A). Stoma shallow, cheilorhabdions as small and sclerotized structures at anterior end (Fig. 2A), sometimes indistinct. Excretory pore near nerve ring (Fig. 2A), located mostly anterior to basal bulb. Esophagus with cylindrical procorpus, metacorpus absent or slightly swollen, isthmus present, nerve ring around isthmus, basal bulb distinct. Esophago-intestinal valve present, usually weak. Gonad monorchic, reflexed. Distance from base of esophagus to anterior end of testis variable. Spicules (Fig. 3) paired, brown in color. Head (manubrium) of spicules elongate, the ratio length/width from 1.4 to 2.0 (averaging 1.7 ± 0.2) in some specimens (4/20), twice as long as wide; shaft (calomus) very short or absent; blade (lamina) thick, tapering gradually posteriorly, blade terminus blunt with a longitudinal depression slit in ventral side (Fig. 3C,D); velum present. Each spicule with two internal ribs. Gubernaculum boat-shaped in lateral view, anterior part usually with one or two ventral projections (Fig. 2C,G). Eleven pairs [occasionally 12 (Fig. 1B)] and one single precloacal genital papillae distributed as in Fig. 1B. Tail conoid; tail terminus without mucron (Figs. 1B,2B).

General morphological characteristics of males collected from *D. abbreviatus* appear similar to those collected from *G. mellonella,* but they differ morphometrically (Table 1). Most measurements of first-generation males from *D. abbreviatus* are shorter than those collected

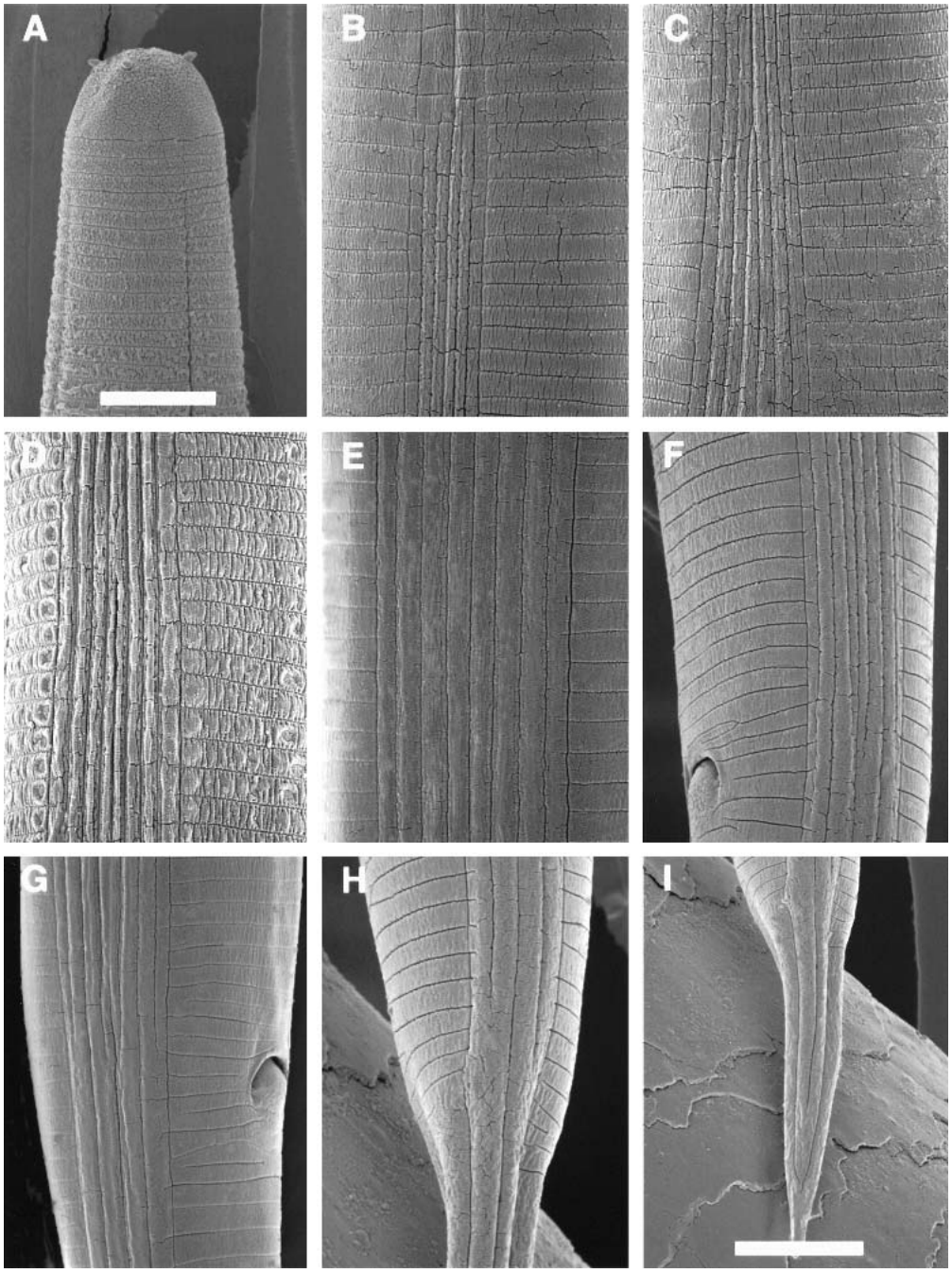

FIG. 6. *Steinernema diaprepesi* n. sp., lateral field of infective juvenile. A) Anterior region showing prominent cephalic papillae. B) Lateral field with two ridges anteriorly, six ridges posteriorly. C) Lateral field showing the seventh ridge arising in the middle of lateral field. D) Lateral field showing the middle ridge dividing into two making a total of eight. E) Eight ridges in lateral field. F,G) Left and right lateral fields showing the disappearance of two marginal and two central ridges making the lateral fields become four. H) Number of ridges in lateral field reduce to two. I) Tail showing the end of lateral field. Scale bars: A–H = 7.5 µm, I = 12 µm.

from *G. mellonella*. This result concurs with the previous suggestion by Nguyen and Smart (1995b) that, when possible, *Steinernema* species should be reared in *G. mellonella* for nematode description and (or) identification.

Males, second generation: Measurements of 20 males reared in *Galleria* are given in Table 1. Second-generation male similar to that of the first generation except body, spicule, and gubernaculum shorter and thinner, excretory pore much more anterior, and isthmus more distinct (Table 1). Gubernaculum short. Mucron on tail terminus sometimes present (Fig. 2D).

Females, first generation

Allotype (Female, first generation): Length 5,242 µm; width 211 µm; stoma width 9.1 µm; stoma length 7.6 µm; distance from anterior end to excretory pore 109 µm, to nerve ring 132 µm, to end of esophagus 182 µm; anal body width 61 µm; tail length 32 µm; V% = 52.

TABLE 1. Measurements (in µm) of males of *Steinernema diaprepesi* n. sp. from *Galleria mellonella* and *Diaprepes abbreviatus* (n = 20).

	Reared using *Galleria* (First generation)			Reared using *Diaprepes* (First generation)			Reared using *Galleria* (Second generation)		
Character	Means	SD	Range	Means	SD	Range	Means	SD	Range
Body length	1,735	156	1,506–2,078	1,403	185	1,060–1,693	1,176	117	1,036–1,343
Greatest width	113	15	90–145	78	11	61–100	64	7	54–78
EP	115	9	100–130	100	13	74–117	83	8	71–108
NR	119	6	109–129	121	11	100–139	110	8	100–136
ES	150	7	136–162	148	11	127–168	138	12	129–168
Testis reflexion	391	68	241–542	289	51	211–380	264	16	187–428
T	25	3	20–32	25	3.2	21–32	24	5	18–30
ABW	42	11	36–50	40	4	30–45	34	6	29–42
Spicule length	79	5	71–90	66	5	57–76	69	8	61–76
Spicule width	16	2	14–20	12.3	1.7	9–15	12.5	3	11–15
Gub. length	54	5	45–61	44	3.8	38–53	40	6	30–53
Gub. width	8.7	1.4	6–12	6.7	1.3	4.6–9.1	67	1.4	5–9
D = EP/ES (%)	80	6	68–86	68	8	52–80	60	8	54–67
SW	1.8	1.3	1.5–2.0	1.7	0.3	1.2–2.1	2	0.2	1.66–2.33
GS	0.69	0.1	59–79	0.66	0.04	0.62–0.74	0.59	0.07	0.46–0.73

EP = distance from anterior end to excretory pore.
NR = distance from anterior end to nerve ring.
ES = distance from anterior end to end of esophagus.
T = tail length.
ABW = anal body width.
Gub. = gubernaculum.
SW = spicule length/ABW.
GS = gubernaculum length/spicule length.
SD = standard deviation.

Measurements of 20 females are presented in Table 2. Body cuticle smooth or with faint annules. Lateral fields and phasmids absent. Head rounded, continuous with body; six labial papillae, four cephalic papillae (Fig. 4A,B). Lips indistinct. Amphids present. Stoma shallow, subtriangular anteriorly; triradiate internally (Fig. 4A,B). Cheilorhabdions (Fig. 5A) well sclerotized but small. A smaller sclerotized structure posterior to cheilorhabdions (presumably the prorhabdions), observed in other species, indistinct in this species. Esophagus with procorpus cylindrical, muscular; metacorpus swollen; isthmus distinct; basal bulb valvate (Fig. 5A) as in other Steinernematids. Nerve ring surrounds isthmus, just anterior to basal bulb. Esophago-intestinal valve present. Excretory pore located just anterior to, or at the middle of, basal bulb (Fig. 5A). Gonads amphidelphic, reflexed, often containing many eggs. Vulva, a transverse slit; protruding or not; low double-flapped epiptygma present (Fig. 4E,F). Vagina sclerotized, short. Body width greater anterior to vulva than posterior to vulva. Tail shape variable (Fig. 5B,C), ventral postanal swelling present, tail shorter than anal body width. Most females with five papillae-like structures on tail tip (Fig 4C,D). Size of these structures is variable

TABLE 2. Measurements (in µm) of females of *Steinernema diaprepesi* n. sp. collected from *Galleria mellonella* (n = 20).

	First generation			Second generation		
Character	Means	SD	Range	Means	SD	Range
Body length	6,508	1,512	4,061–9,878	2,433	260	2,030–3,030
Stoma length	10.3	2.2	7.6–13.6	10.6	1.1	9.1–12.1
Stoma width	12.1	2.8	9–18.2	10.4	1.5	9.1–13.6
Greatest width	272	44	190–343	158	12	137–180
EP	156	39	98–214	116	15	94–139
NR	150	21	105–179	144	12	120–153
ES	200	25	119–232	182	11	161–211
T	52	10	38–68	78	6	67–91
ABW	94	22	59–138	50	7	39–59
V%	50	2.8	44–57	54	3	49–60
D = EP/ES (%)	80	19	46–105	64	8.2	47–74

EP = distance from anterior end to excretory pore.
NR = distance from anterior end to nerve ring.
ES = distance from anterior end to end of esophagus.
T = tail length.
ABW = anal body width.
SD = standard deviation.

depending on age of females, usually longer in young females (Fig. 5C), becoming smaller and short as the female bodies enlarge, disappearing in fully mature females (Fig. 5D).

Females, second generation: Measurements of 20 females reared in *G. mellonella* are given in Table 2. Similar to first-generation female but smaller (length = 2,385 µm, width = 149 µm compared to 6,512 µm and 264 µm, respectively, for first-generation female). Vulva less protruding, epiptygma (Fig. 5D) usually more prominent than that of first-generation females. Tail, tapering to a pointy end, longer than anal body width; ventral postanal swelling present (Fig. 5E).

Infective juveniles: Measurements of 50 juveniles are given in Table 3. Body elongate. Sheath (second-stage cuticle) present immediately after harvesting, but many IJ will lose their sheath in storage (in our study, all IJ observed have sheath). Labial region smooth, continuous, rounded anteriorly (Fig. 6A). Labial papillae not seen; four cephalic papillae prominent (Fig. 6A). Amphids slit-shaped but not prominent. Cuticle marked with prominent transverse striations. Lateral field begins anteriorly with one line (Fig. 6A). Two additional lines appear at annules 14 to 16 to form two ridges (Fig. 6B). Near excretory pore level, the number of ridges in lateral fields increases from two to six (Fig. 6B). A new central ridge appears more posteriorly, about a body width posterior to excretory pore, making a total of seven ridges in the lateral field (Fig. 6C). Near the end of the esophagus, the central ridge divides into two, making a total of eight ridges—the maximum number in the lateral field (Fig. 6D,E). The portion with eight ridges is the longest part (compared to portions with 2, 6, 7, 4 ridges) of the lateral field. At the level of the anus the two marginal and two central ridges disappear (Fig. 6F,G), only four ridges remain in the lateral field. At about mid-tail the four ridges in the lateral field become two ridges (Fig. 6H). Near the tail terminus, the two marginal lines in the lateral field converge (Fig. 6I) and the central line disappears before reaching the end of the lateral field.

Esophagus with thin corpus (Fig. 5F), basal bulb more or less elongate with visible valve. Tail attenuate, tapering gradually (Fig. 5G). Hyaline portion occupies about 57% (50–63) of tail length, sometimes up to 70% (Fig. 5G).

The morphometrics of IJ reared from *G. mellonella* and from *D. abbreviatus* appear different (Table 3).

Cross hybridization tests: Males and females of *S. diaprepesi* n. sp. did not interbreed with *S. feltiae, S. glaseri,* and *S. oregonense.* In the control, males and females of the each species mated and produced offspring normally.

Molecular diagnosis: Because PCR-RFLP analysis should be used with caution in determining affinities among species (Dowling et al., 1996), a sequencing approach was undertaken. The alignment of the sequences of the ITS regions of four species (*S. feltiae, S. oregonense, S. glaseri,* and *S. diaprepesi* n. sp.) is presented in Figure 7. The partial 18S (nucleotides 1–12), 5.8S gene sequence (319–466), and 28S portion (792–814) show little variation among the four species. The ITS1 (13–318) and ITS2 (467–791) regions are much more variable and provide most of the base differences (Table 4) that are important for species diagnosis (Adams et al., 1998). The lengths of the amplified sequences and sequence similarity (Table 5) also are different among the four species (Table 4) (Nguyen et al., 2001). The phylogenetic relationships among the 11 studied *Steinernema* species are presented in Figure 8. *Steinernema diaprepesi* n. sp. appears as the sister to *S. glaseri,* and the clade *S. glaseri* + *S. diaprepesi* n. sp. is well supported (100%) by bootstrap analysis.

Habitat and biology: Steinernema diaprepesi n. sp. was isolated from the larvae of *D. abbreviatus* that were buried in cages beneath citrus trees as part of an experiment to evaluate the insecticidal efficacy of commercially available entomopathogenic nematodes. During 2 years of the experiment, indigenous populations of *S. diaprepesi* n. sp. infected and killed 13% to 50% of the buried insects within 7 days, with higher rates of parasitism during the summer months compared to spring and autumn months. The nematode was isolated with much lower frequency from the rhizosphere of native plants growing between citrus tree rows than from the rhizosphere of citrus trees.

The topotype locality is an irrigated, commercial citrus orchard c. 6 km east of Bartow, Florida, and immediately southeast of the intersection of Cowpen Road and 80-Foot Road. Mean monthly temperatures range from 16.1 to 27.9 °C, and cumulative annual precipitation averages 1,364 mm with most rainfall occurring

TABLE 3. Measurements (in µm) of infective juveniles of *Steinernema diaprepesi* n. sp. collected from *Galleria mellonella* and *Diaprepes abbreviatus* (n = 50).

Character	Reared using *Galleria*			Reared using *Diaprepes*		
	Means	SD	Range	Means	SD	Range
Body length	1,002	53	880–1,133	957	31	807–1,030
Greatest width	34	3.6	30–42	36	6	29–42
EP	74	6.4	66–83	69	8	55–77
NR	102	6	74–109	105	5	98–114
ES	138	7.4	111–152	144	5	136–156
T	83	5	65–91	81	9	76–88
ABW	23	2.3	21–27	22	1.8	18–24
a	30	2.6	23–35	26.5	1.6	23–29
b	7.3	0.5	6.5–8.3	6.6	0.4	6.1–7.6
c	12.1	0.7	10.4–13.2	11.7	0.7	10–13
D = EP/ES (%)	54	5	30–70	48	2.2	47–56
E = EP/T (%)	90	6.5	78–114	85	9	71–96

EP = distance from anterior end to excretory pore.
NR = distance from anterior end to nerve ring.
ES = distance from anterior end to end of esophagus.
T = tail length.
ABW = anal body width.
a = body length/greatest width; b = body length/ES; c = body length/T.
SD = standard deviation.

```
                    1                                                  50
Steinernema feltiae GGAAGGATCA TTATTGAGCT TATCCATTTA CTTG---GAT TCAAATGAAT
S. oregonense       GGAAGGATCA TTATTGAGCT TATCCATTTA CTTG---GAT TCAAATGAAT
S. glaseri          GGAAGGATCA TTATTGAGCC TACTCTCATA CATGTGAGTA TTATGATCAC
S. diaprepesi       GGAAGGATCA TTATTGAGCT TA-ACACTTC GTTGTTTATA CGTTACGTAT
                    ********** *********  **  *   *    **               *

                    51                                                 100
Steinernema feltiae CGAGCTGAAT -TTTCGCTGT TCGTTTCAAA GCGTTGTATT CTCTCAACTA
S. oregonense       CGAGCTGAAT CGTTTGCTGT TTGTCTCGAG GCAATGTATT CTCTCATCTA
S. glaseri          TGTTCGGAAC GCGGCACT-G TCGTTTCTAG GTGTCGCGAC CGTTCG-ACA
S. diaprepesi       CGTTCGGAAC GACACTGTCC ACGTTTCTAA GTGTCGATTC CGTTCA-CTA
                     *  * ***         *     ** ** *  *    *     *  **    *

                    101                                                150
Steinernema feltiae ACGGCTATGA ATGGTTTCTA TAGGTGTCTG GAGCAGTTGT ATGAGCGTGA
S. oregonense       ACGGCTATGA ATGGTTTCTA TAGGTGTCTG GAGCAGTTGT ATGAGCGTGA
S. glaseri          ACGGCTTTGA ATGGTTTCTA TAGGTGTCTG GAGCAGCTGT ATGAGCGTGG
S. diaprepesi       ACGGCTTCGA ATGGTTTCTA TAGGTGTCTG GAGCAGCTGT ATGAGCGTGG
                    ******  ** ********** ********** ****** *** *********

                    151                                                200
Steinernema feltiae CTGTGGTGAT GGACATTTTG ---------- ---------- -GTGGCTCCT
S. oregonense       CTGTGGTGAT GGACATTTGA ---------- ---------- -GT-------
S. glaseri          CTGTGGTGAA GGACATTTGA CATCGCGT-- ---------- ----------
S. diaprepesi       CTGTGATGAA GGACATTTAA CATCCTATGC CAGACGTCTA GCTGTCTCTT
                    ***** ***  ********

                    201                                                250
Steinernema feltiae TAGTCGGGTC ACTAGAATTA AAGAAGTCTG TTATGACTCG CCGTTCTT-A
S. oregonense       ---TCTTGTG ACTAGAATTA AAGAAGTCTG ATACGACTCG CCGTTCTTAA
S. glaseri          -CTCGACGCG GTGAGAATTG AAGAGGTCAG -TCGGAGACC CGCCGTTCAC
S. diaprepesi       GCGTTTGGTG ATGAGAATTA AAGAGGTCAG -TCGGAGACC CGCCGTTCA-
                           *      ******  **** *** *  *  **  *  *     *

                    251                                                300
Steinernema feltiae AAAAACTTCA ATTAACGTTT GATCAATTTG ACTGCACCAG CCGT----AG
S. oregonense       AAAAACTTCA ATTAACGTTT GATCAATTTG ACTGCACCAG CCGT----AG
S. glaseri          AAACCCTACC ATTAACAATT TTACACACGA TGACAAGCAT CGTTGATGCT
S. diaprepesi       AAAACCTACC ATTAACATTT TCCATACTAA GCTCCATATG TATTTATGGT
                    ***  ** *  ******  **                  *       *

                    301                                                350
Steinernema feltiae GTGTACTTAA AGATTTATCA AGTCTTGT-C GGTGGATCAC TCGGTTCGTA
S. oregonense       GTGTACTTAA AGATTTATCA AGTCTTGT-C GGTGGATCAC TCGGTTCGTA
S. glaseri          GTGTTATACA ACTGTTACCA AGTCTTAT-C GGTGGATCAC TCGGTTCGTA
S. diaprepesi       GGCGAAAACA ATGTTATCCA AGTCTTATCC GGTGGATCAC TCGGTTCGTA
                    *        * *   *   ** ****** * * ********** **********

                    351                                                400
Steinernema feltiae GTTCGATGAA AAACGGGGCA AAAACCGTTA TTTGGCGTGA ATTGCAGACA
S. oregonense       GTTCGATGAA AAACGGGGCA AAAACCGTTA TTTGGCGTGA ATTGCAGACA
S. glaseri          GTTCGATGAA AAACGGGGCA AAAACCGTTA TTTGGCGTGA ATTGCAGACA
S. diaprepesi       GTTCGATGAA AAACGGGGCA AAAACCGTTA TTTGGCGTGA ATTGCAGACA
                    ********** ********** ********** ********** **********
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FIG. 7. Alignment rDNA sequences from four species of *Steinernema: S. diaprepesi* n. sp., *S. feltiae, S. glaseri,* and *S. oregonense.* Asterisk (*), no differences found among nematode species. Hyphen (-), gap.

May through September and frequent periods of drought from mid-autumn to late-spring. Soil texture is astatula sand (97:1:2, sand:silt:clay). *Steinernema diaprepesi* n. sp. has been isolated from other orchards located on the central ridge of Florida, which is characterized by deep sandy soils. Attempts to isolate the nematode in heavier-textured soils off of the central ridge have been unsuccessful; however, no comprehensive survey has yet been conducted.

Type host and locality

The nematode was collected from an infested citrus root weevil, *Diaprepes abbreviatus* (L), in a commercial citrus orchard in Polk County, Florida.

Type specimens

Holotype (male, first generation): Isolated from hemocoel of *Galleria mellonella* deposited in the U.S. Depart-

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                        401                                                450
Steinernema feltiae     TATTGAACGC TAAAATTTTG AACGCAAATG GCACTATCAG GTTTATATCT
S. oregonense           TATTGAACGC TAAAATTTTG AACGCAAATG GCACTATCAG GTTTATATCT
S. glaseri              TATTGAACGC TAAAATTTTG AACGCAAATG GCACTATCAG GTTTATATCT
S. diaprepesi           TATTGAACGC TAAAATTTTG AACGCAAATG GCACTATCAG GTTAATATCT
                        ********** ********** ********** ********** *** ******

                        451                                                500
Steinernema feltiae     GTTAGTATGT TTGGTTGAGG GTCGATTAAT TCGTAACCTG CAGTCTGCTG
S. oregonense           GTTAGTATGT TTGGTTGAGG GTCGATTAAT TCGTAACTTG CAGTCTGCTG
S. glaseri              GATAGTATGT TTGGTTGAGG GTCGACTAAC ACGTTACTTG CAGTCAG---
S. diaprepesi           GATAGTATGT TTGGTTGAGG GTCGATTAAC TCGTTACTTG CAGTCAGCTT
                        * ******** ********** ***** ***   *** ** ** ***** *

                        501                                                550
Steinernema feltiae     TGACTGTTTT TTCGATTAGT TA----TTTG GTTTTTTTAT CGAGTACCTT
S. oregonense           TGACTGTTTT TCCGATTAGT TACTCGATTG GCTCGCTGAT CGAGTACCTT
S. glaseri              CGACTGTTTT TTCGACGAGC TATGTAC--G TTCGTATGTA CCTCGTTCGG
S. diaprepesi           CGACTGTTTA TTCGATAAGC TACTTTCGAG CTGCGAAAGT ACCTTTTCGG
                         ********  * ***  **  **       *                    *

                        551                                                600
Steinernema feltiae     TTTGGAATGT GAA-TTTGAT TGTCTAATTC GTTTCCTAAT CGAA---ACG
S. oregonense           CTAGGTATGT GAATTTTGAT AGTCTAATTC GTTTCCTAAT CGAA---ACG
S. glaseri              TGTGAACGTT CCCCCGGCAC TGGGGGCGAT AGTGCAATGG ACAAGGCTTT
S. diaprepesi           TGTGAACGCT TCAATGCGAT AGGCTAATGG AGGTCGTTAG GCGAGTGTCT
                           *     *          *   *             *         *

                        601                                                650
Steinernema feltiae     AGCTATTTTT TATTTCT-GT GCAATGTATT TTTGGTGTTT CGGCGTTTTT
S. oregonense           AGCTATCTTT GAATTCTGGT GCGTTGTATC TTTGGTGTTT CGGCGCGTTT
S. glaseri              -------TGT CGTGTCCGCT ATCACATCGG TTCCGTGCGT T-GATGGCTT
S. diaprepesi           CTTTCGCTAA CGCTTCTGCT ATCATATCGG TTCTGTGCGT TACGTGGCTA
                               *       **   *       *    **  ***  *         *

                        651                                                700
Steinernema feltiae     CTTGCCGACT GATTGGTACA AACTTAACAG TTCGTATATT TTTCAGAATT
S. oregonense           CTTGCCGACT GATCTGTACG TA-------- ACCGTATATG CTTCA-ATTT
S. glaseri              TGGCGTGTCT CTTGCCAGCT GACTTGTACG TAATTTTTTG CGTATGTAAG
S. diaprepesi           TGGCGTGTCT CTTGCCAGTT GACTTGTACG CAGACGTAAC TGTCTCGTAT
                              * **   *         *                *      *

                        701                                                750
Steinernema feltiae     TTTCAGAGGC CCTTACAATA CATCACTTGA CACAACACGT ATCGTTTGTC
S. oregonense           GATCAGATGC CCTTAGCTTA CTTCACTCGA CACAACACGT TTCGTTTGTT
S. glaseri              CTTCTTGAAG TCAGTGTTGC CAGCAAGCGT TTGAGCCTGT ACGGTTCGGC
S. diaprepesi           GTAAGCTTCT TGA-AGTCGG CTGCCACATG TTCGACCTTT GCGGGTTGAC
                                             *  *            *   *    * * *

                        751                                                800
Steinernema feltiae     GAGGAATTGC GCAAGAA--- -----AGAAA CTTTTCGTTT TACGACCTCA
S. oregonense           GAGTAATCGC GCAAAAA--- -TTGTAAACT TTTTCGTTTT TACGACCTCA
S. glaseri              GCGCGACGTA GCTGGGACTT CGTGTTCGAT GTTTTCGAAT GACGACCTCA
S. diaprepesi           GAACGCAACT GGAACTTGCT CG-ATTCGAT GTTTTCGAAT TACGACCTCA
                        *          *                     ***     *  *********

                         801         814
Steinernema feltiae      ACTCAAGCAA GATT
S. oregonense            ACTCAAGCAA GACT
S. glaseri               ACTCAAGCAA GACT
S. diaprepesi            ACTCAAGCAA GACT
                         ********** ** *
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* no differences found among nematode species.
\- gaps.

FIG. 7. CONTINUED.

TABLE 4. Sequence length of ITS regions, composition, and diagnostic characters of four species of *Steinernema.*

Species (Seq length)	ITS1 (bp)	5.8S (bp)	ITS2 (bp)	A (%)	C (%)	G (%)	T (%)	Diagnostic character
S. diaprepese (808 bp)	301	158	313	24.1	20.6	24.1	31.2	144
S. feltiae (766 bp)	275	157	298	25.5	16.7	21.5	36.3	45
S. glaseri (774 bp)	279	157	302	22.5	21.2	26.7	29.6	136
S. oregonense (759 bp)	267	157	297	24.9	18.1	22.1	34.9	41

Diagnostic characters = numbers of characters (in the same column of the alignment) present in one sequence but not in others.

ment of Agriculture Nematode Collection (USDANC), Beltsville, Maryland.

Allotype (female, first generation): Same data as holotype, deposited in the USDANC, Beltsville, Maryland.

Paratypes (first-generation males and females, and third-stage IJ): Same data as holotype. Many males and females of the first generation and several third-stage IJ in TAF deposited in USDANC, Beltsville, Maryland; several males, and females of the first generation, and several third-stage IJ deposited in the Florida Collection of Nematodes, Florida Department of Agriculture and Consumer Services, Gainesville, Florida; one male and one female of the first generation, and several IJ deposited in the California Collection of Nematodes, University of California Davis Nematode Collection, Davis, California.

Diagnosis. Males: Spicule averaging 79 (71–90) μm; D% about 80; the ratio SW about 1.8. Lateral field with one narrow ridge. *Females:* Vulva with short, low double-flapped epiptygma; tail terminus usually with 5 papillae-like structures. *Infective juveniles:* Body length averaging 1,002 (880–1,133) μm, EP = 74 (66–83) μm; tail length = 83 (65–91) μm, and E% = 89.6 (78–114). Lateral field pattern variable; the formula for the arrangement of ridges from head to tail is: 2, 6, 7, 8, 4, 2 (Fig. 6). The portion with eight ridges is the longest. The new species is characterized genetically by sequence lengths of ITS region (808 bp), ITS1 (301 bp), ITS2 (313 bp), and composition of its sequence.

Relationships: Steinernema diaprepesi n. sp. body length of IJ (1,002 μm) is intermediate between that of the *S. glaseri* group (*S. arenarium, S. cubanum, S. glaseri, S. longicaudum, S. puertoricense,* body length > 1,000 μm) and *S. kraussei* group (*S. feltiae, S. kraussei, S. neocurtillae, S. oregonense,* 800 μm < body length < 1,000 μm). The new species can be distinguished from these *Steinernema* species by several morphological characters of males, females, and IJ. Males can be differentiated from other Steinernematids by shape and length of spicules and gubernaculum (Fig. 3; Nguyen and Smart, 1997), D% = 80, and SW = 1.8. Females can be distinguished from other species by the presence of five papillae-like structures on the tail tip (Figs. 4,5) and vulva with low double-flapped epiptygma (Figs. 4,5). Infective juveniles of the new species can be recognized by body length, distance from anterior end to excretory pore, tail length, and E%. Finally, *Steinernema diaprepesi* n. sp. can be differentiated from the three closest species (*S. feltiae, S. oregonense, S. glaseri*) by sequence analysis and by reproductive incompatibility, as previously stated.

TABLE 5. Pairwise similarity among four species of *Steinernema* expressed as the number of matches between each sequence pair divided by sequence length.

	S. fel	*S. ore*	*S. gla*	*S. dia*
S. feltiae	1.0000	0.8972	0.6097	0.6332
S. oregonense		1.0000	0.6008	0.6271
S. glaseri			1.0000	0.7429
S. diaprepese				1.0000

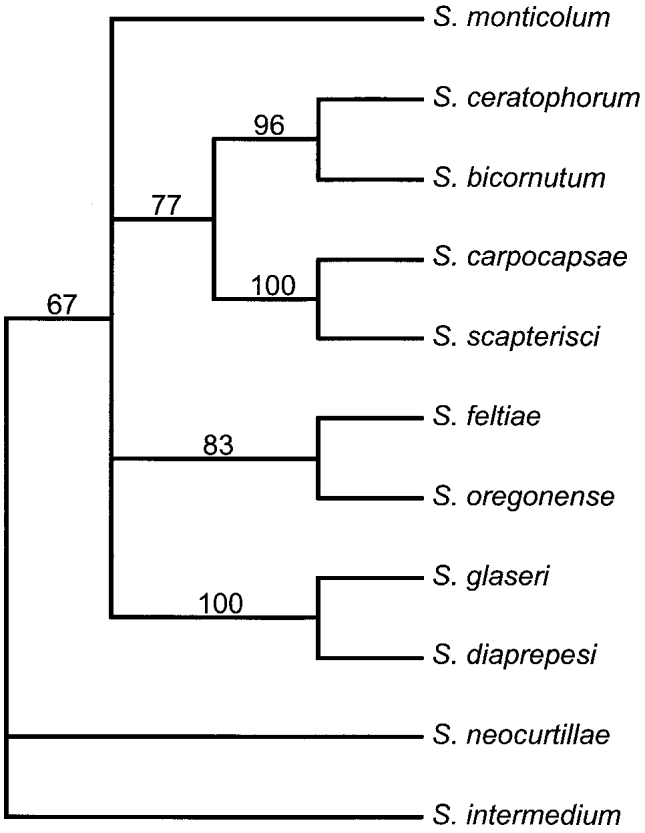

FIG. 8. Phylogenetic relationship between 11 species of *Steinernema.* Numbers over branches indicate bootstrap proportion. The clade *S. glaseri* + *S. diaprepese* n. sp. is well supported (100%) by bootstrap analysis.

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