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Molecular Characterization and Phylogenetic Analysis of *Hoplolaimus stephanus*

Xinyuan Ma,[1] Paula Agudelo,[1] John D. Mueller,[2] Halina T. Knap[1]

Abstract: Three *Hoplolaimus stephanus* populations were characterized morphologically, both by morphometrics and by SEM observations. These populations were used to develop a rapid and accurate molecular identification method for the species, which is useful because of the high level of morphological similarity between *H. stephanus* and *H. galeatus*. Species-specific primers for *H. stephanus*, amplifying a distinct fragment (260 bp) of the nuclear ribosomal internal transcribed spacer1 (ITS1), can be used in multiplex PCR along with previously developed primers for other common *Hoplolaimus* species. We also infer phylogenetic relationships among *H. stephanus*, the closely-related *H. galeatus*, and several other *Hoplolaimus* species, using sequences of the actin gene, ITS1 and LSUD.

Key words: actin, DNA taxonomy, Hoplolaimidae, *Hoplolaimus stephanus*, lance nematodes, plant-parasitic nematodes.

Hoplolaimus stephanus Sher, 1963 was first described from swamp soil in Nicols, South Carolina, USA (Sher, 1963). Vovlas *et al.* (1991) later provided additional scanning electron microscopy (SEM) observations and supplementary morphometric data from a population collected in Raleigh, North Carolina, USA. The host plants were not identified in either of these two publications, which are also the only two published references available for this species.

We identified three populations of *H. stephanus*, one in Pennsylvania and two in South Carolina, USA. To ensure accurate identification, combined morphological and molecular analyses were initiated. *H. stephanus* morphologically resembles *H. galeatus*, but it can be distinguished from *H. galeatus* by the 24 to 28 longitudinal striations on the basal annule of the lip region compared to 32-36 in *H. galeatus*, shorter spicules, less areolation of the lateral field, and shorter body length (Sher, 1963). There are no publicly available DNA sequences for *H. stephanus* and this species has not been genetically characterized.

Hoplolaimus spp. reported from the southeastern USA include *H. columbus* Sher, 1963, *H. galeatus* (Cobb, 1913) Thorne, 1935, *H. magnistylus* Robbins, 1982, *H. stephanus*, *H. seinhorsti* Luc, 1958 and *H. tylenchiformis* von Daday, 1905 (Lewis and Fassuliotis, 1982). *H. columbus*, *H. galeatus* and *H. magnistylus*, are considered to be economically important and can cause serious damage to agronomic crops, including cotton (*Gossypium hirsutum* L.), corn (*Zea mays* L.) and soybean (*Glycine max* L.) (Fassuliotis, 1974; Nyczepir and Lewis, 1979; Robbins *et al.*, 1987, 1989; Henn and Dunn, 1989; Noe, 1993).

Hoplolaimus galeatus feeds in both the cortex and vascular tissues in cotton and causes extensive damage in the vascular cylinder (Krusberg and Sasser, 1956). It is also a prevalent pathogen of turf grasses such as St. Augustinegrass (*Stenotaphrum secundatum*) and bermudagrass (*Cynodon dactylon*) in Florida (Henn and Dunn, 1989; Giblin-Davis *et al.*, 1990, 1995). It has a wide distribution range in the United States (Wrather *et al.*, 1992; Lewis *et al.*, 1993; Martin *et al.*, 1994; Gazaway and McLean, 2003), and there are records from Canada, South America, Central America, and India on a variety of hosts (Fortuner, 1991). Because the morphometric values of *H. stephanus* and *H. galeatus* overlap considerably (Sher 1963; Vovlas et al., 1991; Handoo and Golden, 1992) and because the useful morphological characters for their discrimination require high magnification and considerable diagnostic time, it is important to improve our ability to accurately diagnose these species. Our finding *H. stephanus* populations associated with two woody hosts, birch tree and dogwood tree (*Betula nigra* L. and *Cornus florida* L.) and one grass host (*Poa pratensis* L.) indicates that the host range for *H. stephanus* overlaps that of *H. galeatus*, and evidences overlap in the geographic distribution as well.

Species identification is a prerequisite to developing resistant varieties and to determining basic biology and behavior of lance nematodes. Molecular diagnostic techniques based on sequence variation in the ITS region have been developed to discriminate the species in *Hoplolaimus*. Species-specific primers to identify *H. columbus*, *H. galeatus* and *H. magnistylus* were recently developed by Bae *et al.* (2009). A number of ITS and LSU sequences are publicly available in GenBank for these and a few more species of *Hoplolaimus*, but not for *H. stephanus*. Our objectives were to develop a rapid and accurate diagnostic molecular identification method for *H. stephanus* and to infer phylogenetic relationships among this species and the closely-related *H. galeatus*.

Materials and Methods

Nematode samples and DNA extraction: The species used in this study are listed in Table 1, along with the geographic origin and host plant of the populations. Three populations of *H. stephanus*, including one from Dillsburg, Pennsylvania (on Kentucky bluegrass) and two from the campus of Clemson University, Clemson,

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TABLE 1. Species, site of initial recovery, plant host, and GenBank Accession number for populations used in this study.

Isolate	Hosts	Location	GenBank Accession Number		
			Actin	ITS	LSUD
**H. stephanus* PAST01	Kentucky bluegrass (*Poa pratensis*)	Dillsburg, PA	HQ678694	HQ678733	HQ678718
H. stephanus PAST02	Kentucky bluegrass (*Poa pratensis*)	Dillsburg, PA	HQ678695	HQ678734	HQ678717
H. stephanus PAST03	Kentucky bluegrass (*Poa pratensis*)	Dillsburg, PA	-	HQ678735	HQ678720
H. stephanus PAST04	Kentucky bluegrass (*Poa pratensis*)	Dillsburg, PA	HQ678696	HQ678736	HQ678719
H. stephanus SCST01	Birch tree (*Betula nigra*)	Clemson University, SC	HQ678705	HQ678737	HQ678721
H. stephanus SCST03	Birch tree (*Betula nigra*)	Clemson University, SC	HQ678706	HQ678738	HQ678722
H. stephanus SCST04	Birch tree (*Betula nigra*)	Clemson University, SC	-	HQ678739	-
H. stephanus SCST05	Dogwood tree (*Cornus florida*)	Clemson University, SC	HQ678707	-	HQ678723
H. stephanus SCST07	Dogwood tree (*Cornus florida*)	Clemson University, SC	HQ678708	HQ678740	HQ678724
**Hoplolaimus sp.1*	Unknown	Smoky Mountain, TN	-	EU515329	EU626793
**Hoplolaimus sp.2*	Turf grass	University of Illinois, IL	-	EU515330	EU626794
**Hoplolaimus sp.2*	Turf grass	Manhattan, KS	-	EU515331	EU626795
**Hoplolaimus sp.3*	Birch Tree	Clemson University, SC	-	EU515332	EU586798
**Hoplolaimus sp.3*	Cotton	Belle Mina, Limestone County, AL	-	EU515333	EU586797
H. galeatus SCGA11	St. Agustine grass (*Stenotaphrum secondatum*)	Pee Dee R. E. C., Florence, SC	HQ678701	-	-
H. galeatus SCGA12	St. Agustine grass (*Stenotaphrum secondatum*)	Pee Dee R. E. C., Florence, SC	HQ678702	HQ678725	HQ678709
H. galeatus SCGA13	St. Agustine grass (*Stenotaphrum secondatum*)	Pee Dee R. E. C., Florence, SC	HQ678703	HQ678728	-
H. galeatus SCGA14	Bermuda grass (*Cynodon dactylon*)	Pee Dee R. E. C., Florence, SC	HQ678704	HQ678726	HQ678712
H. galeatus SCGA15	Bermuda grass (*Cynodon dactylon*)	Pee Dee R. E. C., Florence, SC	-	HQ678727	HQ678711
H. galeatus SCGA16	Bermuda grass (*Cynodon dactylon*)	Pee Dee R. E. C., Florence, SC	-	-	HQ678710
**H. galeatus*	Cotton	B.P.I., FL	-	EU515322	EU626786
**H. galeatus*	Bermuda grass	Fort Lauderdale Research and Education center, FL	-	EU515323	EU626787
**H. galeatus*	Augustine grass	Fort Lauderdale Research and Education center, FL	-	EU515324	EU626788
**H. magnistylus*	Cotton	Portland Ashley County, AR	-	EU515325	EU626789
**H. magnistylus*	Willow tree	Hope County, AR	-	EU515326	EU626790
**H. concaudajuvencus*	Hackberry	Perry County, AR	-	EU515328	EU626792
**H. seinhorsti*	Unknown	Israel	-	-	DQ328752
**H. seinhorsti*	Peanut	IFAS Experiment Station, Jay, FL	-	EU515327	EU626791
H. columbus SCCO01	Soybean (*Glycine max*)	Edisto R. E. C., Blackville, SC	HQ678699	-	-
H. columbus SCCO02	Soybean (*Glycine max*)	Edisto R. E. C., Blackville, SC	HQ678697	-	-
H. columbus SCCO03	Soybean (*Glycine max*)	Edisto R. E. C., Blackville, SC	HQ678700	-	-
H. columbus SCCO04	Soybean (*Glycine max*)	Edisto R. E. C., Blackville, SC	HQ678698	-	-
H. columbus SCCO12	Cotton (*Gossypium hirsutum*)	Edisto R. E. C., Blackville, SC	-	-	HQ678715
H. columbus SCCO13	Cotton (*Gossypium hirsutum*)	Edisto R. E. C., Blackville, SC	-	HQ678730	HQ678714
H. columbus SCCO14	Cotton (*Gossypium hirsutum*)	Edisto R. E. C., Blackville, SC	-	HQ678731	HQ678713
H. columbus SCCO15	Cotton (*Gossypium hirsutum*)	Edisto R. E. C., Blackville, SC	-	HQ678732	HQ678716
H. columbus SCCO16	Cotton (*Gossypium hirsutum*)	Edisto R. E. C., Blackville, SC	-	HQ678729	-
**H. columbus*	wheat	Tianmen, Hubei, China	-	FJ766014	-
**H. columbus*	Cotton	Johnston, NC	-	FJ485639	-
**H. columbus*	Cotton	Johnston, NC	-	FJ485638	-
**H. columbus*	Cotton	Johnston, NC	-	-	EU554676
**H. columbus*	Cotton	UGA research station Midville, GA	-	-	EU554675
**H. columbus*	Soybean	Blackville, SC	-	-	EU554674
**Heterodera glycines* (Out-group)			AF318603	HM560783	HM560853
**Globodera rostochiensis* (Out-group)			AF539593	EF622532	AY592993

*the asterisk indicates sequences from other researchers or publicly available on GenBank.
**the code "PAST" means *H. stephanus* from PA; "SCST" means South Carolina stephanus; the same as SCGA for *H. galeatus* and SCCO for *H. columbus* in SC.

South Carolina (one on dogwood and one on river birch) were used for the morphological and molecular characterizations. Two populations of *H. columbus* from South Carolina (one from a cotton field and one from a soybean field), and two populations of *H. galeatus* from South Carolina (one on St. Augustine grass and one on bermudagrass) were also included in the analyses. Forty-seven original sequences generated in this study were added to GenBank, but publicly available sequences from other authors for several *Hoplolaimus* species and two outgroup species were included for comparison. The accession numbers for all sequences included in this study are listed in Table 1.

DNA was extracted from individual nematodes handpicked from each population, using Sigma Extract-N-Amp kit (XNAT2) (Sigma, St. Louis, MO). The manufacturer's protocol was modified by reducing all volumes to one eighth of the recommended amounts. One nematode was placed into a 0.2 ml centrifuge tube containing 12.5 µl of the kit's Extraction Solution. The nematode was then crushed using the tip of a <10 µl pipette tip, followed by adding 3.5 µl of the kit's Tissue Prep solution to the tube. The tube was then vortexed, followed by a brief centrifugation to collect contents. Tubes were then incubated at 55 °C for 10 minutes, followed by incubation at 95 °C for 3 minutes. Next, 12.5 µl of the kit's Neutralization Solution was added to each tube. The extracted DNA was used for PCR or stored at -20°C.

Morphological characterizations: At least 8 females and 4 males from each population were used for the morphological diagnosis. Measurements and observation of

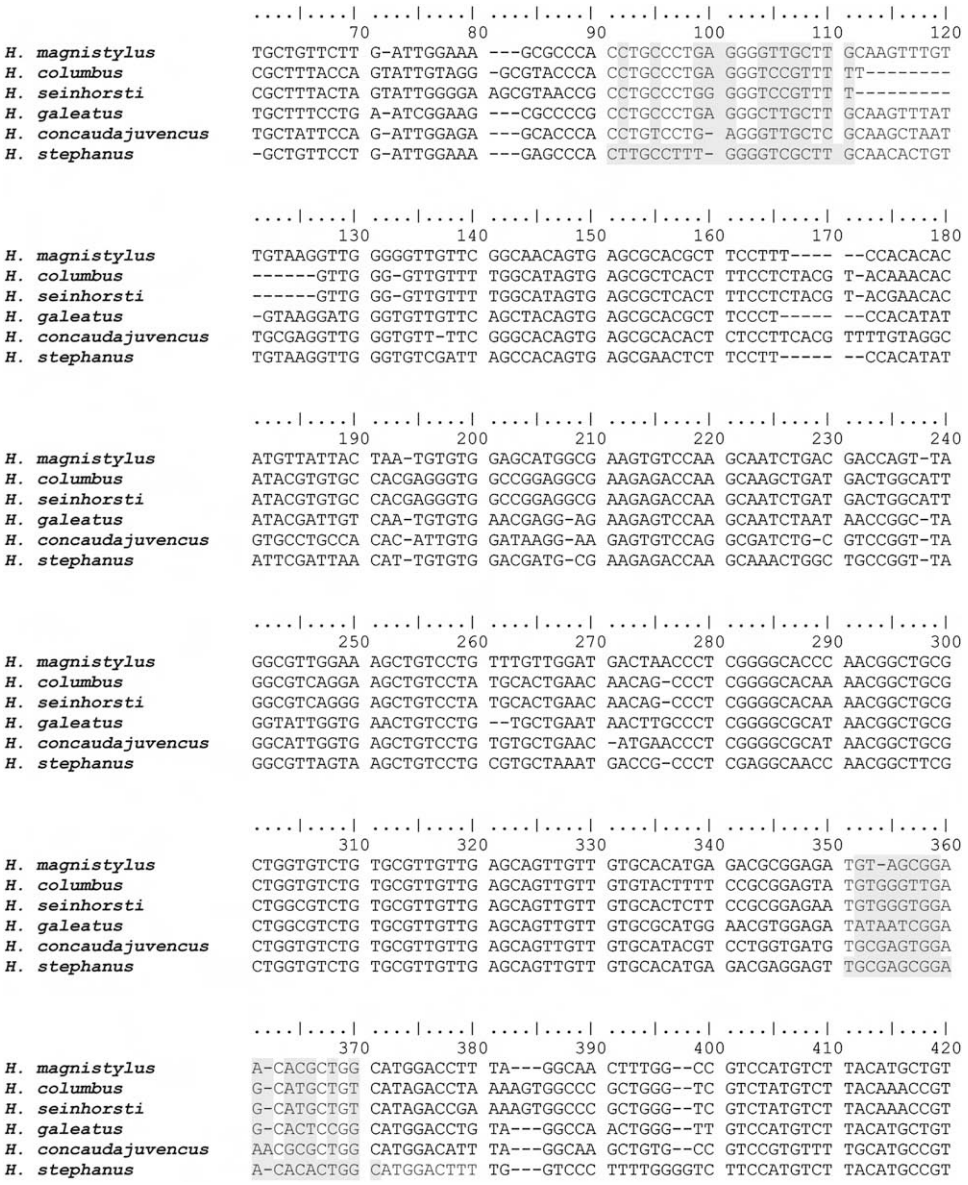

FIG. 1. Partial ITS1 region sequence alignment of five *Hoplolaimus* species. The highlighted portions indicate variation among the five species, used to design *Hoplolaimus stephanus* species-specific primer pair.

morphological diagnostic characters were made using temporary mounts in water and with an Olympus BX60 microscope equipped with the software iSolution Lite (Image and Microscope Technology i-Solution, Inc.).

Scanning electron microscopy (SEM) observations were conducted on a Hitachi Analytical Tabletop Microscope TM3000. Male and female specimens of *H. stephanus* and *H. galeatus* were fixed in 2.5% glutaraldehyde for at least 2 hours, then passed through a graded series of ethanol dehydration (25%, 50%, 75%, 90%, 95% , 100% ethanol, 15 min each), followed by critical point drying with hexamethyldisilazane (HMDS) and platinum coating prior to examination.

Molecular characterization: Molecular characterization of populations was conducted using species-specific primers and methodology as described by Bae *et al.* (2009) for discrimination of *H. galeatus, H. columbus,* and *H. magnistylus.*

H. stephanus species-specific primer design: The species-specific primers for *H. stephanus* were designed using comparative ITS1 region sequence alignments (Fig. 1) of *Hoplolaimus columbus, H. galeatus, H. magnistylus, H. seinhorsti, H. concaudajuvencus* and *H. stephanus* (gi226431011, gi186920200, gi186920202, gi186920203, gi186920204, *H. stephanus*SCST01). Five putative sets of primers were designed with the Primer-Blast application on NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast). After testing under a range of annealing temperatures (54-61°C), species-specific primer pair Hs-1f (5'- CCTGCCTTGGGGGTCGCTTG-3') and Hs-1r (5'- GCC AGTGTGTTCCGCTCGCA-3') were chosen and optimal PCR reactions with these primers were performed with annealing temperature of 60°C.

PCR Amplification of actin, ITS1 and LSUD genes: The actin region was amplified by the primers Act1-f (5'-CCAA ATCATGTTCGAGACGTT-3') and Act1-r (5'-GAACATA GCCTCTGGGCAAC-3'). These primers were designed using comparative sequence alignments of *Heterodera cynodontis, Heterodera avenae, Heterodera schachtii, Heterodera latipons, Heterodera glycines* and *Caenorhabditis elegans* from GenBank (Accession numbers gi167472950, gi41387727, gi41387723, gi41387721, gi26422171, gi18314322, gi133952034). The best putative primers were selected using the Primer-Blast application of NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Amplifications were performed in 20µl reactions, each containing: 5 µl PCR-grade water, 10 µl of ReadyMix TAq PCR Reaction Mix with $MgCl_2$ (Sigma, St. Louis, MO) (20 mM Tris-HCl pH 8.3, 100 mM KCl, 3 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTP mixture (dATP, dCTP, dGTP, and dTTP), and 0.06 units of Taq DNA Polymerase/µl), 1.0 µl of each primer (20 µM), and 2 µl of DNA template. The PCR reactions were performed on a PTC-100 Peltier thermal cycler (MJ Research, Inc. Watertown, Massachusetts) with the following run parameters: one initial denaturation cycle at 95°C for 3 min, followed by 36 cycles at 95°C for 45s, 46°C for 1.5

TABLE 2. Morphological characters and morphometric data of *Hoplolaimus stephanus* populations and of *H. galeatus.*

species, (source), and (number of specimens examined)	Length (mm)	lateral incisures	esophageal gland nulcei	stylet length (µm)	labial annules	longitudinal striae on basal lip annule	position of excretory pore	phasmids in relation to vulva	tail annules	males	spicule length (µm)
H. stephanus (from Sher, 1963) (n = 20 ♀, 10 ♂)	1.01-1.45	4	3	43-50	4	24-28	posterior to hemizonid	1 anterior 1 posterior	12	present	30.0-38.0
H. stephanus (from Vovlas *et al.*, 1991) (n = 10 ♀, 4 ♂)	1.01-1.43	4	3	41-48	4	30-36	posterior to hemizonid	1 anterior 1 posterior	11-15	present	35.0-42.0
H. stephanus (Birch population, this study) (n = 10 ♀, 4 ♂)	1.15-1.57	4	3	42-51	4	24-26	posterior to hemizonid	1 anterior 1 posterior	12	present	35.5-39.8
H. stephanus (Grass population, this study) (n = 8 ♀, 4♂)	1.23-1.52	4	3	47-49	4	24-26	posterior to hemizonid	1 anterior 1 posterior	12	present	30.1-32.6
H. stephanus (Dogwood population, this study) (n = 8 ♀, 4♂)	1.28-1.67	4	3	47-52	4	24-26	posterior to hemizonid	1 anterior 1 posterior	12	present	37.0-41.0
H. galeatus (from Sher, 1963) (n = 20 ♀, 10 ♂)	1.24-1.94	4	3	43-52	5	32-36	posterior to hemizonid	1 anterior 1 posterior	10-16	present	40.0-52.0

min, 72°C for 2 min and final extension at 72°C for 10 min.

The ITS1 and LSU-D region were amplified using the primers Hoc-1f (5'-AACCTGCTGCTGGATCATTA-3'), Hoc-2r (5'-CCGAGTGATCCACCGATAA-3'), LSUD-f (5'-ACCCGCTGAACTTAAGCATAT-3') and LSUD-r (5'-TTTCGCCCCTATACCCAAGTC-3'), respectively, designed by Bae *et al.* (2008). PCR reactions were performed as described above, using the following parameters: an initial denaturation cycle at 95°C for 3 min, 36 cycles at 95°C for 45s, 59°C for 1.5 min, 72°C for 2 min, and a final extension cycle at 72°C for 10 min. Each reaction included a negative control without DNA template. After amplification, 5 µl of each reaction were loaded onto a 1.5% agarose gel (120 V, 50 min) and photographed under UV light. At least three replicates were performed for each population-primer set combination. PCR products were purified and concentrated with Bio-Rad PCR Kleen Spin Columns (Bio-Rad, Hercules, CA). Purified DNA was sent to the Clemson University Genomics Institute (Clemson, SC) for direct sequencing in both directions. Amplification primers were used as sequencing primers. The sequences were edited and aligned using BioEdit 7.0 (Hall, 1999).

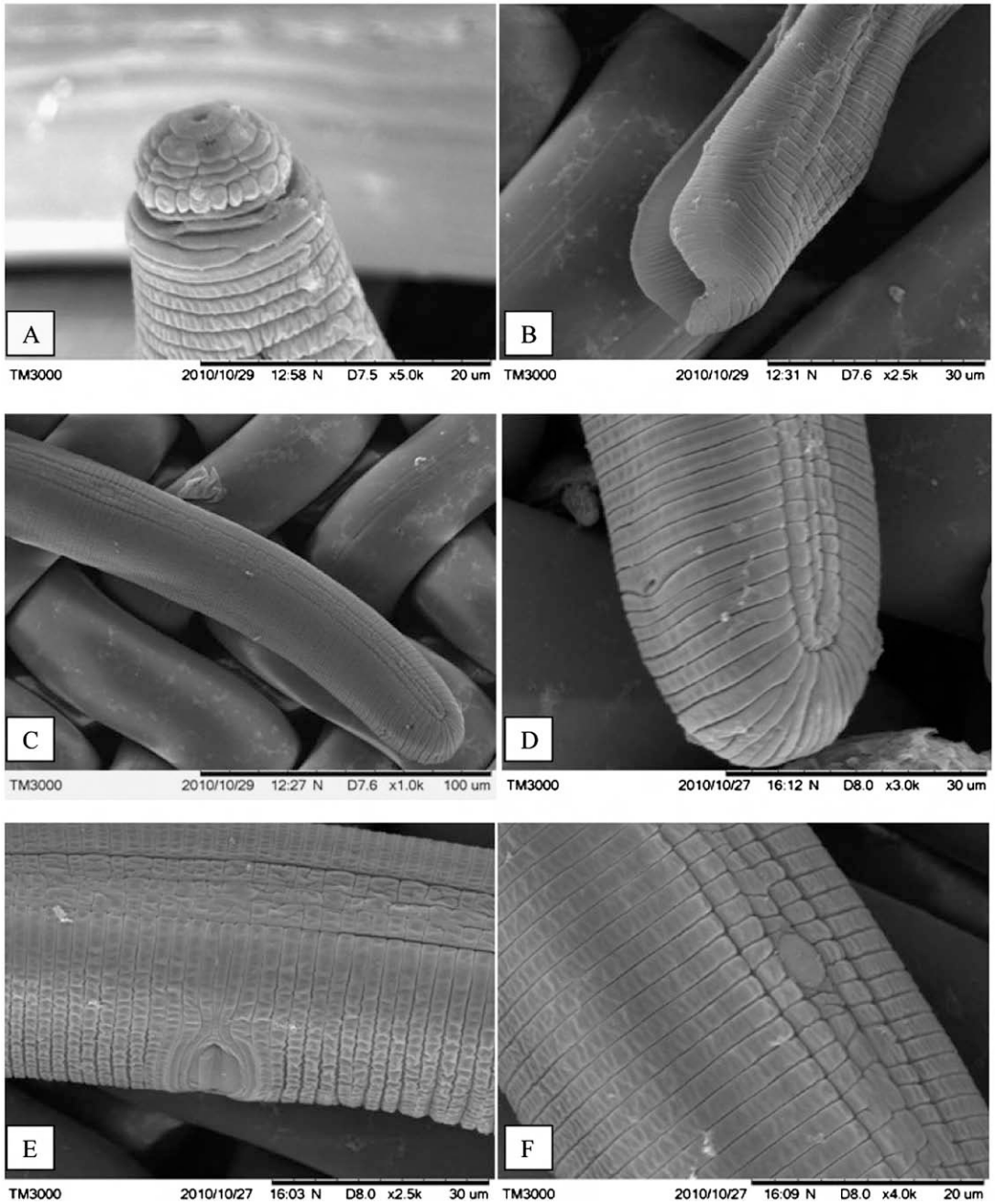

Fig. 2. Scanning electron micrographs of *Hoplolaimus stephanus*. A. anterior body portion; B. male tail region ; C. female posterior body portion; D. female tail region; E. vulval region; F. posterior phasmid and areolated lateral field.

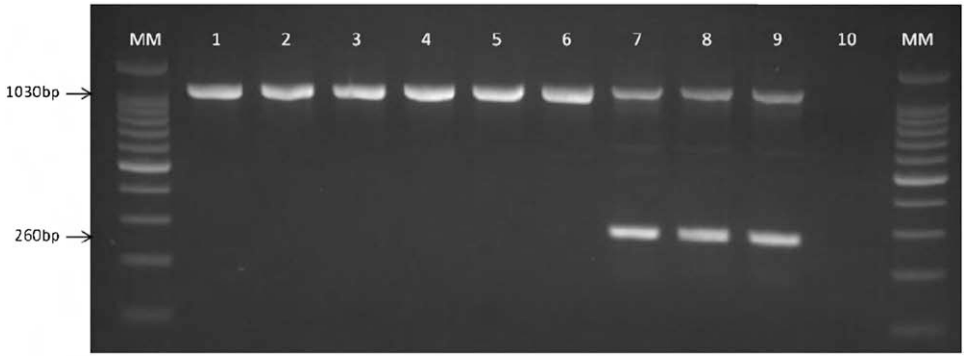

FIG. 3. Multiplex PCR products amplified from *Hoplolaimus columbus, H. galeatus* and *H. stephanus* using *H. stephanus* species-specific primer sets and resolved using agarose gel electrophoresis. Multiplex primers used were LSUD-1f, LSUD-2r, HS-1f, and HS-1r. LSUD fragment was amplified as internal positive control. Lane 1-3 are *H. columbus* and lanes 4-6 are *H. galeatus* (both species only amplify the positive control). Lanes 7-9 are *H. stephanus*, which amplified both the positive control and the species-specific fragment (260 bp). Lane 10 is the negative control.

Phylogenetic analysis: Sequences of ingroup and outgroup taxa were aligned using ClustalW (Thompson *et al.*, 1994). Sequences of *Globodera rostochiensis* and *Heterodera glycines* were used as the outgroup taxon for actin, ITS1 and 28S region (Table 1). The files were converted from FASTA to NEXUS format using DNA Sequence Polymorphism Version 5.10.01 (DnaSPv5) (Librado and Rozas, 2009). A best fit model of nucleotide substitution was selected using the GTR+I+G model with the Akaike Information Criterion (AIC) among 56 different models using ModelTest v 3.7 (Posada and Crandall, 1998). The Akaike-supported model, the base frequencies, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses. Bayesian inference was implemented for each gene separately using MrBayes 3.1.2 program (Huelsenbeck and Ronquist, 2001) running the chain for 100,000 generations with the Markov Chain Monte Carlo (MCMC) method, a sample frequency of 10 and burn-in value of 250. We estimated the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule. The phylogenentic trees were viewed with TreeView1.6.6 (Page, 1996).

RESULTS

Morphological characterization: The measurements and diagnostic morphological characters for the three *H. stephanus* populations included in this study are presented in Table 2. We include a comparison to the measurements and characters of *H. stephanus* published by Sher (1963) and by Vovlas *et al.* (1991), and a comparison to morphometrics and characters of a *H. galeatus* population published in Sher's (1963) and Handoo and Golden's (1992) revisions of the genus. Our measurements and morphological characters for the three populations conform to the original description of *H. stephanus*, except for the larger size of females (1.28-1.67mm vs 1.01-1.45mm) of the dogwood population. The grass and birch populations were very similar to Sher's (1963). We found greater differences with the observations by Vovlas *et al.* (1991), who report more longitudinal striations on the basal lip annule (30-36), and also larger variation of the number of tail annules (11-15). Our observations suggest that tail annules (12) are a stable character, as are the number of lip annules (4).

Our observations confirm the diagnostic characters for *H. stephanus*, including four lip annules (Fig. 2A), basal lip annule with 24-28 longitudinal striae (Fig. 2A), presence of males (Fig. 2B), four lateral incisures (Figs. 2C-F), presence of epiptygma (Fig. 2E), and incomplete areolation of the lateral field (Fig. 2E, 2F). Our observations on the presence of epiptygma coincide with those reported by Volvas *et al.* (1991).

Molecular identification: Multiplex PCR products amplified from *H. columbus, H. galeatus* and *H. stephanus*

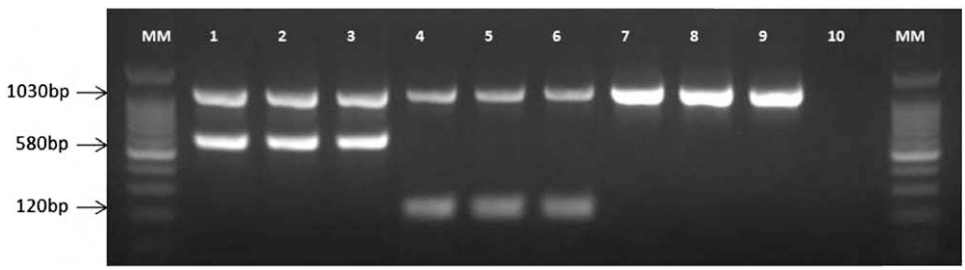

FIG. 4. Multiplex PCR products amplified from *Hoplolaimus columbus, H. galeatus* and *H. stephanus* using species-specific primer sets and resolved using agarose gel electrophoresis. Multiplex primers used were HC-1r, HG-2r, HM-3r (reverse primer) and Hoc-1f (forward primer). LSUD fragment was amplified as internal positive control for each reaction. Lanes 1-3 are *H. columbus* (580 bp), lanes 4-6 are *H. galeatus* (120 bp); lanes 7-9 were *H. stephanus*; lane 10 is the negative control. No amplicons were observed for the HM-3r primer (Bae *et al.*, 2009).

templates using our *H. stephanus* species-specific primer set and resolved using agarose gel electrophoresis are shown in Figure 3. An internal positive control for each reaction was included by using primers LSUD-1f and LSUD-2r (amplicon size of 1030bp). Primers HS-1f and HS-1r do not amplify *H. columbus* (lanes 1-3) or *H. galeatus* (lanes 4-6). These primers only amplify *H. stephanus* (lanes 7-9, 260bp fragment).

The species-specific primers designed by Bae *et al.* (2009) accurately discriminate *H. columbus, H. galeatus* and *H. magnistylus*. In Figure 4, multiplex PCR products amplified from *H. columbus, H. galeatus* and *H. stephanus* templates using these species-specific primer sets are shown. An LSUD fragment was amplified as internal positive control for each reaction. Lanes 1-3 show *H. columbus* (580 bp fragment), lanes 4-6 show *H. galeatus* (120 bp fragment), and lanes 7-9 show *H. stephanus* (amplification of only the internal control).

Phylogenetic analysis: All three *H. stephanus* populations considered in this study were grouped together in Clade I (Figures 5-7), with high posterior probability (100% in ITS1 and actin trees, 99% in LSUD tree), which supports our morphological identification and characterization of the populations. The unknown species *Hoplolaimus* sp.3, from GenBank and Bae *et al.* (2008) was grouped into the same clade with *H. stephanus* in both ITS1 and LSUD phylogram trees. The BLAST search of the sequences of both ITS1 and LSUD fragments from the three populations of *H. stephanus* in this study result in high sequence similarity (99%) with this unknown species. We are confident that the specimens reported as unknown species 3 are *H. stephanus*.

Unknown species *Hoplolaimus* sp.1 and *Hoplolaimus* sp.2 from Bae *et al.* (2008) are very close to *H. stephanus* (96% and 98% sequence similarity, respectively), but likely different species. *In silico* analysis indicate that our proposed *H. stephanus*-specific primers will not amplify

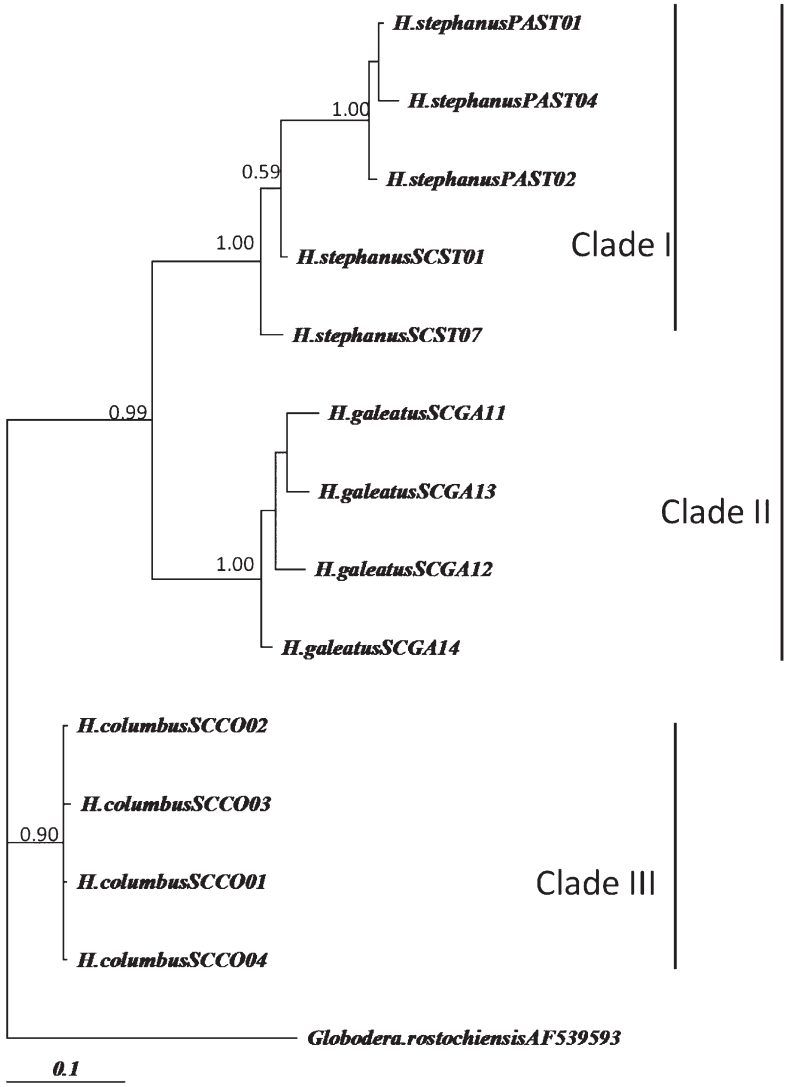

Fig. 5. Bayesian inference 50% majority rule consensus tree of actin region of *Hoplolaimus* specimens. Numbers at nodes are Bayesian posterior probabilities.

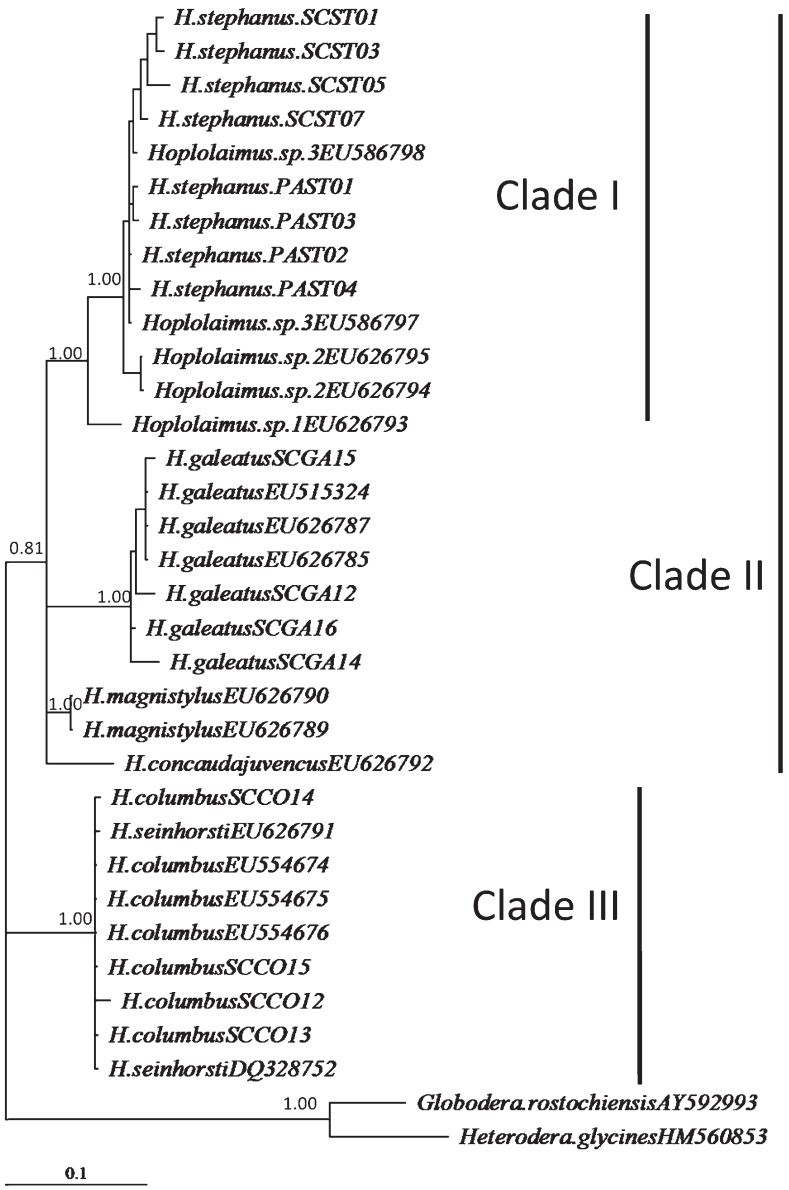

FIG. 6. Bayesian inference 50% majority rule consensus tree of LSUD region of *Hoplolaimus* species specimens. Numbers at nodes are Bayesian posterior probabilities.

these two species, but actual specimens will be required to validate this and to characterize the species morphologically. Until we are able to obtain more of these specimens, these two species will remain uncertain.

Phylogenetic analysis of Bayesian Inference using actin, ITS1 and LSUD sequences produced phylogram trees with similar topological relations among the *Hoplolaimus* species. The actin gene tree in Fig.5 indicates a close relationship between *H. stephanus* and *H. galeatus*. The LSUD tree in Fig. 6 groups *H. columbus* and *H. seinhorsti* into the same Clade II; and *H. magnistylus, H. galeatus, H. concaudajuvencus* and *H. stephanus* into a big Clade III, supported by 0.81 posterior probability. These two clades coincide with the proposal by Siddiqi (2000) of divisions into two subgenera: i) subgenus *Hoplolaimus* Daday, 1905 that includes species with lateral field with four incisures, such as *H. galeatus*, *H. stephanus*, *H. concaudajuvencus,* and *H. magnistylus*; and ii) subgenus *Basirolaimus* Shamsi, 1979 that includes species with one to three incisures, such as *H. columbus* and *H. seinhorsti.*

DISCUSSION

H. stephanus is more commonly occurring than currently reflected in the literature, and this leads us to believe that it is very probable that some reports of *H. galeatus* could in fact be *H. stephanus*, especially considering the overlap in host plants and geographic distribution. Our phylogenetic analysis supports *H. stephanus* as a valid phylogenetic species that can be easily discriminated genetically. However, it can be challenging to

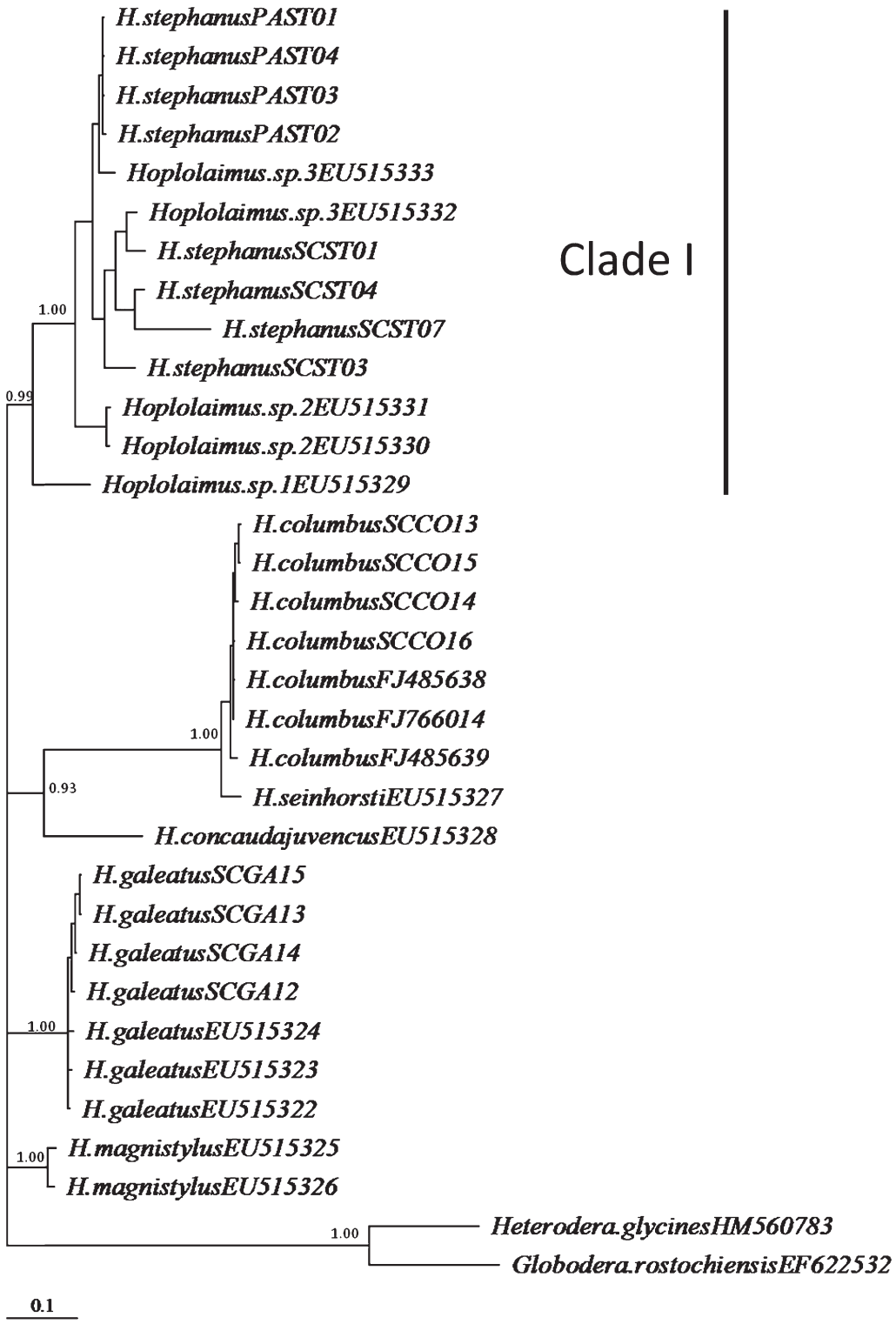

FIG. 7. Bayesian inference 50% majority rule consensus tree of ITS1 region of *Hoplolaimus* species specimens. Numbers at nodes are Bayesian posterior probabilities.

differentiate *H. galeatus* from *H. stephanus* by morphology. Our species-specific primers will therefore prove very useful for identification.

The actin gene has been widely used in phylogenetic analysis of other nematode groups, like *Globodera* and *Heterodera* species (Kovaleva *et al.*, 2005). We believe that using this gene together with the ribosomal sequences will increase the robustness of phylogenetic analyses of relationships among *Hoplolaimus* species. In this study, the application of three genetic markers provided a higher resolution analysis of taxonomic relationships and better support for the delimitation of *H. stephanus* (Figure 5-7).

It is necessary to undertake studies that will compare the ecology and biology of *H. stephanus* and *H. galeatus*. Pathogenicity studies on potential common hosts, such as grasses and woody species, could reveal the need to develop different threshold levels for these two species. Edaphic factors studies, mainly those concerning observations on correlations with soil texture and levels of disturbance, could help predict the presence and geographic distribution of these two closely-related species.

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