Molecular Characterization and Phylogenetic Relationships of Plant-Parasitic Nematodes Associated with Turfgrasses in North Carolina and South Carolina, United States

Yongsan Zeng, Department of Plant Protection, Zhongkai University of Agriculture and Engineering, Guangzhou, 510225, People's Republic of China; Department of Plant Pathology, North Carolina State University, Raleigh 27695; **Weimin Ye**, Nematode Assay Section, Agronomic Division, North Carolina Department of Agriculture and Consumer Services, Raleigh 27607; **James Kerns** and **Lane Tredway**, Department of Plant Pathology, North Carolina State University, Raleigh; **Samuel Martin**, School of Agricultural, Forest and Environmental Sciences, Pee Dee Research & Education Center, Florence, SC 29506; and **Matt Martin**, Crop Science Department, North Carolina State University, Castle Hayne 28429

Abstract

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The near-full-length 18S ribosomal DNA (rDNA) gene and internal transcribed spacer 1 region were amplified and sequenced from 52 nematode populations belonging to 28 representative species in 13 families recovered from turfgrasses in North Carolina (38 populations) and South Carolina (14 populations). This study also included 13 nematode populations from eight other plant hosts from North Carolina for comparison. Nematodes were molecularly characterized and the phylogenetic relationships were explored based on 18S rDNA sequences. Phylogenetic analysis using Bayesian inference was performed using five groups of the plant-parasitic nematode populations Tylenchids, Criconeematids, Longidorids,

Xiphinematids, and Trichodorids. The 65 nematode populations were clustered correspondingly within appropriate positions of 13 families, including Belonolaimidae, Caloosiidae, Criconeematidae, Dolichodoridae, Hemicycliophoridae, Hoplolaimidae, Heteroderidae, Longidoridae, Meloidogynidae, Paratylenchidae, Pratylenchidae, Telotylenchidae, and Trichodoridae. This study confirms previous morphological-based identification of the plant-parasitic nematode species found in turfgrasses and provides a framework for future studies of plant-parasitic nematodes associated with turfgrasses based upon DNA sequences and phylogenetic relationships.

Turfgrass (multiple species) beautifies home lawns and provides safe playing surfaces on athletic fields, outdoor recreation, and economic opportunities for tens of thousands of seed and sod producers, lawn care operators and landscapers. There are approximately 35,850 km² of managed turfgrasses in the United States (Milesi et al. 2005). This places turfgrass third in total agricultural crop acreage nationwide (<http://www.ntep.org/pdf/turfinitiative.pdf>). Turfgrass is the number one or two agricultural commodity in many states such as Maryland, Pennsylvania, Florida, New Jersey, and North Carolina (<http://www.ntep.org/pdf/turfinitiative.pdf>). However, maintenance of turfgrass is very challenging due to economic pressures, damage by pests, and changes in the availability of pesticides. Of the pests that impact turfgrass, plant-parasitic nematodes are often overlooked and, therefore, have become a serious problem in turfgrass. During a survey from 2010 to 2013, 29 species of plant-parasitic nematodes belonging to 22 genera in 15 families were found associated with the turfgrasses bermudagrass (*Cynodon dactylon*), creeping bentgrass (*Agrostis stolonifera*), and zoysiagrass (*Zoysia japonica*) in North Carolina and South Carolina (Zeng et al. 2012b). Of the plant-parasitic nematodes found in the survey, *Belonolaimus longicaudatus*, *Cactodera* sp., *Dolichodorus heterocephalus*, *Hemicycliophora* spp., *Hoplolaimus galeatus*, *Meloidogyne* spp., *Mesocriconeema* spp., *Paratrichodorus* spp., and *Xiphinema* spp. were the most frequently encountered and damaging species (Ye et al. 2012). From this research, damage thresholds where control actions should be taken were proposed (Ye et al. 2012).

Morphological identification of plant-parasitic nematodes associated with turfgrass is difficult because of the presence of many similar morphological characteristics in species such as *Cactodera* sp., *Hemicriconeemoides* spp., *Hemicycliophora* spp., *Helicotylenchus* spp., *Meloidogyne* spp., *Mesocriconeema* spp., *Ogma* spp., and *Pratylenchus* spp. Moreover, the diagnostic life stages, such as females for *Meloidogyne* spp. and *Cactodera* sp., are usually not available in communities extracted from soil samples. However, accurate identification and pathogenic characterization of nematodes attacking turfgrasses are needed as an initial step in designing effective control measures or in searching for possible sources of host-plant resistance against plant-parasitic nematodes found in turfgrass.

Molecular systematics is a valuable tool in modern nematode taxonomy (Subbotin et al. 2005, 2006; Ye et al. 2007). Studies of phylogenetic relationships among nematodes are not only essential to taxonomy but also allow for a more complete understanding of the biology of nematodes as agricultural pests (Subbotin et al. 2001). DNA sequence data from the 18S ribosomal DNA (rDNA), internal transcribed spacer (ITS), and 28S rDNA, as well as mitochondrial DNA (mtDNA), are the most frequently used genomic regions in phylogenetic studies of plant-parasitic nematodes due to ubiquitous occurrence and essential functions (Blaxter et al. 1998; Duarte et al. 2010; Gutiérrez-Gutiérrez et al. 2013; Kumari and Subbotin 2012; McClure et al. 2012; Subbotin et al. 2001, 2005, 2006; Van Megen et al. 2009; Vovlas et al. 2008; Ye et al. 2007). The 18S and ITS regions are frequently used for both phylogenetic analyses and diagnostic purposes because the 18S region is conserved while the ITS region is more variable. The conserved region allows for sequences from divergent taxa to be easily aligned, provides a valuable tool for distinguishing taxa, and also enables the design of universal primers (Dorris et al. 1999). Near-full-length sequences of the 18S and ITS for nematodes can be amplified by polymerase chain reaction (PCR). For this reason, these regions are very popular among molecular systematists for phylogenetic investigation

Corresponding author: J. Kerns; E-mail: jpkerns@ncsu.edu

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of many nematodes (Kanzaki and Giblin-Davis 2014; Turbeville et al. 1991; Ye et al. 2007).

Currently, molecular information on plant-parasitic nematodes associated with turfgrass is limited. Nischwitz et al. (2013) characterized two species of *Meloidogyne* (namely, *Meloidogyne fallax* and *M. minor*) using 28S D2/D3 and ITS regions and analyzed, based upon heat shock protein 90 (Hsp90), phylogenetic relationships among five species of *Meloidogyne*, including *M. fallax*, *M. minor*, *M. chitwoodi*, *M. naasi*, and *M. hapla* from golf course putting greens in the western United States. McClure et al. (2012) molecularly identified *Meloidogyne* spp. from 110 golf course greens by phylogenetically analyzing sequences from 18S, 28S D2/D3, ITS rDNA, mtDNA cytochrome oxidase subunit I, and 16S rRNA gene sequences. Molecular data has been applied in a few studies such as those describing a new species or for diagnosing nematodes associated with turfgrass (Inserra et al. 2013b; Salazar et al. 2013; Shaver et al. 2013; Zeng et al. 2012a; Zhuo et al. 2011).

In North Carolina and South Carolina, with more than 1,000 golf courses, turfgrass is a major industry. There were approximately 30 plant-parasitic nematode species recovered during our previous survey (Zeng et al. 2012b) but no data on molecular characterization of and phylogenetic relationships among these nematodes are available. The objectives of this study were to (i) molecularly characterize 52 plant-parasitic nematode populations belonging to 28 species collected from turfgrasses in North Carolina and South Carolina using the near-full-length 18S rDNA gene and ITS1 rDNA region and (ii) investigate the phylogenetic relationships among these plant-parasitic nematode species and verify previous morphological-based identification of these species (Zeng et al. 2012b) with analysis of 18S rDNA sequences.

Materials and Methods

Plant-parasitic nematode populations. In total, 282 soil samples were collected during a plant-parasitic nematode survey of 111 golf courses in 39 counties in North Carolina and South Carolina during summer 2011 (Zeng et al. 2012b). Each sample consisted of 12 soil cores (1.5 cm in diameter by 20 cm deep) sampled at roughly equal intervals in a zig-zag pattern across an area of 1,000 m² or less. Soil samples were placed in sealed plastic bags, which were then placed in sample boxes and stored at 4°C until analysis. Nematodes were extracted from soil samples by a combination of elutriation (Byrd et al. 1976) and centrifugation (Jenkins 1964) methods. The present study selected 52 representative plant-parasitic nematode populations belonging to 20 genera and 28 species in 13 families for molecular characterization. Two families (Tylenchidae and Aphelenchoidae) were not characterized due to limited specimens and lack of DNA. Thirteen populations of species within the Tylenchidae and Aphelenchoidae families from other hosts in North Carolina were included for phylogenetic comparison (Table 1).

DNA extraction. For molecular analysis, a single nematode or up to 10 nematodes from the same sample were hand picked into 10 µl of AE buffer (10 mM Tris-Cl and 0.5 mM EDTA, pH 9.0) on a glass microscope. Nematodes were identified to species using a compound microscope and keys proposed by Siddiqi (2000) and Decraemer (1995). Identified nematodes were then macerated with a pipette tip, collected in 50 µl of AE buffer, and stored at -20°C.

DNA amplification, cleaning, and sequencing. The primers used for PCR and DNA sequencing are presented in Table 2. Primers SSUF07/SSUR26 (Floyd et al. 2002), 18S965/18S1573R (Mullin et al. 2005), and 18SnF/18SnR (Kanzaki and Futai 2002) were used to amplify the 18S gene. The primers rDNA2 (Vrain et al. 1992) and rDNA1.58S (Cherry et al. 1997) were used to amplify the ITS1 rDNA region. A 25-µl PCR was performed using Apex Taq Red Master Mix DNA polymerase (Genesee Scientific Corporation, San Diego, CA) according to the manufacturer's protocol. The thermal cycler program for PCR was as follows: denaturation at 95°C for 6 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min (Ye et al. 2007). PCR products were cleaned using ExoSap-IT (Affymetrix, Inc., Santa Clara, CA) according to the

manufacturer's protocol. DNA sequencing was performed using PCR primers for direct sequencing by dideoxynucleotide chain termination using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) in an Applied Biosystems 3730 XL DNA Analyzer (Applied Biosystems) by the Genomic Sciences Laboratory (North Carolina State University, Raleigh).

DNA sequencing and phylogenetic analyses. DNA sequences were edited with ChromasPro1.5 2003-2009 (Technelysium Pty. Ltd., Helensvale, Australia) and aligned using ClustalW (<http://workbench.sdsc.edu>; Bioinformatics and Computational Biology group, Department of Bioengineering, University of California, San Diego). The sequences obtained in this study were deposited into the GenBank database under the accessions numbers listed in Table 1. Outgroup species and other species from GenBank were also used for phylogenetic analysis. In order to reduce the ambiguous sites and include more species in multiple DNA sequence alignments in one dataset, phylogenetic analysis using Bayesian inference of the populations was divided into the five groups: Tylenchids, Criconematids, Longidorids, Xiphinematids, and Trichodorids. The models of base substitution in the 18S sequence data were evaluated using MODELTEST version 3.06 (Posada and Crandall 1998). ITS1 is highly variable across genera and is not suited for phylogenetic analysis above the genus level. Thus, ITS1 sequence data were used for specific confirmation only, not for constructing phylogenetic relationships. The Akaike-supported model (Arnold 2010), the proportion of invariable sites, and the γ distribution shape parameters and substitution rates were used in phylogenetic analyses using 18S sequence data. Bayesian analysis was performed to confirm the tree topology for each gene separately using MrBayes 3.1.0 (Huelsenbeck and Ronquist 2001), running the chain for 1,000,000 generations and setting the burn-in at 1,000. Markov Chain Monte Carlo methods were used within a Bayesian framework to estimate the posterior probabilities (pp) of the phylogenetic trees (Larget and Simon 1999) using the 50% majority rule. The λ^2 test for homogeneity of base frequencies and phylogenetic trees was performed using PAUP* (version 4.0; Sinauer Associates, Inc. Publishers, Sunderland, MA).

Results

Near-full-length 18S rDNA (60 populations, including ITS1) sequences from 65 plant-parasitic nematodes were obtained in this study (Table 1). These nematode populations from turfgrass in North Carolina and South Carolina belonged to 28 species from 13 families (Zeng et al. 2012b). Phylogenetic analysis of the 18S sequence included 67, 48, 27, 42, and 24 nematode populations within the groups Tylenchid, Criconematid, Longidorid, Xiphinematid, and Trichodorid, respectively. The proportion of constant characters of these datasets was 62, 71, 91, 85, and 81%, respectively. The λ^2 test showed that homogeneity of base frequencies across taxa was not rejected for any of the datasets. The trees produced by Bayesian analysis of 18S sequence data are presented in Figures 1 through 5.

Eleven nematode species within the Tylenchid group (Table 1; *B. longicaudatus*, *Cactodera* sp., *D. heterocephalus*, *Helicotylenchus dihystra*, *Heterodera* sp., *Hoplolaimus galeatus*, *M. graminis*, *M. naasi*, *Pratylenchus penetrans*, *Scutellonema brachyurum*, and *Tylenchorynchus claytoni*) were found during the survey (Zeng et al. 2012b). The blastn search of 31 of these populations matched well with data of corresponding species found in GenBank.

Three *B. longicaudatus* populations (T5, T10, and T63) from turfgrass were analyzed in this study. The sequences of the near-full-length 18S plus ITS1 sequence from these three populations had 2,239 identical nucleotides (identity = 2,239/2,242 = 99%). The blastn search of the 18S sequence of three *B. longicaudatus* populations (DQ912919, AY633449, and EU130838) in GenBank revealed high similarity (99% identities) with the three populations found in this study. When the ITS1 sequence was considered for these same *B. longicaudatus* populations, they shared 464 to 466 (99%) identical nucleotides with four populations of *B. longicaudatus* (GQ896549, DQ494792, DQ672369, and DQ672368) in GenBank.

Two *Cactodera* sp. populations (T42 and 12-33402) were analyzed in this study. The 18S sequences of these populations shared 624 identical nucleotides (100%). The 18S sequence alignment of T42, 12-33402, and *Cactodera* sp. (AY911929) in GenBank yielded 624 total characters with 623 identical nucleotides (99%).

One population of *D. heterocephalus* (T76) and one population of *Helicotylenchus dihystra* (T35) were analyzed. The blastn search of the 18S sequence of *D. heterocephalus* (T76) revealed the highest match with *Dolichodorus* sp. (EF025336). Alignment of these two sequences yielded 1,723 identical nucleotides (100%). From the 18S sequence alone, the *H. dihystra* population from this study (T35) was identical to *H. dihystra* (AJ966486) in GenBank (1,763/1,763 = 100%).

An unidentified population of *Heterodera* sp. (T75) was found (Zeng et al. 2012b). Alignment of partial 18S plus ITS1 sequences from T75 and two other species—*H. mani* (EU669916) and *H. avenae* (FJ040403)—in GenBank yielded 1,477 total characters with 1,472 and 1,470 identical nucleotides (99%).

Four populations of *Hoplolaimus galeatus* (T2, T29, T62, and T65) were analyzed in this study. The 18S plus ITS1 sequences from these populations had 2,308 identical nucleotides (2,308/2,308 = 100%). The blastn search of the ITS1 sequence of these four populations was similar to *H. galeatus* (EU515321 and EU515322) in GenBank. Alignment of these six sequences yielded 529 total characters with 528 identical nucleotides (100%). Two populations of *H. columbus*, one from cotton (11-16545) and other from soybean (12-22307), were used for comparison. The 18S sequences (1,587 bp) of these two populations and the four turfgrass populations of *H. galeatus* shared 95% identities.

The 18S plus ITS1 sequences of five populations of *M. graminis* (T44, T54, T56, T60, and T61) had 1,891 identical nucleotides (1891/1950 = 97%). The blastn search of the 18S sequence had a high match with *M. graminis* (JN241838), with 99% identity. Three populations of *M. naasi* were sequenced (T51, T53, and T74), with the 18S plus ITS1 sequences (2,015 bp) among these populations having 0 to 5 nucleotide differences. The blastn search of the 18S sequence matched those of *M. naasi* (AY593900 and AY593901). Sequence alignment of these five populations yielded 1,687 total characters (99%), with 0 to 5 nucleotide differences and four insertions or deletions. Three *M. naasi* populations from this study (T51, T53, and T74) had 322 identical nucleotides (322/322 = 100%) and one insertion or deletion (0%) in the ITS1 region. These populations shared 322 identical nucleotides with two other populations of *M. naasi* (EU910042 and EU910045).

P. penetrans (T6), *S. brachyurum* (T58), and *T. claytoni* (T11 and T38) were found in the survey. The 18S sequence alignment of *P. penetrans* T6 with *P. penetrans* (EU669925) was 99% identical. Alignments of the 18S plus ITS1 sequences showed 99% identities between *S. brachyurum* (T58) and *S. brachyurum* (DQ316097) and between populations of *T. claytoni* (T11 and T38) and *T. claytoni* (EU368587).

Phylogenetic analysis inferred from the 18S sequences revealed four distinct clades of tylenchid species recovered from turfgrass and other crops (Fig. 1). Clade I (pp = 100%) comprised nematodes in the families Hoplolaimidae and Heteroderidae and had 30 populations, including 14 from the current study: *Helicotylenchus dihystra* (T35), *S. brachyurum* (T58), *Cactodera* sp. (T41, T42, and 12-33402), *Heterodera* sp. (T75), *Heterodera glycines* (T70

Table 1. Details of plant-parasitic nematode populations from a survey of turfgrass and other hosts in North Carolina and South Carolina included in phylogenetic study

Genera, species	Host ^a	Locality (county, state)	Isolate code (DNA & lab ID)	Accession number ^b
Tylenchid				
<i>Belonolaimus longicaudatus</i>	Bermudagrass	Lenoir, NC	T5 11-2030	KJ934129
<i>B. longicaudatus</i>	Turfgrass	Moore, NC	T10 11-3366	KJ934128
<i>B. longicaudatus</i>	Bermudagrass	Beaufort, SC	T63 11-30676	KJ934136
<i>B. longicaudatus</i>	Peach	Montgomery, NC	11-29875	KJ934139
<i>B. longicaudatus</i>	Tobacco	Sampson, NC	12-25409	KJ934140
<i>Cactodera</i> sp.	Turfgrass	Cumberland, NC	T42 11-30146	KJ934135
<i>Cactodera</i> sp.	Turfgrass	Cumberland, NC	12-33402	KJ934187
<i>Cactodera</i> sp.	Watermelon	Sampson, NC	T41 10-27762	KJ934145
<i>Dolichodorus heterocephalus</i>	Turfgrass	New Hanover, NC	T76 12-675	KJ934144
<i>Helicotylenchus dihystra</i>	Turfgrass	Wake, NC	T35 11-29935	KJ934127
<i>Heterodera glycines</i>	Soybean	Gates, NC	T70 11-30739	KJ934138
<i>H. glycines</i>	Soybean	Gates, NC	T70b 11-30739	KJ934142
<i>Heterodera</i> sp.	Bermudagrass	Horry, SC	T75 11-30748	KJ934134
<i>Hoplolaimus columbus</i>	Cotton	Scotland, NC	11-16545	KJ934149
<i>H. columbus</i>	Soybean	Edgecombe, NC	12-22307	KJ934150
<i>H. galeatus</i>	Centipede grass	Cumberland, NC	T2 11-1848	KJ934131
<i>H. galeatus</i>	Bermudagrass	Beaufort, SC	T65 11-30676	KJ934146
<i>H. galeatus</i>	Bermudagrass	Beaufort, SC	T62 11-30688	KJ934147
<i>H. galeatus</i>	Turfgrass	New Hanover, NC	T29 11-29594	KJ934148
<i>Meloidogyne graminis</i>	Bermudagrass	Kershaw, SC	T44 11-30365	KJ934143
<i>M. graminis</i>	Bentgrass	Avery, NC	T54 11-30385	KJ934153
<i>M. graminis</i>	Bermudagrass	Burke, NC	T56 11-30389	KJ934154
<i>M. graminis</i>	Bermudagrass	Beaufort, SC	T60 11-30673	KJ934152
<i>M. graminis</i>	Bermudagrass	Beaufort, SC	T61 11-30688	KJ934155
<i>M. naasi</i>	Bentgrass	Avery, NC	T51 11-30383	KJ934133
<i>M. naasi</i>	Bentgrass	Avery, NC	T53 11-30385	KJ934132
<i>M. naasi</i>	Turfgrass	Greenville, SC	T74 12-44	KJ934151
<i>Pratylenchus penetrans</i>	Bermudagrass	Craven, NC	T6 11-2088	KJ934156
<i>Scutellonema brachyurum</i>	Turfgrass	New Hanover, NC	T58 11-29736	KJ934137
<i>Tylenchorhynchus claytoni</i>	Turfgrass	Moore, NC	T11 11-3366	KJ934130
<i>T. claytoni</i>	Turfgrass	New Hanover, NC	T38 11-29736	KJ934141

(continued on next page)

^a When the specific turfgrass species was not known, 'Turfgrass' was used as the host designation.

^b Accession numbers under which deposited in GenBank. Deposited sequences were from 18S and internal transcribed spacer ribosomal DNA.

^c Sequences deposited in GenBank only included 18S sequence data.

and T70b from soybean), *Hoplolaimus galeatus* (T2, T29, T62, and T65), and *H. columbus* (11-16545 and 12-22307). Within this clade, six *Hoplolaimus* populations were in a highly supported (pp = 100%) monophyletic clade and sister to two populations of *Rotylenchus* (AJ966503 and EU306356), also with high support (pp = 99%). *Heterodera* sp. (T75) and *Heterodera glycines* (T70 and T70b) were in a highly supported (pp = 100%) monophyletic clade with six other *Heterodera* populations. Three other Heteroderidae populations (*Cactodera* sp. T41, T42, and 12-33402) appeared close to a clade consisting of five *Helicotylenchus* populations (T35, AJ966486, FJ969124, AY284606, and T58); however, this clade was weakly supported (pp < 50%).

Clade II (pp = 100%), belonging to the family Belonolaimidae, contained eight populations, including the five turfgrass populations of *Belonolaimus* (T5, T10, T63, 11-29875, and 12-25409; Fig. 1). All sequenced *Belonolaimus* populations were within a monophyletic clade with 100% support. Clade III (pp = 55%) consisted of 27 populations belonging to four families: Meloidogynidae, Tylenchidae, Pratylenchidae, and Telotylenchidae. All 15 *Meloidogyne* populations, including *M. naasi* (T51, T53, and T74) and *M. graminis* (T44, T60, T54, T56, and T61), were all within a highly supported (pp = 100%) monophyletic clade and sister to a clade with three populations of Pratylenchidae (FJ969136, KJ934156, and AF442189), including *P. penetrans* (T6), with 98% support. This population was also in a highly supported clade (pp = 100%) with *P. convallariae* (FJ969136). The clade containing *Meloidogyne* and *Pratylenchus* spp. was sister to *Zygotylenchus* (AF442189). Five populations of

Telotylenchidae, including *T. claytoni* (T11 and T38, both from turfgrass) as well as *T. claytoni* (EU368587, AY993979, and AY593905), were grouped in a moderately (pp = 79%) supported clade but in a weakly supported (pp = 67%) clade with *Hirschmanniella loofi* (EU306353). Two turfgrass populations of *T. claytoni* (T11 and T38) and another population of *T. claytoni* (EU368587) were in a clade with 100% support. Clade IV (pp = 100%) included two populations of Dolichodoridae. The turfgrass population *D. heterocephalus* (T76) was identical to an unidentified *Dolichodorus* sp. (EF025336) in GenBank.

Eleven nematode species, including *Hemicaloosia graminis*, *Hemicriconemoides wessoni*, *Hemicylophora wyei*, *H. thienemanni*, *Hemicylophora* sp., *Mesocriconema* sp., *Mesocriconema sphaerocephalum*, *M. xenoplax*, and *Paratylenchus goldeni*, were found during this survey (Zeng et al. 2012b). The blastn search of 26 populations from the current study matched well with data of corresponding species found in GenBank. Two populations of *Hemicaloosia graminis* (T14 and T77) were analyzed in this study. The 18S plus ITS1 sequence alignment of these populations yielded 1,961 total characters with 1,953 identical nucleotides (99%) and one insertion or deletion (0%). The 18S blastn search of these two populations revealed the highest match as *H. vagisclera* (JQ246426). Alignment of these three sequences yielded 1,495 total characters with 1- to 2-bp differences.

The 18S plus ITS1 sequences of four *Hemicriconemoides wessoni* populations (T12, T49, T59, and 09-24185) shared 1,182 identical nucleotides (98 to 99%). From the 18S sequence alone (only partial

Table 1. (continued from preceding page)

Genera, species	Host ^a	Locality (county, state)	Isolate code (DNA & lab ID)	Accession number ^b
Criconeematid				
<i>Hemicaloosia graminis</i>	Turfgrass	New Hanover, NC	T77 10-27720	JQ446376
<i>H. graminis</i>	Centipede grass	New Hanover, NC	T14 11-3379	KJ934167
<i>Hemicriconemoides chitwoodi</i>	Boxwood	Cumberland, NC	T32 11-29905	KJ934162
<i>H. wessoni</i>	Bermudagrass	Brunswick, NC	09-24185	KJ934163
<i>H. wessoni</i>	Centipede grass	New Hanover, NC	T12 11-3379	KJ934164
<i>H. wessoni</i>	Bermudagrass	Kershaw, SC	T49 11-30365	KJ934165
<i>H. wessoni</i>	Bermudagrass	Beaufort, SC	T59 11-30664	KJ934166
<i>Hemicylophora wyei</i>	Turfgrass	New Hanover, NC	T39 11-29736	KJ934172
<i>H. wyei</i>	Turfgrass	New Hanover, NC	T46 11-30321	KJ934173
<i>H. wyei</i>	Turfgrass	New Hanover, NC	T57 11-30316	KJ934174
<i>Hemicylophora</i> sp.	Bermudagrass	New Hanover, NC	T15 11-3357	KJ934170
<i>Hemicylophora</i> sp.	Turfgrass	New Hanover, NC	T28 11-29594	KJ934169
<i>H. thienemanni</i>	Bermudagrass	Charleston, SC	T72 11-30769	KJ934168
<i>H. thienemanni</i>	Corn	Greene, NC	M62 08-4103	KJ934171 ^c
<i>Mesocriconema sphaerocephalum</i>	Bermudagrass	Beaufort, SC	T64 11-30664	KJ934182 ^c
<i>M. xenoplax</i>	Turfgrass	Wallace, NC	T3 11-1978	KJ934176
<i>M. xenoplax</i>	Turfgrass	Wake, NC	T34 11-29935	KJ934177
<i>M. xenoplax</i>	Bermudagrass	Nash, NC	T37 11-29404	KJ934178
<i>M. xenoplax</i>	Turfgrass	Greenville, SC	T73 12-44	KJ934180
<i>M. xenoplax</i>	Peach	Montgomery, NC	T33 11-29849	KJ934179 ^c
<i>Mesocriconema</i> sp.	Centipede grass	New Hanover, NC	T13 11-3379	KJ934185
<i>Mesocriconema</i> sp.	Centipede grass	New Hanover, NC	T27 11-29593	KJ934184 ^c
<i>Mesocriconema</i> sp.	Bermudagrass	Dare, NC	T8 11-2734	KJ934181
<i>Ogma</i> sp.	Boxwood	Columbus, NC	12-23998	KJ934161
<i>Ogma</i> sp.	Daylily	Brunswick, NC	13-9959	KJ934175
<i>Paratylenchus goldeni</i>	Centipede grass	Cumberland, NC	T36 11-29913	KJ934186
Longidorid				
<i>Longidorus paralongicaudatus</i>	Turfgrass	Brunswick, NC	12-24191	KJ934123 ^c
Xiphinematid				
<i>Xiphinema americanum</i>	Turfgrass	Moore, NC	T30 11-29987	KJ934159
<i>X. americanum</i>	Turfgrass	New Hanover, NC	T40 11-29736	KJ934158
<i>X. chambersi</i>	Zoysiagrass	New Hanover, NC	T4 11-1995	KJ934157
<i>X. chambersi</i>	Daylily	Robeson, NC	10-23191	KJ934160
Trichodorid				
<i>Paratrichodorus allius</i>	Bentgrass	Avery, NC	T52 11-30383	KJ934124
<i>Nanidorus minor</i>	Bermudagrass	Kershaw, SC	T43 11-30365	KJ934125
<i>N. minor</i>	Bermudagrass	Rio Grande, Puerto Rico	T20 11-6013	KJ934126

for T12 and T49), alignment of these four populations with *H. wesoni* (JF972467) in GenBank revealed 99 to 100% identities.

Six turfgrass populations of *Hemicycliophora* were analyzed in this study, including three populations of *Hemicycliophora wyei* (T39, T46, and T57), one population of *H. thienemanni* (T72), and two unidentified populations of *Hemicycliophora* (T15 and T28). The 18S plus ITS1 sequences (partial 18S for T57) from of the three populations of *H. wyei* (T39, T46, and T57) shared 1,169 identical nucleotides (98 to 99%). The ITS1 blastn search of the same three populations were identical (560/560 = 100%) to *H. wyei* (KF430528) from GenBank. The 18S blastn search of *H. thienemanni* (T72) revealed the highest match (99%) with *H. thienemanni* (EU306341).

Nine populations of *Mesocriconema* were analyzed in this study, including *M. sphaerocephalum* (T64), *M. xenoplax* (T3, T33, T34, T37, and T73), and three unidentified populations of *Mesocriconema* (T8, T13, and T27). A blastn search showed that no 18S sequence of *M. sphaerocephalum* was available for comparison in GenBank. *M. sphaerocephalum* (T64) had 1,631 (1,631/1,710 = 95%) and 1,529 (1,529/1,603 = 95%) identical nucleotides with *M. xenoplax* (AY284625 and AY284626). Alignment of the 18S sequences from *M. xenoplax* (T3, T33, T34, T37, and T73) with one population of *M. xenoplax* (AY284625) from GenBank revealed 99% identities.

One population of *P. goldeni* (T36) was recovered during the survey (Zeng et al. 2012b). No sequence data for *P. goldeni* were available for comparison in GenBank. The 18S sequence shared 1,543 to 1,671 identical nucleotides (96 to 98%) with *Paratylenchus* spp. (AJ966496, AY284630, AY284633, and AY284634).

Phylogenetic analysis inferred from the 18S sequence revealed six distinct clades of Criconeematid species (Fig. 2). In total, six major clades (five highly and one weakly supported) were identified within this group. Clade I (pp = 57%) consisted of 14 populations, including six from the present study: *Ogma* sp. (12-23998) and *Hemicriconemoides chitwoodi* (T32) from boxwood, and four populations of *Hemicriconemoides wesoni* (T12, T49, T59, and 09-24185) from turfgrass. Within this clade, relationships between the genera *Hemicriconemoides*, *Ogma*, *Criconema*, and *Crossonema*, were unresolved. Two populations of *Ogma* (13-9959 from boxwood and 12-23998 from daylily) were not identified to species level due to lack of sufficient specimens. Bayesian analysis based on the 18S sequence showed that these two populations were clustered in two distinct clades. *Ogma* sp. (13-9959) was in a highly supported (pp = 100%) clade (clade IV; Fig. 2) with *Ogma serratum* (HM116031), which was in a highly supported (pp = 100%) clade with the genus *Hemicycliophora*. *Ogma* sp. (12-23998) was in a highly supported (pp = 100%) clade with *Crossonema* sp. (FJ969120) in clade I (Fig. 2), with species in the genera *Criconema*, *Crossonema*, and *Hemicriconemoides*, indicating that the genus *Ogma* included six selected populations appearing to be paraphyletic. Clade II (pp = 100%) included *Caloosia longicaudata* (GU989624), *Hemicaloosia graminis* (T14 and T77), and *H. vagisclera* (JQ246426). *C. longicaudata* formed a highly supported clade (pp = 100%) with three populations of *Hemicaloosia* as a sister taxon. Clade III (pp = 100%) consisted of 12 populations, including 7 populations from this survey: *Hemicycliophora thienemanni* (T72 and M62), *Hemicycliophora* sp. (T15), *Hemicycliophora* sp. (T28), and *H. wyei* (T39,

T46, and T57). Twelve populations of *Hemicycliophora* were in a highly supported (pp = 100%) monophyletic clade. Five populations of *H. thienemanni* (T72, M62, EU306341, AY284628, and AY284629) formed a highly supported clade (pp = 98%) with two unidentified *Hemicycliophora* populations (T15 and T28). *H. wyei* was associated with *H. conida* (AJ966471), with moderate support (pp = 82%). The highly supported (pp = 100%) clade IV consisted of two populations of *Ogma*, including the study population 13-9959 from daylily. Clade V (pp = 100%) included nine populations of *Mesocriconema* from turfgrass and three other populations of *M. xenoplax* from GenBank in a highly supported (pp = 100%) monophyletic clade. Six of our *M. xenoplax* populations, three *M. xenoplax* from GenBank, and one population of *M. sphaerocephalum* (T64) were in a highly supported (pp = 100%) clade and were sister taxa of two unidentified populations of *Mesocriconema* (T13 and T27). Clade VI (pp = 100%) contained five populations of *Paratylenchus*, including one from turfgrass (*P. goldeni*; T36).

There was one population of a Longidorid recovered during our survey (Zeng et al. 2012b). The blastn search of the near-full-length 18S sequence of this population (12-24191) revealed the highest match as *Longidorus paralongicaudatus* (AY283160), with 99% identity. Based on Bayesian analysis, *L. paralongicaudatus* (12-24191) is in a strongly supported clade (pp = 100%) with *L. paralongicaudatus* (AY283160) from hickory in Washington, AR (Fig. 3). This clade was in a well-supported (100%) monophyletic clade with five other species in the genus *Longidorus* from different hosts in the United States. The study population was sister to *L. bififormis* (AY283171) and *L. americanus* (AY283168).

Two Xiphinematid nematode species, *Xiphinema americanum* and *X. chambersi*, were detected during our survey (Zeng et al. 2012b). Two populations of *X. americanum* from turfgrass (T30 and T40) and two populations of *X. chambersi* from turfgrass (T4) and daylily (10-23191) were analyzed. A blastn search of these four populations matched with their corresponding species. From the 18S sequence alone, the two populations of *X. chambersi* (T4 and 10-23191) and *X. chambersi* (AY283174) in GenBank yielded 1,739 total characters with 99% identities. One survey population, *X. americanum* (T30), shared 1,752 (1,752/1,753 = 99%) identical nucleotides with *X. americanum* (AM086672), 1,761 (1,761/1,763 = 99%) with *X. americanum* (AM086684), and 1,757 (1,757/1,762 = 99%) with *X. americanum* (AY283170). The other survey population, *X. americanum* (T40), shared 1,750 (1,750/1,752 = 99%) with *X. americanum* (AM086672), 1,759 (1,759/1,762 = 99%) with *X. americanum* (AM086684), and 1,755 (1,755/1,761 = 99%) identical nucleotides with *X. americanum* (AY283170). However, both T30 and T40 shared 98% identical nucleotides with a Portugal population of *X. americanum* (AM086679; 1,717/1,754 = 98% for T30, and 1,715/1,753 = 98% for T40). T30 shared 99% identical nucleotides with T40 (1,767/1,768 = 99%).

Phylogenetic analysis inferred from the 18S sequence revealed two distinct clades of *Xiphinema* spp., representing *X. americanum*-group and non-*X. americanum*-group nematodes (Fig. 4). *X. chambersi* (T4) and *X. chambersi* (10-23191) were grouped into one clade (non-*X. americanum*-group) and two study populations of *X. americanum*

Table 2. Primers used for polymerase chain reaction and DNA sequencing of plant-parasitic nematodes recovered during a survey of turfgrass in North Carolina and South Carolina^a

Primer	Marker	Sequence (5' to 3')	Reference
SSUF07	18S	AAAGATTAAGCCATGTCATG	Floyd et al. (2002)
SSUR26	18S	CATTCTTGCGAAATGCTTTCG	Floyd et al. (2002)
18S965	18S	GGCGATCAGATACCGCCCTAGTT	Mullin et al. (2005)
18S1573R	18S	TACAAAGGGCAGGGACGTAAT	Mullin et al. (2005)
18SnF	18S	TGGATAACTGTGGTAATTCTAGAGC	Kanzaki and Futai (2002)
18SnR	18S	TTACGACTTTTGCCC GGITC	Kanzaki and Futai (2002)
rDNA2	ITS1	TTGATTACGTTCCCTGCCCTTT	Vrain et al. (1992)
rDNA1.58S	ITS1	ACGAGCCGAGTGATCCACCG	Cherry et al. (1997)

^a rDNA = ribosomal DNA and ITS = internal transcribed spacer.

(T30 and T40) were clustered within another clade (*X. americanum* group). The two *X. chambersi* populations (T4 and 10-23191) were in a strongly supported (100%) monophyletic clade with *X. chambersi* (AY283174) from maple (Lawrence, AR). This species is closer to *X. turcicum* (GU725086), *X. paritaliae* (AY297831), and *X. ifacolum* (AY297826) than other species in the non-*X. americanum*-group. The 2 *X. americanum*

populations in this study (T30 and T40) were in a highly supported (100%) monophyletic clade with 12 other *Xiphinema* populations, including 3 *X. americanum* (AY283170, AM086672, and AM086684), *X. floridiae* (AM086687), and *X. georgianum* (AM086688) from the United States; *X. cf. americanum* (AM086679) from Portugal; *X. brevicolle* (AY297822) and *X. oxycaudatum* (AY297835) from

Fig. 1. Phylogenetic relationships among 18 genera and 67 species of Tylenchid nematodes collected from turfgrass sites in North Carolina and South Carolina inferred from the 10,001 replicate of Bayesian analysis deduced from 18S rDNA sequences. Two Criconematid species sequences are outgroups. Numbers at nodes indicate bootstrap scores >50%. The phylogenetic tree was generated using MrBayes 3.1.0 (Huelsenbeck and Ronquist 2001).

Brazil; *X. diffusum* (AM086685) from Australia; *X. incognitum* (AM086670) from China; *X. simile* (AM086681) from Serbia; and *X. taylari* (AM086675) from Slovakia. Both study populations were closer in relationship to the three U.S. populations (AY283170, AM086672, and AM086684) of *X. americanum* than to the *X. cf. americanum* from Portugal (AM086679).

Two Trichodorid nematode species, *Paratrichodorus allius* and *Nanidorus minor*, were recovered during our survey (Zeng et al. 2012b). The blastn search of three turfgrass trichodorid populations (T20, T43, and T52) matched with their corresponding species. Alignment of 18S and ITS1 sequences from the survey population *P. allius* (T52) and *P. allius* (AM087124) in GenBank yielded

Fig. 2. Phylogenetic relationships among nine genera and 49 species of Cricematid nematodes collected from turfgrass sites in North Carolina and South Carolina inferred from the 10,001 replicate of Bayesian analysis deduced from 18S rDNA sequences. Two *Paratylenchoides* spp. sequences are outgroups. Numbers at nodes indicate bootstrap scores >50%. The phylogenetic tree was generated using MrBayes 3.1.0 (Huelsenbeck and Ronquist 2001).

2,280 total characters with 2,258 identical nucleotides (99%) and seven insertions or deletions (0%). From the 18S sequence alone, the survey population *P. allius* (T52) shared 1,731 (1,731/1,736 = 99%) identical nucleotides with *P. allius* (AM269895) and 1,717 (1,717/1,724 = 99%) with *P. allius* (AJ439572). Using ITS1 sequence data, the survey population *P. allius* (T52) shared 540 (540/546 = 99%) identical nucleotides with *P. allius* (AM087124). The 18S sequences of two populations of *N. minor* (T20 and T43) had 1,685 identical nucleotides (1,685/1,696 = 99%) and three insertions or deletions (3/1,696 = 0%). The 18S sequences from the survey population *N. minor* (T20) shared 1,725 (1,725/1,734 = 99%) and 1,659 (1,659/1,664 = 99%) identical nucleotides with *N. minor* (AM269897) and *N. minor* (DQ345526), respectively. The 18S sequences from another survey population of *N. minor* (T43) shared 1,689 (1,689/1,703 = 99%) identical nucleotides with *N. minor* (AM269897) and 1,654 (1,654/1,664 = 99%) with *N. minor* (DQ345526).

Phylogenetic analysis inferred from the 18S sequence revealed two distinct clades of Trichodorid (Fig. 5). *P. allius* (T52) and *N. minor* (T20 and T43) were in two different clades. *P. allius* (T52) was in a highly supported (97%) monophyletic clade with three U.S. *P. allius* populations (AJ439572, AM087124, and AM269895). This clade was sister to three populations of *P. teres* (AJ439575, AM087125, and AM269896). One *P. allius* population (AJ439623) from Utah was in a basal position in this clade. Two survey populations of *N. minor* (T20 and T43) were in a highly supported (100%) monophyletic clade with populations of *N. minor* from Greece (AM269897) and from

Portugal (DQ345526). This species appeared to be sister to *P. porosus*, with 100% support.

Discussion

Previous studies revealed intraspecific variability in the 18S and 28S D2/D3 rDNA as well as the mtDNA in nematodes (Kumari and Subbotin 2012; Lazarova et al. 2006; Neilson et al. 2004; Oliveira et al. 2004). In the present study, there were minor DNA sequence differences within the detected plant-parasitic nematode populations. However, morphological examination of the populations matched well with the original or previous descriptions. Thus, these minor DNA sequence differences were considered to be intraspecific variation. These results are similar to intraspecific divergence reported for other nematodes such as *L. diadecturus* (Neilson et al. 2004), the *X. setariae/vulgare* complex (Oliveira et al. 2004), and *X. americanum*-group populations from North America (Lazarova et al. 2006). This study included 13 sequences of plant-parasitic nematode populations from other plant hosts to compare with nematode sequences from turfgrasses; no significant sequence differences was observed for these species from different hosts.

There are thousands of 18S nematode sequences in the GenBank database, providing an extensive and growing resource for comparison (Griffiths et al. 2006). The 18S rDNA sequences have been used extensively in phylogenetic studies (Bert et al. 2008; Blaxter et al. 1998; Gutiérrez-Gutiérrez et al. 2010, 2012, 2013; Kumari and Subbotin 2012; Oliveira et al. 2004; Subbotin et al. 2008; Ye et al. 2007). However, the vast majority of nematode species have not been

Fig. 3. Phylogenetic relationships among two genera and 28 species of Longidorid nematodes collected from turfgrass sites in North Carolina and South Carolina inferred from the 10,001 replicate of Bayesian analysis deduced from 18S rDNA sequences. A *Xiphinema* sp. sequence is an outgroup. Numbers at nodes indicate bootstrap scores >50%. The phylogenetic tree was generated using MrBayes 3.1.0 (Huelsenbeck and Ronquist 2001).

sequenced. For example, *M. sphaerocephalum* (T64) and *Paratylenchus goldeni* (T36) were sequenced in this study but sequences for these nematodes were not available in GenBank. Molecular identification of nematodes is still a challenge due to the degree of sequence conservation and the availability of deposited 18S sequences for comparison. In the present study, several cyst or cystoid nematode populations were not identified to the species level, mainly because very limited sequences of *Cactodera* were available in GenBank. In our survey (Zeng et al. 2012b), only the second-stage juveniles of these genera were observed in soil samples, and no adult females or cysts were recovered for species identification. Five criconematid populations were also found during the survey; however, they were not identified to species level because of lack of sufficient specimens for morphological identification and no identical matches in GenBank. Within some nematode genera, such as *Heterodera*, the 18S gene sequence is highly conserved among

different species. The sequences are nearly identical among *Heterodera glycines*, *H. hordecalis*, *H. trifolii*, *H. betae*, and *H. schachtii*. Other gene sequences, including the ITS region, D2/D3 expansion segments of the 28S rDNA gene, and the mtDNA gene, for species discrimination and phylogenetic studies are needed to discriminate *Heterodera* spp. In one case, *D. heterocephalus* (T76) was identical to an unidentified *Dolichodorus* sp. (EF025336) from GenBank based upon 18S sequence data; these two nematodes are likely the same species.

The *Hemicycliophora* populations T39, T46, and 11-30286 were morphologically identified as *Hemicycliophora conida* (Zeng et al. 2012b), having a lateral field with transverse anastomoses or breaks of the striae, elongated continuous conical tails with distinct annulations and rounded tips, long vulval lips with a vulval sleeve, a stylet of 78.3 to 90.4 μm long, V (% distance of vulva from anterior) = 81.5 to 85.1%, R (total annulus number) = 248 to 269, and RVan (annulus

Fig. 4. Phylogenetic relationships among 42 species of *Xiphinema* nematodes collected from turfgrass sites in North Carolina and South Carolina inferred from the 10,001 replicate of Bayesian analysis deduced from 18S rDNA sequences. Two Dorylaimid species sequences are outgroups. Numbers at nodes indicate bootstrap scores >50%. The phylogenetic tree was generated using MrBayes 3.1.0 (Huelsenbeck and Ronquist 2001).

The present molecular phylogenetic analysis revealed that clade I within the criconematid group is weakly supported (pp = 57%), consisting of 14 populations belonging to four genera: *Criconema*, *Crossonema*, *Hemicriconemoides*, and *Ogma*. These genera are not well resolved and available sequence data are very limited. Neither *Hemicriconemoides* nor *Ogma* appear to be monophyletic. This is similar to results based on 28S D2/D3 reported by Subbotin et al.

(2005). This issue may be resolved by including additional species of the genus in new phylogenetic analysis and reexamining the species identification.

Conclusions

Analysis of DNA sequences is of importance in confirmation of classical morphology-based identifications of nematode species and in reconstruction of their phylogenetic relationships with available sequences in GenBank. In the present study, the 18S and ITS1 sequence data were employed successfully to support our previous morphological identification of nematodes associated with turf-grasses. Through blastn search based on 18S or ITS1 sequence data, the species found during this survey and identified morphologically matched well with data of corresponding species found in GenBank. This study provides basic information on DNA sequence and a comprehensive framework of phylogenetic relationships of plant-parasitic nematodes associated with turfgrass. This is the first report on molecular characterization and phylogeny of plant-parasitic nematodes associated with turfgrasses in North Carolina and South Carolina. Several isolates have not been identified to species level due to lack of sufficient specimens for DNA sequencing and morphological study; thus, more sampling is desired.

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