

PRIMER NOTE

Development of taxon-specific markers for high-throughput screening of microbial-feeding nematodes

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Abstract

In an effort to assess the taxonomic identity of large-scale samplings of nematodes from the Konza Tallgrass Prairie, we sequenced a portion of the 18S rRNA gene and its associated internally transcribed spacer (ITS1) from 74 nematodes encompassing four taxonomic families. From these sequences, we have developed a series of molecular probes to distinguish 16 distinct microbivorous nematode taxa. Using a combination of low power microscopy and taxon-specific real-time probes, the 74 nematodes were correctly assigned to their respective taxonomic groups. This optimized method provides a high-throughput assay to determine nematode identities across larger data sets.

Keywords: 18S rRNA, 5' nuclease assay, Nematoda, real-time PCR, Taqman

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The Konza Prairie Biological Station is a 3487-ha area of native tallgrass prairie in the Flint Hills of northeastern Kansas that is the primary site for the Konza Prairie LTER Program. The Konza Prairie LTER is a comprehensive, multidisciplinary program designed to address long-term research questions relevant to tallgrass prairie ecosystems. Since 1981, the Konza Prairie LTER research program has encompassed studies ranging from the organismic to the population, community, ecosystem and landscape ecology levels. Volumes of research on the Konza have shown that nitrogen addition and reduced frequency of burning greatly impacts the tallgrass prairie ecosystem (Knapp *et al.* 1998). In addition to changes in plant communities, these perturbations have also been shown to alter Konza nematode communities. Todd (1996) showed that microbial-feeding nematode densities increased with nitrogen addition and decreased with the removal of burning. This and other ecological studies for nematodes have traditionally been based on taxonomic assessments at the family level. With morphological identification to genus or below either too cumbersome or limited by systematic expertise (Yeates 2003), molecular studies utilizing the conserved 18S rRNA gene to assess the phylogenetic/taxonomic similarity across taxa have become a viable alternative to morphometric

assay (Blaxter *et al.* 1998). This study describes the development of molecular methods to assess nematode taxonomic identity within the trophic group of microbial-feeding nematodes on the Konza Tallgrass Prairie.

Two 5 cm diameter × 20 cm deep soil cores were collected from each of 16 experimental plots designed to determine the long-term effects of burning and fertilization on tallgrass ecosystems. Nematodes were extracted from a 100-cm³ subsample using a standard centrifugal-flotation technique (Jenkins 1964). Microbial-feeding nematodes were subsequently identified to large-scale morphological groupings using a Wild M8 dissection scope (50× magnification) and placed singly into 40 µL worm lysis solution [1 mg/mL proteinase K, 10 mM Tris (pH 8.2), 50 mM KCl, 2.5 mM MgCl₂, 0.45% Tween-20, 0.45% NP-40, 0.05% gelatin]. Nematodes were incubated at 60 °C for 1 h to liberate DNA and denature cellular proteins. A final incubation for 15 min at 95 °C was performed to ensure proteinase K inactivity. After incubation, the lysate was stored at –80 °C.

From a randomized subset of each taxonomic family identified, a 900-bp fragment consisting of the 5' 500 bases of the 18S rRNA gene and the entire internally transcribed spacer region (ITS1) was amplified for 74 nematodes using the 18s1.2 primer of Mullin *et al.* (2003) (3'-GGCGATCAGATACCGCCCTAGTT-5') and the rDNA1.58 s primer of Cherry *et al.* (1997) (3'-ACGAGCCGAGTGATCCACCG-5'). Polymerase chain reactions (PCRs; 1× Promega *Taq*

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Table 1 Real-time assays were run in multiplexed groups using taxon-specific probe and primer sets

Multiplexed probe sets	Taxon (GenBank Accession no.)	No. of nematodes	Primers	Probes	Annealing temperature for multiplexed reaction
Cephalobidae 1	<i>Acrobeles</i> sp. 1 (DQ275360)	6	GACTGGTGGACTGTAGCCTCT GCCGTTGGGTCACCTTGTGG	5'-/FAM/CAGTCGTGCATCAAACAATGCAC/BHQ2/-3'	67 °C
	<i>Acrobeles</i> sp. 2 (DQ275361)	5		5'-/TEX/AGTCGTGCATCAACAACAATGCA/BHQ2/-3'	
Cephalobidae 2	<i>Acrobeloides</i> sp. (DQ275355)	18	TCGTAACAAGGTAGCCGTAGGT GCAAACGCACCGCACATAST	5'-/FAM/CTGCCACTACTATAGCGCCGAAGC/BHQ2/-3'	60 °C
	<i>Chiloplacus</i> sp. (DQ275354)	4	GTAACAAGGTAGCCGTAGGTGAA CCGTGCAAGACGTACATCAAG	5'-/TEX/TGATCCTACCACGATCTACTAGTGCC/BHQ2/-3'	
Cephalobidae 3	<i>Cephalobidae</i> sp. 1 (DQ275365)	1	AACGAGCGAGACTCTAGCCTA GGCATCACAGACCTGTTAATTGC	5'-/TEX/TCACGATTGTCCTTTTGTGTCTTCTT/BHQ2/-3'	63 °C
	<i>Cephalobidae</i> sp. 2 (DQ275362)	1		5'-/FAM/CACGATTGTCTTTTCGGTTGCTTCTT/BHQ2/-3'	
	<i>Eucephalobus</i> sp. (DQ275366)	13		5'-/TEX/CGGATTACTAAAGTCCGGTCCGC/BHQ2/-3'	
Panagrolaimidae	<i>Panagrolaimus</i> sp. 1 (DQ275363)	1	GTCTGGTTAATTCGATAACGAAC AACGCCGAAATCCCATTTAA	5'-/TEX/CGCTTTCAACCGTAAGGAAGAAAG/BHQ2/-3'	60 °C
	<i>Panagrolaimus</i> sp. 1 (DQ275364)	1		5'-/FAM/CGCTTTCTCCATAAAAGAAGAAAGG/BHQ2/-3'	
Plectidae 1	<i>Plectus</i> sp. 1 (DQ275356)	4	GAAGGAATCAGCGTGCTAAA CCTCGTTCAAGGAAATAATTG	5'-/FAM/CGTAGGATTTTGTATGGGTTTACCAA/BHQ2/-3'	63 °C
	<i>Plectus</i> sp. 2 (DQ275357)	1	ATTACTGTCYTGATACTTCT CTCGTTCAAGGAAATAATTG	5'-/TEX/CGTAGGATTTTAAATGGGTTTACCAA/BHQ2/-3'	
Plectidae 2	<i>Plectus</i> sp. 3 (DQ275358)	3	GCTGTCCTGGTTACTTCTTAGAG AATTCCTCGTTCAAGGAAATAATT	5'-/FAM/CCCGATCACGGAAGATTTTCTT/BHQ2/-3'	63 °C
	<i>Wilsonema</i> sp. (DQ275359)	4	TTACTGTCTGGATTCTTCTTAGAG CCTCGTTCAAGGAAATAATTGC	5'-/TEX/CGTAGGATTTTGTAGGGGTTTACCAA/BHQ2/-3'	
Rhabditidae	<i>Mesorhabditis</i> sp. 1 (DQ275367)	1	TGTGTTCCCTGCGAGTGGTGAA CAGTAGCCGTCGGGCTT	5'-/TEX/TAGCTGCTTATAGCTGTGTGCTAGCA/BHQ2/-3'	63 °C
	<i>Mesorhabditis</i> sp. 2 (DQ275369)	1	CGAGTGGTGAAGTGGCTTGAT TGTAGACACCAACGGTTTCCG	5'-/HEX/CGGTGGGCTTTACTAACTGCTTAT/BHQ2/-3'	
	<i>Oscheius</i> sp. (DQ275368)	10	GACTGTGTTCCCTGCGAGTGGT TGTAGACACCAACGGTTTCCG	5'-/FAM/CGGGCGTTACTAGCTGCTTTGAAGT/BHQ2/-3'	

polymerase reaction buffer (Promega), 3.75 mM MgCl₂, 200 μM dNTP, 0.5 μM of each forward and reverse primers, 1 U Promega *Taq* polymerase and 0.05 μL worm lysis per 1 μL final volume) were run for 40 cycles at 95 °C for 1 min, 55 °C for 1 min, 74 °C for 2 min on an MJ Research PTC-100 thermocycler (Bio-Rad Laboratories) and the amplicons sequenced using an ABI PRISM 3700 DNA sequencer (Applied Biosystems). Sequences were grouped by taxonomic family and aligned using CLUSTAL_x (Thompson *et al.* 1997). Alignments were screened for regions of sequence that would elucidate taxonomic the differences within each family. From these sequence differences, BEACON DESIGNER 3 (PREMIER Biosoft) was used to produce probe and primer sequences that positively identified only members of a specific taxon (Table 1). Dual-labelled fluorescent probes (e.g. Taqman probes) were synthesized and used for real-time detection of taxonomic identity.

Nematode lysates previously used for 18S rRNA sequencing were subjected to real-time assays (1× Bio-Rad IQ Supermix, 0.5 μM of each primer, 0.25 μM of each probe and 0.05 μL worm lysis per 1 μL final volume) run for 40 cycles at 95 °C for 30 s, 60–67 °C for 30 s, 74 °C for 30 s in multiplexed taxa sets (Table 1) on an ICYCLER IQ real-time thermocycler (Bio-Rad Laboratories). Probe detection was visualized using ICYCLER IQ real-time detection software. For all nematodes where sequence was known, taxonomic information gained from the initial low-power morphological identification was used to narrow the focus for the real-time analysis. After nematodes were identified by morphology to a higher order taxonomic category, one of the seven multiplexed probe and primer sets was used to determine lower-order taxonomy. Microscopy along with taxon-specific probes allowed for proper taxonomic assignment of all nematodes.

Although high-power microscopy is sufficient to assign taxonomic identification to adult nematodes, the time and expertise needed to properly identify individual nematodes would make a large study unfeasible. Additionally, as many taxa are identifiable only through morphological features found in adult specimens, morphological identification of juveniles provides exceptional difficulty. As low-power microscopy is necessary to isolate individual nematodes into microtubes for DNA analysis, gross morphological identification to family, or at times genus, can provide a first-pass filter on nematode taxonomy. Once an initial assessment of morphology has been made, real-time PCR

using multiplexed probes and primers specific to that subset of taxa can be used to further diagnose nematode taxonomy down to species. Although 18S probing could have successfully assigned all individuals to a specific taxon group, the use of microscopy to narrow the focus to a smaller subset of probes allows identification of most nematodes in one or two PCRs, rather than randomly probing with 16 different probes. Thus, the combination of microscopy and real-time PCR is currently the most efficient methodology for assessing nematode taxonomy across a large data set. This methodology, however, still relies on a working knowledge of nematode taxonomy. Future work will produce family specific probes to allow researchers with limited taxonomic knowledge the ability to complete large and taxonomically diverse projects.

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