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Article | DOI: 10.21307/jofnem-2018-037

JOURNAL OF NEMATOLOGY
Issue 0 | Vol. 0

Discovery and Identification of *Meloidogyne* Species Using COI DNA Barcoding

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This paper was edited by XXXXXX.

Received for publication XXXXXXX.

Abstract

DNA barcoding with a new cytochrome oxidase c subunit 1 primer set generated a 721 to 724 bp fragment used for the identification of 322 *Meloidogyne* specimens, including 205 new sequences combined with 117 from GenBank. A maximum likelihood analysis grouped the specimens into 19 well-supported clades and four single-specimen lineages. The "major" tropical apomictic species (*Meloidogyne arenaria*, *Meloidogyne incognita*, *Meloidogyne javanica*) were not discriminated by this barcode although some closely related species such as *Meloidogyne konaensis* were characterized by fixed diagnostic nucleotides. Species that were collected from multiple localities and strongly characterized as discrete lineages or species include *Meloidogyne enterolobii*, *Meloidogyne partityla*., *Meloidogyne hapla*, *Meloidogyne graminicola*, *Meloidogyne naasi*, *Meloidogyne chitwoodi*, and *Meloidogyne fallax*. Seven unnamed groups illustrate the limitations of DNA barcoding without the benefit of a well-populated reference library. The addition of these DNA sequences to GenBank and the Barcode of Life Database (BOLD) should stimulate and facilitate root-knot nematode identification and provide a first step in new species discovery.

Key words

COI, DNA barcoding, *Meloidogyne*, Plant parasitic nematodes, Root-knot nematodes, Taxonomy.

The term DNA-barcoding has multiple definitions. The earliest mention of barcoding in nematology was in 1998 by Dr Mark Blaxter, then of Edinburgh University, referring to the "(d)evelopment of a molecular barcode system for soil nematode identification" in the first volume of the Natural Environment Research Council Soil Biodiversity Newsletter (http://soilbio.nerc.ac.uk/newsletters.htm). The barcode he was referring to was the 18S nuclear (small subunit) ribosomal gene. Other gene regions proposed for DNA-barcoding soon followed, creating a broader definition that generally applied to the use of DNA sequences for species identification (Floyd et al., 2002; Blaxter, 2004; Powers, 2004;). In 2003 a widely cited paper by Hebert et al. (2003) proposed a standardization of the barcode definition linked to the amplification of a 658 bp gene region within the cytochrome oxidase subunit 1 mitochondrial gene. The goal of this conceptual paper was the development of a global bioidentification system for animals. Considerable controversy immediately followed this publication with criticism ranging from theoretical concerns about the use of a single gene, the ability of an organelle gene to track species boundaries, and barcoding's impact on the process of taxonomic investigation (DeSalle et al., 2005; Will et al., 2005). Practical concerns were expressed about lack of amplification with some groups, the designation of types, taxonomic resolution, and economic cost at the expense of traditional taxonomic approaches (Meyer and Paulay, 2005; Rubinoff et al., 2006; McFadden et al., 2011). Now, 15 years later, DNA-barcoding has become a component within the broader scope of integrated taxonomy and a routine tool for identification (Hodgetts et al., 2016;

Janssen et al., 2016). As a diagnostic and discovery enterprise, DNA barcoding has generated thousands of publications, features biennial international conferences, has a dedicated database – BOLD, the Barcode of Life Database – and has multiple administrative structures such as the International Barcode of Life (IBOLD) and its affiliates (www.boldsystems.org/index.php/default).

Nematology was slow to adopt this formalized version of barcoding, perhaps due to poor amplification with the original "Folmer" primer sets (Folmer et al., 1994). Now multiple primer sets for amplification of nematode cytochrome oxidase c subunit 1 (COI) are available (Derycke et al., 2005, 2010; Prosser et al., 2013; Kiewnick et al., 2014, Powers et al., 2014; Janssen et al., 2016). These primer sets typically have limited taxonomic scope with amplifications specific for genera or in some cases extending across families and superfamilies (Powers et al., 2014). The objective of this study is to present a primer set used for the amplification of 721 to 724 bp of COI sequence from *Meloidogyne*. A maximum likelihood (ML) tree is provided to illustrate the ability of this gene region to discriminate among many described *Meloidogyne* species. The primers also function as a means to amplify DNA from juvenile stages in community analyses, possibly leading to new species discoveries. Contributions to a COI reference library should aid future taxonomic and ecological research in the genus.

Materials and methods

Nematode collection

Most of the specimens DNA barcoded in this study were either specimens submitted to the UNL Nematology Diagnostics Clinic, specimens contributed by colleagues, or specimens collected during grant funded surveys (NSF projects DEB-1145440; USDA Multistate Project W3186).

Primer sequences

The primer set for amplification of the COI gene region were:

COI-F5-Mel – 5′-TGATTGATTTAGGTTCTGGAACT-KSWTGAAC-3′

COI-R9-Mel – 5′-CATAATGAAAATGGGCAACAA-CATAATAAGTATC-3′

After removal of the primer sequences, amplification products from the *Meloidogyne* specimens were either 721 or 724 bp. GenBank sequences used in this study generally were 100 to 300 nucleotides shorter than sequences generated with the new primer set.

Amplification conditions

Nematodes amplified at the UNL Nematology Laboratory were individually smashed in 18 ul of sterile H20 with a transparent microfuge micropipette tip on a coverslip and added to a 0.5 ml microfuge tube. Nematode lysate was either amplified immediately or stored at –20°C. Amplification conditions were as follows: denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 48.0°C for 30 sec, and extension at 72°C for 90 sec with a 0.5° per second ramp rate to 72°C. A final extension was performed at 72°C for 5 min as described by Powers et al. (2014) and Olson et al. (2017). Polymerase chain reaction (PCR) products were separated and visualized on 1% agarose using 0.5XTBE and stained with ethidium bromide. PCR products of sufficiently high quality were cleaned and sent for sequencing of both strands by University of California–Davis DNA sequencing facility.

Data storage

Nucleotide sequences have been submitted to GenBank (accession numbers MH128384–MH128585) and the Barcode of Life Database (BOLD).

Phylogenetic analysis

Phylogenetic trees were constructed under ML and Neighbor Joining (NJ) criteria in MEGA version 6. Sequences were edited using CodonCode Aligner version 7.1 (www.codoncode.com/) and aligned using Muscle within MEGA version 6 (Tamura et al., 2013). Gap opening penalty was set at –400 with a gap extension penalty of –200. The General Time Reversible Model with Gamma distributed rates (GTR+G) was determined to be the best substitution model by Bayesian Information Criterion using the Best Fit Substitution Model tool in MEGA 6.0. ML trees used a use all sites option for gaps and 200 bootstrap replications to assess clade support.

Results

Figure 1 displays a ML tree of 322 *Meloidogyne* sequences including 117 sequences from GenBank and 205 sequences from the University of Nebraska–Lincoln Nematology Laboratory. ML partitions these sequences into 19 groups with bootstrap support values from 93 to 100 (Tables 1, 2, Fig. 1).

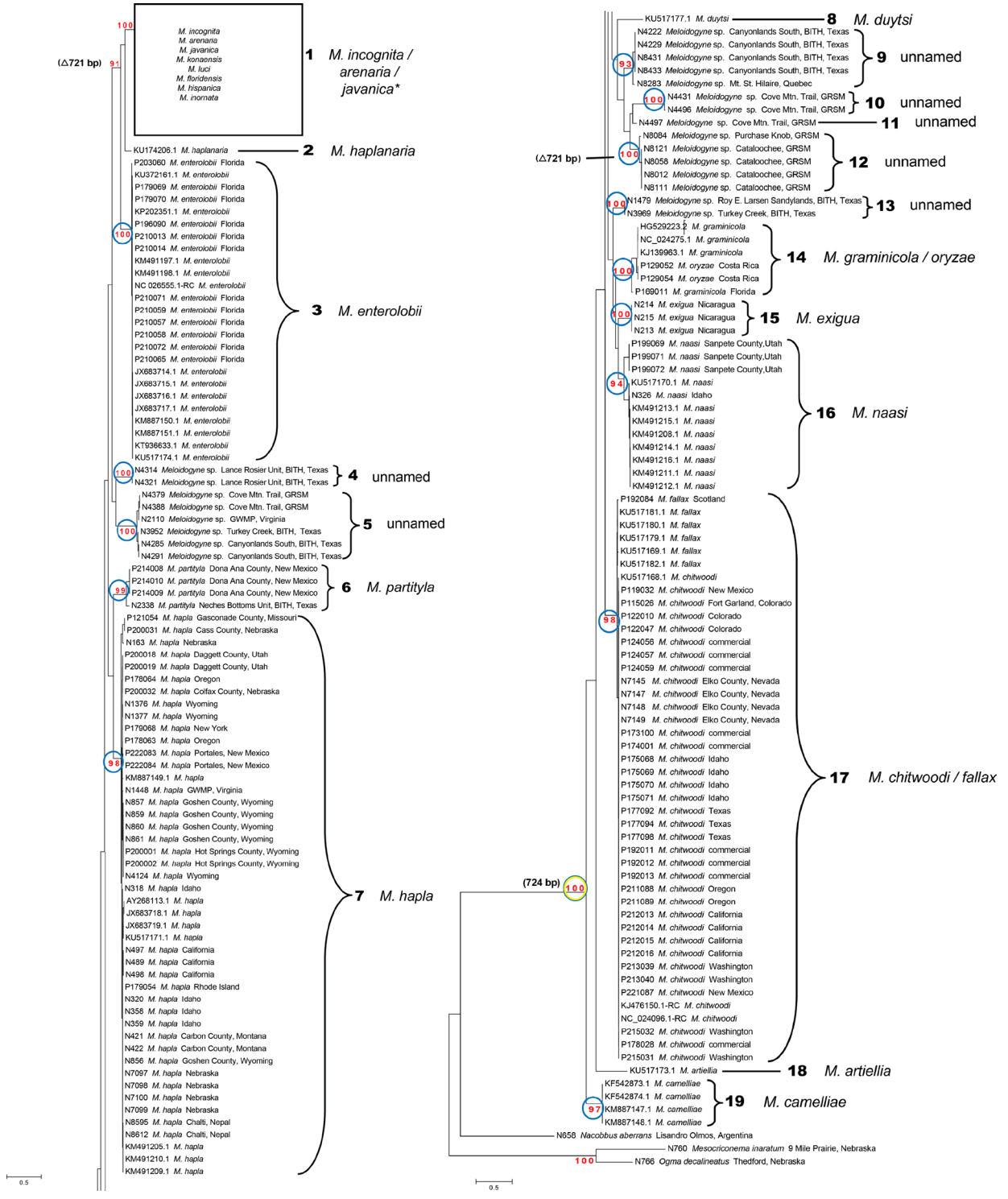

Figure 1: Maximum likelihood tree of 322 *Meloidogyne* COI sequences created in MEGA 6.06 using GTR+G substitution model, with 200 bootstraps and a gap treatment of use all sites. Support values that designate clades and haplotype groups are circled. Clades that correspond to named and unnamed species or haplotype groups are numbered. Clades that include specimens with a single amino acid deletion are denoted by (Δ721 bp). Group 1 has been reduced to a box of species names. Sequences within Group 1 are presented in Table 2. A list of GenBank accession numbers for specimens included in Group 1 are found in supplementary Table 1.

Table 1. COI sequence collection data for groups 2 to 19.

NID	Group	Species	Locality	Host/habitat	GenBank accession #
P203060	3	*Meloidogyne enterolobii*	Florida	Ornamentals-Nursery	MH128522
P179069	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128519
P179070	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128520
P196090	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128521
P210013	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128523
P210014	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128524
P210071	3	*M. enterolobii*	Florida	Ornamentals-ursery	MH128529
P210059	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128527
P210057	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128525
P210058	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128526
P210072	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128530
P210065	3	*M. enterolobii*	Florida	Ornamentals-Nursery	MH128528
N4314	4	*Meloidogyne* sp.	Lance Rosier Unit, BITH[a]	Loblolly pine	MH128531
N4321	4	*Meloidogyne* sp.	Lance Rosier Unit, BITH[a]	Loblolly pine	MH128532
N4379	5	*Meloidogyne* sp.	Cove Mtn. Trail, GRSM[b]	Chestnut	MH128537
N4388	5	*Meloidogyne* sp.	Cove Mtn. Trail, GRSM[b]	Chestnut	MH128538
N2110	5	*Meloidogyne* sp.	GWMP[c]	Fort Marcy earthworks	MH128533
N3952	5	*Meloidogyne* sp.	Turkey Creek, BITH[a]	Baygall community	MH128534
N4285	5	*Meloidogyne* sp.	Canyonlands South, BITH[a]	Magnolia	MH128535
N4291	5	*Meloidogyne* sp.	Canyonlands South, BITH[a]	Magnolia	MH128536
P214008	6	*Meloidogyne partityla*	Dona Ana County, New Mexico	Pecan	MH128540
P214010	6	*M. partityla*	Dona Ana County, New Mexico	Pecan	MH128542
P214009	6	*M. partityla*	Dona Ana County, New Mexico	Pecan	MH128541
N2338	6	*M. partityla*	Neches Bottoms Unit, BITH[a]	Sandbar	MH128539
P121054	7	*Meloidogyne hapla*	Gasconade County, Missouri	Peony	MH128568
P200031	7	*M. hapla*	Cass County, Nebraska	Nursery	MH128577
N163	7	*M. hapla*	Nebraska	Wheat	MH128543
P200018	7	*M. hapla*	Daggett County, Utah	Grass pasture	MH128575
P200019	7	*M. hapla*	Daggett County, Utah	Grass pasture	MH128576
P178064	7	*M. hapla*	Oregon	Potato	MH128570
P200032	7	*M. hapla*	Colfax County, Nebraska	Nursery	MH128578

N1376	7	*M. hapla*	Fremont County, Wyoming	Red bean	MH128558
N1377	7	*M. hapla*	Fremont County, Wyoming	Red bean	MH128559
P179068	7	*M. hapla*	New York	GH	MH128572
P178063	7	*M. hapla*	Oregon	Potato	MH128569
P222083	7	*M. hapla*	Portales, New Mexico	GH culture	MH128579
P222084	7	*M. hapla*	Portales, New Mexico	GH culture	MH128580
N1448	7	*M. hapla*	GWMP[c]	Waterfowl sanctuary	MH128560
N857	7	*M. hapla*	Goshen County, Wyoming	Potato	MH128554
N859	7	*M. hapla*	Goshen County, Wyoming	Potato	MH128555
N860	7	*M. hapla*	Goshen County, Wyoming	Potato	MH128556
N861	7	*M. hapla*	Goshen County, Wyoming	Potato	MH128557
P200001	7	*M. hapla*	Hot Springs County, Wyoming	Alfalfa	MH128573
P200002	7	*M. hapla*	Hot Springs County, Wyoming	Alfalfa	MH128574
N4124	7	*M. hapla*	Wyoming	–	MH128561
N318	7	*M. hapla*	Idaho	Potato	MH128544
N497	7	*M. hapla*	California	–	MH128551
N489	7	*M. hapla*	California	–	MH128550
N498	7	*M. hapla*	California	–	MH128552
P179054	7	*M. hapla*	Rhode Island	GH culture	MH128571
N320	7	*M. hapla*	Idaho	Potato	MH128545
N358	7	*M. hapla*	Idaho	Potato	MH128546
N359	7	*M. hapla*	Idaho	Potato	MH128547
N421	7	*M. hapla*	Carbon County, Montana	Alfalfa	MH128548
N422	7	*M. hapla*	Carbon County, Montana	Alfalfa	MH128549
N856	7	*M. hapla*	Goshen County, Wyoming	Potato	MH128553
N7097	7	*M. hapla*	Nebraska	Alfalfa	MH128562
N7098	7	*M. hapla*	Nebraska	Alfalfa	MH128563
N7100	7	*M. hapla*	Nebraska	Alfalfa	MH128565
N7099	7	*M. hapla*	Nebraska	Alfalfa	MH128564
N8595	7	*M. hapla*	Chalti, Nepal	Pine forest	MH128566
N8612	7	*M. hapla*	Chalti, Nepal	Pine forest	MH128567
N4222	9	*Meloidogyne* sp.	Canyonlands South, BITH[a]	Beech	MH128581
N4229	9	*Meloidogyne* sp.	Canyonlands South, BITH[a]	Beech	MH128582

N8431	9	*Meloidogyne* sp.	Canyonlands South, BITH[a]	Beech	MH128584
N8433	9	*Meloidogyne* sp.	Canyonlands South, BITH[a]	Beech	MH128585
N8283	9	*Meloidogyne* sp.	Mt. St. Hilaire, Quebec, Canada	Hardwood forest	MH128583
N4431	10	*Meloidogyne* sp.	Cove Mtn. Trail, GRSM[b]	Chestnut	MH128463
N4496	10	*Meloidogyne* sp.	Cove Mtn. Trail, GRSM[b]	Chestnut	MH128464
N4497	11	*Meloidogyne* sp.	Cove Mtn. Trail, GRSM[b]	Chestnut	MH128465
N8084	12	*Meloidogyne* sp.	Purchase Knob, GRSM[b]	Chestnut	MH128468
N8121	12	*Meloidogyne* sp.	Cataloochee, GRSM[b]	Oak	MH128470
N8058	12	*Meloidogyne* sp.	Cataloochee, GRSM[b]	Chestnut	MH128467
N8012	12	*Meloidogyne* sp.	Cataloochee, GRSM[b]	Chestnut	MH128466
N8111	12	*Meloidogyne* sp.	Cataloochee, GRSM[b]	Oak	MH128469
N1479	13	*Meloidogyne* sp.	Roy E. Larsen Sandylands, BITH[a]	Baygall community	MH128471
N3969	13	*Meloidogyne* sp.	Turkey Creek, BITH[a]	Baygall community	MH128472
P129052	14	*Meloidogyne oryzae*	Costa Rica	Rice	MH128473
P129054	14	*M. oryzae*	Costa Rica	Rice	MH128474
P169011	14	*Meloidogyne graminicola*	Florida	Purple nutsedge	MH128475
N214	15	*Meloidogyne exigua*	Nicaragua	Coffee	MH128477
N215	15	*M. exigua*	Nicaragua	Coffee	MH128478
N213	15	*M. exigua*	Nicaragua	Coffee	MH128476
P199069	16	*Meloidogyne naasi*	Sanpete County, Utah	Grass	MH128480
P199071	16	*M. naasi*	Sanpete County, Utah	Grass	MH128481
P199072	16	*M. naasi*	Sanpete County, Utah	Grass	MH128482
N326	16	*M. naasi*	Idaho	Potato	MH128479
P192084	17	*Meloidogyne fallax*	Scotland	Genomic DNA	MH128507
P119032	17	*Meloidogyne chitwoodi*	New Mexico	Culture	MH128488
P115026	17	*M. chitwoodi*	Fort Garland, Colorado	Soil sample	MH128487
P122010	17	*M. chitwoodi*	Colorado	Soil sample	MH128489
P122047	17	*M. chitwoodi*	Colorado	Soil sample	MH128490
P124056	17	*M. chitwoodi*	Commercial	Potato	MH128491
P124057	17	*M. chitwoodi*	Commercial	Potato	MH128492
P124059	17	*M. chitwoodi*	Commercial	Potato	MH128493
N7145	17	*M. chitwoodi*	Elko County, Nevada	Potato	MH128483
N7147	17	*M. chitwoodi*	Elko County, Nevada	Potato	MH128484
N7148	17	*M. chitwoodi*	Elko County, Nevada	Potato	MH128485
N7149	17	*M. chitwoodi*	Elko County, Nevada	Potato	MH128486
P173100	17	*M. chitwoodi*	Commercial	Potato	MH128494
P174001	17	*M. chitwoodi*	Commercial	Potato	MH128495
P175068	17	*M. chitwoodi*	Idaho	Potato	MH128496
P175069	17	*M. chitwoodi*	Idaho	Potato	MH128497
P175070	17	*M. chitwoodi*	Idaho	Potato	MH128498

P175071	17	*M. chitwoodi*	Idaho	Potato	MH128499
P177092	17	*M. chitwoodi*	Texas	Potato	MH128500
P177094	17	*M. chitwoodi*	Texas	Potato	MH128501
P177098	17	*M. chitwoodi*	Texas	Potato	MH128502
P192011	17	*M. chitwoodi*	Commercial	Potato	MH128504
P192012	17	*M. chitwoodi*	Commercial	Potato	MH128505
P192013	17	*M. chitwoodi*	Commercial	Potato	MH128506
P211088	17	*M. chitwoodi*	Oregon	Potato	MH128508
P211089	17	*M. chitwoodi*	Oregon	Potato	MH128509
P212013	17	*M. chitwoodi*	California	Potato	MH128510
P212014	17	*M. chitwoodi*	California	Potato	MH128511
P212015	17	*M. chitwoodi*	California	Potato	MH128512
P212016	17	*M. chitwoodi*	California	Potato	MH128513
P213039	17	*M. chitwoodi*	Washington	Potato	MH128514
P213040	17	*M. chitwoodi*	Washington	Potato	MH128515
P221087	17	*M. chitwoodi*	New Mexico	Potato	MH128518
P215032	17	*M. chitwoodi*	Washington	Potato	MH128517
P178028	17	*M. chitwoodi*	Commercial	Potato	MH128503
P215031	17	*M. chitwoodi*	Washington	Potato	MH128516

[a]BITH=Big Thicket National Preserve, Texas.
[b]GRSM=Great Smoky Mountains National Park, Tennessee and North Carolina.
[c]GWMP=George Washington Memorial Parkway, Virginia.

Three unique GenBank sequences represent *Meloidogyne haplanaria* (Eisenback et al., 2004), *Meloidogyne duytsi* (Karssen et al., 1998), and *Meloidogyne artiellia* (Franklin, 1961) as distinct from other sequences in the dataset, but without dditional supporting sequences.

Groups 1 to 3 form a clade characterized by the loss of a single amino acid (3 bp) resulting in a 721 bp sequenced region. This shared deletion unites *M. haplanaria* and *Meloidogyne enterolobii* (Yang and Eisenback, 1983) with the so-called "major" tropical apomictic species of *Meloidogyne* (Elling, 2013). Included in this group are sequences representing *Meloidogyne arenaria* (Neal, 1889; Chitwood, 1949), *Meloidogyne incognita* (Kofoid and White, 1919; Chitwood, 1949), *Meloidogyne javanica* (Treub, 1885, Chitwood, 1949), as well as *Meloidogyne hispanica* (Hirschmann, 1986), *Meloidogyne floridensis* (Handoo et al., 2004), *Meloidogyne konaensis* (Eisenback et al., 1994), *Meloidogyne luci* (Carneiro et al., 1956; Table 2). The same amino acid deletion is also found in unnamed group 12. Within group 1, the COI sequences are nearly identical with a few notable exceptions. Four substitutions are shared by three specimens identified as *M. konaensis*, including GenBank accession KU372176, identified as *Meloidogyne* sp. 2 TJ-2016 T316 on *Beta vulgaris* from Spain in Janssen et al. (2016). Two substitutions are shared by specimens identified as *Meloidogyne incognita grahami,* originally described as *Meloidogyne grahami* (Golden and Slana, 1978), and considered distinct from *M. incognita* based on reproduction on NC-95 tobacco, a cultivar with resistance to *M. incognita*, plus a greater juvenile length and a distinctive perineal pattern (Golden and Slana, 1978).

Outside of clades 1 to 3 there are 11 other described species represented by a minimum of a single COI sequence. *Meloidogyne hapla* (Chitwood, 1949) is represented by specimens from 10 US states and two specimens from Nepal. There are multiple haplotypes within *M. hapla* and possibly some population substructure within the species. Group 17 identified as *Meloidogyne chitwoodi* (Golden et al., 1980) and *Meloidogyne fallax* (Karssen, 1996) is virtually homogeneous except for a 5-bp difference between the two species. Within group 6, identified as *Meloidogyne partityla* (Kleynhans, 1986), one specimen collected from Big Thicket National Preserve, Texas comes from a native lowland plant community,

Table 2. COI sequences included in group 1.

NID	Species	Locality	Host/Habitat	GenBank accession #
N137	*M. konaensis*	Hawaii	Pineapples	MH128384
N138	*M. konaensis*	Hawaii	Pineapples	MH128385
N7067	*Meloidogyne* sp.	Charleston, Missouri	Soybean	MH128414
N7066	*Meloidogyne* sp.	Charleston, Missouri	Soybean	MH128413
N7065	*Meloidogyne* sp.	Charleston, Missouri	Soybean	MH128412
N5777	*Meloidogyne* sp.	Nebraska	Conservatory	MH128410
N5775	*Meloidogyne* sp.	Nebraska	Conservatory	MH128409
N5771	*Meloidogyne* sp.	Nebraska	*Phoenix dactylifera*	MH128408
N3836	*Meloidogyne* sp.	Nebraska	Banana	MH128407
N2668	*Meloidogyne* sp.	Sonora, Mexico	Grapevine	MH128406
N2667	*Meloidogyne* sp.	Sonora, Mexico	Grapevine	MH128405
N2666	*Meloidogyne* sp.	Sonora, Mexico	Grapevine	MH128404
N2665	*Meloidogyne* sp.	Sonora, Mexico	Grapevine	MH128403
N2664	*Meloidogyne* sp.	Sonora, Mexico	Grapevine	MH128402
N2663	*Meloidogyne* sp.	Sonora, Mexico	Grapevine	MH128401
N2662	*Meloidogyne* sp.	Sonora, Mexico	Grapevine	MH128400
N2661	*Meloidogyne* sp.	Sonora, Mexico	Grapevine	MH128399
N2659	*Meloidogyne* sp.	Florida	Peanuts	MH128397
N7068	*Meloidogyne* sp.	Clarkton, Missouri	Soybean	MH128415
N7069	*Meloidogyne* sp.	Clarkton, Missouri	Soybean	MH128416
N7070	*Meloidogyne* sp.	Clarkton, Missouri	Soybean	MH128417
N7072	*Meloidogyne* sp.	Clarkton, Missouri	Soybean	MH128418
N7073	*Meloidogyne* sp.	Clarkton, Missouri	Soybean	MH128419
N7075	*Meloidogyne* sp.	Clarkton, Missouri	Soybean	MH128420
N8309	*Meloidogyne* sp.	Charleston, Missouri	Soybean	MH128421
P118094	*Meloidogyne incognita*	Missouri	Potato	MH128424
P120058	*M. incognita*	Arizona	Culture	MH128425
P120059	*M. incognita*	Arizona	Culture	MH128426
P121032	*M. incognita*	Mississippi	Potato	MH128427
P121058	*M. incognita*	Gasconade County, Missouri	Daylily	MH128428
P121060	*M. incognita*	Moniteau County, Missouri	Daylily	MH128429
P156046	*Meloidogyne floridensis*	Florida	GH culture	MH128430
P156048	*M. floridensis*	Florida	GH culture	MH128431
P158036	*Meloidogyne arenaria*	Alachua County, Florida	–	MH128432
P158037	*M. arenaria*	Alachua County, Florida	–	MH128433
P160024	*Meloidogyne* sp.	Alachua County, Florida	–	MH128434
P160025	*Meloidogyne* sp.	Alachua County, Florida	–	MH128435
P160075	*M. arenaria*	Alachua County, Florida	–	MH128437

P167014	*Meloidogyne javanica*	–	–	MH128438
P167019	*M. arenaria*	–	–	MH128439
P167020	*M. arenaria*	–	–	MH128440
P167021	*M. arenaria*	–	–	MH128441
P176014	*Meloidogyne* sp.	Missouri	Culture	MH128443
P178075	*M. arenaria*	Texas	Potato	MH128444
P195088	*M. javanica*	–	–	MH128445
P195089	*M. javanica*	–	–	MH128446
P196023	*M. javanica*	–	–	MH128447
P196024	*M. javanica*	–	–	MH128448
P196025	*M. javanica*	–	–	MH128449
P202009	*Meloidogyne* sp.	Israel	Culture	MH128450
P229051	*Meloidogyne* sp.	Florida	Culture	MH128451
P229053	*Meloidogyne* sp.	Florida	Culture	MH128452
P229056	*Meloidogyne* sp.	Florida	Culture	MH128453
P233011	*Meloidogyne* sp.	–	Coffee	MH128457
P233014	*Meloidogyne* sp.	–	Coffee	MH128458
P234004	*Meloidogyne* sp.	Morocco	–	MH128459
P234005	*Meloidogyne* sp.	Morocco	–	MH128460
P234006	*Meloidogyne* sp.	Morocco	–	MH128461
P234007	*Meloidogyne* sp.	Morocco	–	MH128462
P73085	*Meloidogyne incognita*	Bonita, Arizona	Pinto beans	MH128422
P73088	*M. incognita*	Bonita, Arizona	Pinto beans	MH128423
N5796	*Meloidogyne* sp.	Ash Meadows NWR, Nevada	–	MH128411
P230069	*Meloidogyne incognita graham*	West Virginia	Culture	MH128454
P230095	*M. incognita graham*	West Virginia	Culture	MH128456
P230070	*M. incognita grahami*	West Virginia	Culture	MH128455
N2660	*Meloidogyne* sp.	Florida	Peanut	MH128398
P167027	*M. arenaria*	–	–	MH128442
N329	*Meloidogyne* sp.	North Dakota	Potato	MH128386
N330	*Meloidogyne* sp.	North Dakota	Potato	MH128387
N331	*Meloidogyne* sp.	North Dakota	Potato	MH128388
N332	*Meloidogyne* sp.	North Dakota	Potato	MH128389
N333	*Meloidogyne* sp.	North Dakota	Potato	MH128390
N334	*Meloidogyne* sp.	North Dakota	Potato	MH128391
N335	*Meloidogyne* sp.	North Dakota	Potato	MH128392
N336	*Meloidogyne* sp.	North Dakota	Potato	MH128393
N337	*Meloidogyne* sp.	North Dakota	Potato	MH128394
N348	*Meloidogyne* sp.	North Dakota	Potato	MH128395
N351	*Meloidogyne* sp.	North Dakota	Potato	MH128396
P160071	*M. arenaria*	Alachua, Florida	Culture	MH128436

Table 3. Measurements of j2 Meloidogyne specimens from unnamed COI haplotype groups and reference species.

Haplotype group/ species	*N*	Length	Tail length	Stylet length	*a*	*b*	*c*
Unnamed 4	2	441 (430–452)	42	17	24.3 (22.7–25.9)	3.8 (3.7–3.9)	10.5 (10.3–10.8)
Unnamed 5	5	431 (406–460)	47 (40–53)	14 (13–15	25.4 914.4–30.8)	3.9 (3.0–4.8)	9.3 (8.0–10.1)
Unnamed 9	5	393 (380–405)	40 (38–44)	15 (15–16)	26.0 (25.5–26.7)	4.0 (3.3–4.4)	9.7 (9.2–10.1)
Unnamed 11 (Singleton A)	1	384	42	15	27.5	3.5	9.1
Unnamed 12	5	353 (339–379)	41 (38–43)	15 (14–15)	22.7 (21.2–25.0)	3.5 (3.3–4.0)	8.6 (8.0–8.9)
Unnamed 13	2	490 (439–541)	59 (57–62)	14	30.7 (30.4–31)	3.9 (3.8–4.0)	8.2 (7.7–8.6)
Meloidogyne ovalis	10	370 (350–430)	–	–	22 (21–24)	–	8 (8–9)
Meloidogyne pini	30	434 (376–493)	44 (37–53)	12.8 (11.4–14.1)	25.7 (21.8–29.1)	–	9.8 (7.5–11.8)
Meloidogyne camelliae	70	501 (443–576)	47 (40–56)	11.6 (11.2–12)	26 (21–30)	3.1	10.7 (9.5–12
Meloidogyne querciana	70	467 (411–541)	46 (39–52)	11.1 (10.2–11.6)	30 (23–39)	2.6	10 (7–13)
Meloidogyne megatyla	23	416 (392–457)	39.7 (31.6–45.1)	14.6 (13.8–16.6)	26 (22–29)	7.1 (6.7–7.8)	10.5 (9.5–13.5)

compared with other specimens from New Mexico collected from commercial pecan (*Carya illinoinensis* (Wangenh.) K. Koch) production.

There are seven groups labeled as unnamed, all with sequence derived from j2 stage specimens except for N4431 and N4496 which were males collected from native chestnut (*Castanea dentata* (Marshall) Borkh.) in Great Smoky Mountains National Park (GRSM), North Carolina. All specimens in the unnamed groups 4, 5, 9 to 13 were isolated from soil samples within Gulf Coast or Eastern North American forests. Groups 9 and 12 were associated with American beech, (*Fagus grandifolia* Ehrh.) and chestnut or oak, respectively. Measurements of the unidentified juveniles are presented in Table 3, and Fig. 2 illustrates juveniles from three of the unnamed groups.

Discussion

The COI gene region used as a diagnostic marker in this study appears to discriminate many of the described species of *Meloidogyne*. It does not separate the apomictic "major species" and their close relatives, except possibly *M. konaensis* and *M. incognita grahami.* Other mitochondrial genes such as NAD 5 may help resolve some of those species boundaries (Janssen et al., 2016). Aside from an inability to discriminate among the tropical clade 1 species, there are advantages to using COI as a DNA barcode. As a protein coding gene, nucleotide alignment is easier compared with non-protein coding genes. Taxonomic resolution is at the population and species level, although for many genera, mutational saturation, lineage extinctions, or inadequate sampling may obscure deeper relationships that aid in the recognition of species groupings. Nonetheless, COI barcodes in combination with an adequately curated sequence database, provide a powerful tool for identification and discovery. The limitation of DNA barcoding without a corresponding database is illustrated by the unnamed groups in the *Meloidogyne* dataset. For example, there was an expectation that focal samples from soil

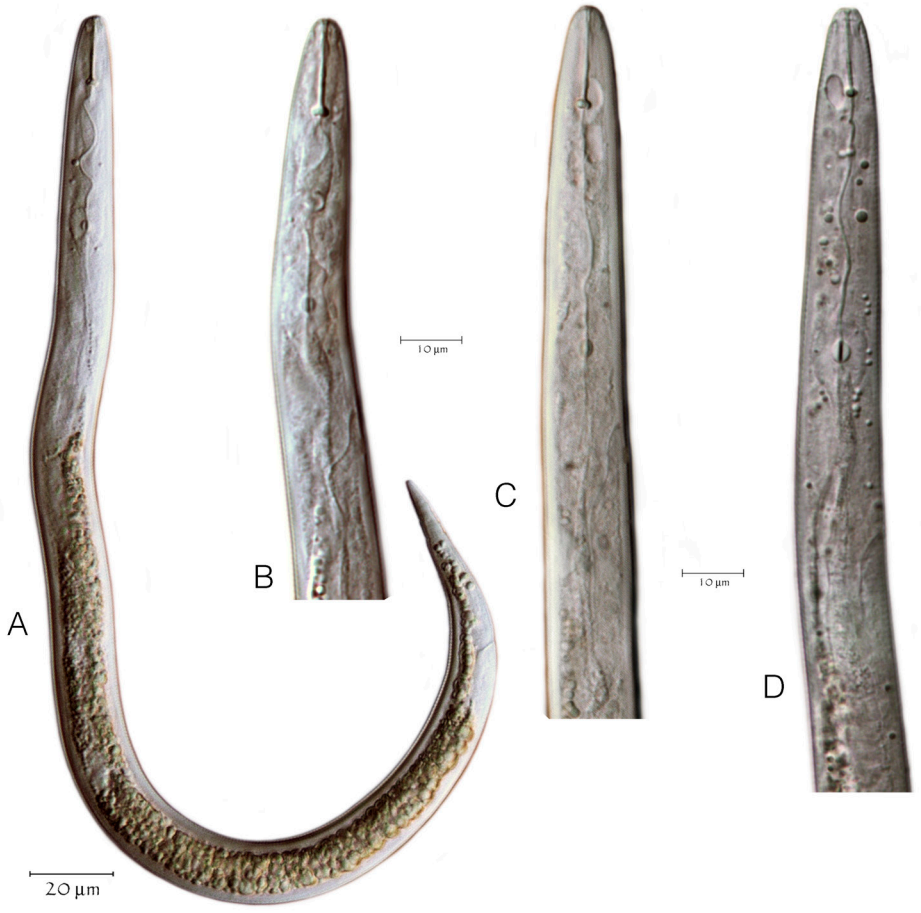

Figure 2: Selected *Meloidogyne* juveniles from unnamed groups. A, Entire body of NID 8084 in Group 12, from chestnut in Great Smoky Mountains National Park (GRSM); B, Anterior region of NID 8012 in Group 12 from chestnut in GRSM; C, Anterior region of NID 8283 from Group 9 from Mt. St. Hilaire, Quebec; D, Anterior region of NID 4379 in Group 5 from chestnut in GRSM.

around individual chestnut and oak trees in GRSM might yield *Meloidogyne querciana* (Golden, 1979) which was described from northern red oak (*Quercus rubra* L.) and chestnut hosts within the same ecoregion. Indeed *Meloidogyne* specimens were found in these samples, however, the barcode data demonstrate that multiple COI lineages were associated with chestnut and oaks in the park. Similarly, unnamed lineages were also discovered associated with American beech and baygall plant communities in Big Thicket National Preserve, Texas (www.nps.gov/bith/plant-communities.htm). These results indicate that considerable *Meloidogyne* diversity exists in the primary and secondary forests of eastern and southern United States. Characterization of this diversity by COI barcoding allows us to rule out described species with representation in the COI database, yet neither COI barcode nor morphometrics of juvenile specimens permits unequivocal assignment of a species name to these specimens. For these unknown specimens a more complete taxonomic analysis that includes obtaining adult stages will be required before a barcode sequence can be linked to a formal Latin binomial.

Acknowledgements

Thanks to the sponsors of nematode surveys, Department of Interior-National Park Service, Discover Life in America, NSF project DEB-1145440, Nebraska Department of Agriculture, Thicket of Diversity, USDA Multistate Project 3186, and University of Nebraska-Lincoln Agricultural Research Division IC-282. Most of all we thank the numerous collaborators and colleagues who have contributed nematode specimens for identification.

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Appendix

Supplementary Table S1

Supplementary Table S1. Accession numbers of specimens from Group 1 acquired from GenBank.

GenBank accession #	Species
KU372176.1	*Meloidogyne* sp.
KM491194.1	*Meloidogyne incognita*
KU372162.1	*Meloidogyne ethiopica*
KU360142.1	*M. incognita*
KM491204.1	*Meloidogyne arenaria*
KU372172.1	*Meloidogyne luci*
KU372173.1	*M. luci*
KM491195.1	*M. incognita*
KM491192.1	*M. incognita*
KM491191.1	*M. incognita*
KM491196.1	*M. incognita*
KM491188.1	*M. incognita*
KM491203.1	*M. arenaria*
KM491201.1	*M. arenaria*
KM491207.1	*M. incognita*
KM491199.1	*M. arenaria*
KM491202.1	*M. arenaria*
KM491200.1	*M. arenaria*
KM491190.1	*M. incognita*
KM491189.1	*M. incognita*
NC_026556.1	*Meloidogyne javanica*
NC_026554.1	*M. arenaria*
KP202352.1	*M. javanica*
KP202350.1	*M. arenaria*
NC_024097.1	*M. incognita*
KJ476151.1	*M. incognita*
KU372158.1	*M. arenaria*
KU372165.1	*M. incognita*
KU372167.1	*Meloidogyne inornata*
KU372159.1	*M. arenaria*
KU372166.1	*M. incognita*
KU372169.1	*M. javanica*
KU372170.1	*M. javanica*
KU372171.1	*M. luci*
KU372174.1	*Meloidogyne* sp.
KU372168.1	*M. inornata*
KU372175.1	*Meloidogyne* sp.
KU372164.1	*M. incognita*
KU372163.1	*M. incognita*
KU517167.1	*M. incognita*
KU360143.1	*M. arenaria*
JX683704.1	*M. arenaria*
KU360144.1	*M. javanica*
JX683696.1	*M. incognita*
JX683698.1	*M. incognita*
JX683699.1	*M. incognita*
JX683700.1	*M. incognita*
JX683706.1	*M. javanica*
JX683701.1	*M. arenaria*
JX683702.1	*M. arenaria*
JX683703.1	*M. arenaria*
JX683705.1	*M. arenaria*
JX683707.1	*M. javanica*
JX683708.1	*M. javanica*
JX683709.1	*M. javanica*
JX683711.1	*M. javanica*
JX683710.1	*M. javanica*
JX683712.1	*Meloidogyne hispanica*
JX683713.1	*M. hispanica*
KM887153.1	*M. arenaria*
KM887152.1	*M. incognita*
KM887155.1	*M. arenaria*
KM887154.1	*M. incognita*
JX683697.1	*M. incognita*
KM887156.1	*M. javanica*
KU517172.1	*M. javanica*
KU517176.1	*M. arenaria*
KU372160.1	*M. arenaria*
KM491193.1	*M. incognita*
KM491206.1	*M. incognita*