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Molecular Characterization of *Meloidogyne christiei* Golden and Kaplan, 1986 (Nematoda, Meloidogynidae) Topotype Population Infecting Turkey Oak (*Quercus laevies*) in Florida

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Abstract: Meloidogyne christiei isolated from turkey oak, *Quercus laevies*, from the type locality in Florida was characterized using isozyme profiles and ribosomal and mitochondrial gene sequences. The phenotype N1a detected from a single egg-laying female of *M. christiei* showed one very strong band of malate dehydrogenase (MDH) activity; however, no esterase (EST) activity was identified from macerate of one or even 20 females per well. Phylogenetic relationships within the genus *Meloidogyne* as inferred from Bayesian analysis of partial 18S ribosomal RNA (rRNA), D2-D3 of 28S rRNA, internal transcribed spacer (ITS) rRNA, and cytochrome oxidase subunit II (COII)-16S rRNA of mitochondrial DNA (mtDNA) gene fragments showed that *M. christiei* formed a separate lineage within the crown group of *Meloidogyne* and its relationships with any of three *Meloidogyne* clades were not resolved.

Key words: 18S rRNA, 28S rRNA, isozymes, ITS rRNA, *Quercus laevis*, *Meloidogyne christiei*, mtDNA, oak, phylogeny, root-knot nematodes.

Meloidogyne christiei was first reported infecting turkey oak (*Q. laevies*) in 1986 in Florida (Golden and Kaplan, 1986), the only locality where it is known to occur. Turkey oak is a deciduous plant native to the Atlantic and Gulf Coastal Plains of the United States, including southeastern Virginia, southern Florida, and southeastern Texas. Three root-knot nematode species, *M. christiei*, *M. querciana* (Golden, 1979), and *M. partytila* (Brito et al., 2013) have been reported as pathogens of *Quercus* spp. Nonetheless, only *M. christiei* and *M. partytila* have been found infecting turkey oak and laurel oak (*Q. laurifolia*), respectively, in Florida. The root galling induced by *M. christiei* resembles nodule-like galls, which occur individually or in clusters (two to five) on one side of the roots and without adjacent swelling. *Meloidogyne christiei* establishes a unique host–parasite relationship by inducing the formation of spiral ducts in the parenchyma apparently during the migration toward the vascular tissue, which open to the rhizosphere, and by the presence of tubular, coiled egg masses. Ducts are lined with several layers of cork cells where the gelatinous matrix, eggs, second-stage juveniles, and males may be found (Golden and Kaplan, 1986).

Isozymes, particularly EST and MDH (Dickson et al., 1970; Esbenshade and Triantaphyllou, 1985) have been employed for identification and characterization of many root-knot nematode species in several laboratories around the world (Fargette, 1987; Pais and Abrantes, 1989; Carneiro et al., 1996; Xu et al., 2004; Cofcewicz et al., 2005; Brito et al., 2008, 2010); however, it is life stage dependent, only egg-laying females can be used. DNA-based methods, which can be performed with any nematode life stage have been used in combination with isozyme analyses to aid in the identification and study of the phylogenetic relationships among the root-knot nematode species (Blok and Powers, 2009). DNA markers, that have proved to be useful in such studies include the mtDNA marker, a region of variable size in *Meloidogyne* between the mitochondrial COII and the large (16S) rRNA gene (Powers and Harris, 1993; Tigano et al., 2005), the small subunit 18S rRNA gene (De Ley et al., 2002; Tigano et al., 2005), the large subunit 28S D2-D3 expansion segments of the rRNA gene (Tenente et al., 2004), and the ITS of rRNA gene (Landa et al., 2008). Since its description in 1986, *M. christiei* has not been reported infecting any other host or location, therefore little information is available other than that provided in the original description. The objectives of this study were to (i) molecularly characterize *M. christiei* topotype population using isozyme profiles and ribosomal and mitochondrial gene sequences and (ii) reconstruct the phylogenetic relationships between *M. christiei* with other *Meloidogyne* spp.

Materials and Methods

Nematode origin: All nematode isolates, except *M. christiei*, used in this study belong to the root-knot nematode collection, Division of Plant Industry, DPI/FDACS, Gainesville, FL. Topotype specimens of *M. christiei* were collected from infected roots of Turkey oak growing in Sanlando Park, Altamonte Springs, FL. Prior to the molecular analyses, nematode species was confirmed using morphology of selected characters of the second-stage juveniles (J2) (body, stylet, tail, and hyaline tail terminous length), perineal patterns of females, and examination of both unique root galling and presence of the coiled egg masses.

Isozyme analyses: Egg-laying females (13 females in duplicate test) were dissected directly from infected oak roots and a single specimen was placed in a 0.6-ml microfuge tube containing 5 µl deionized water and an

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TABLE 1. GenBank accession numbers for gene sequences of *Meloidogyne* species used in this phylogenetic analysis.

Species	Gene fragment			
	18S rRNA	D2-D3 28S rRNA	ITS rRNA	COII-16S rRNA
M. arabicida	AY942625	KF993624	-	AY942852
M. ardenensis	AY593894	-	-	-
M. arenaria	AY942623	JX987332	AF387092	AY635610
M. artiellia	KC875391	AY150369	KC545880	-
M. baetica	-	AY150367	AY150366	-
M. camelliae	-	KF542869	JX912885	JX912887
M. chitwoodi	AY593884	AF435802	AY281852	JQ041535
M. christiei	KR082316	KR082317	KR082319	KR082320
M. coffeicola	HE667739	-	-	-
M. cruciani	HE667740	-	-	-
M. dunensis	-	EF612712	EF612711	-
M. duytsi	KJ636385	-	-	-
M. enterolobii	AY942629	KJ146862	KM046989	AJ421396
M. ethiopica	KC551945	KF482372	KF482366	AY942848
M. exigua	AY942627	AF435795	-	HQ709105
M. fallax	AY593895	KC241969	AY281853	JN241952
M. floridensis	AY942621	-	-	DQ228697
M. graminicola	KF201168	KJ728847	KM111531	KM111533
M. graminis	JN241838	JN019326	JN157866	JN241898
M. hapla	AY942628	DQ145641	EU908052	KP306538
M. haplanaria	-	-	-	KP001589
M. hispanica	HE667741	EU443607	EU443613	JN673274
M. ichinohei	KJ636350	EF029862	-	-
M. incognita	AY284621	JX100425	KJ739707	KP306535
M. inornata	-	KF482374	KF482368	-
M. izalcoensis	HE667743	KF993621	-	-
M. javanica	AY268121	KC953092	KJ739709	KC287197
M. konaensis	HE667744	AF435797	-	-
M. kralli	KJ636370	-	-	-
M. lopezi	KF993642	KF993616	-	KF993629
M. luci	-	KF482371	KF482365	-
M. mali	KJ636400	KF880398	JX978228	KC112913
M. maritima	EU669944	-	-	-
M. marylandi	JN241856	JN019333	JN157854	KC473863
M. microtyla	AF442198	-	-	-
M. minor	AY593899	JN628436	KC241953	-
M. morocciensis	AY942632	-	-	AY942849
M. naasi	AY593900	KC241979	KJ934132	JN241897
M. oryzae	AY942631	-	-	-
M. panyuensis	-	-	AY394719	-
M. paranaensis	AY942622	AF435799	-	AY942851
M. partityla	-	-	-	AY672412
M. silvestris	-	EU570214	EU570216	-
M. spartelensis	-	KP895293	KP896294	KP895297
M. spartinae	EF189177	-	-	-
M. thailandica	-	EU364890	AY858795	EU364883
M. trifoliophila	-	AF435801	JX465593	-
Meloidogyne sp.	EU669950	-	-	-
P. vulnus	KC875389	EU130885	FJ713011	NC_02043
P. penetrans	KJ934156	EU130860	FJ712971	-

equal volume of sample buffer (BioRad, Hercules, CA). Individual females were macerated, and 10 µl of the extract was transferred into each well of a polyacrylamide gel with a 4% stacking (pH = 6.8) and 8% separating gel (pH = 8.8) with tris-glycine buffer (pH = 8.3) in the BioRad Mini-protean III (BioRad). Extracts from two *M. javanica* females (N05-1404-20B) were added separately to individual wells on each gel as standards. One set (two gels) was submitted to polyacrylamide gel electrophoresis at the same time. Electrophoresis was carried as previously reported (Brito et al., 2004). One gel was stained for both MDH and EST, whereas the second one was stained only for EST (Esbenshade and Triantaphyllou, 1985). Relative mobility of isozymes was calculated (Esbenshade and Triantaphyllou, 1985; Brito et al., 2008), and phenotype designations were assigned according to Esbenshade and Triantaphyllou (1985). Because no EST activity was observed when using extract from a single female, the number of specimens for each well was increased to 20 females. A total of 120 females was used in a duplicate test, totaling 240 female examined.

Fig. 1. Roots collected from Turkey oak infected with *Meloidoygne christiei*. Note the typical root-galling symptom, known as a nodule-like galls, and clusters on one side of the roots.

Molecular analyses: DNA was extracted from single females (at least 13 females from each nematode species) or from 5 to 15 specimens of second-stage juveniles from one isolate of each, *M. arenaria* (N06-543-13B), *M. christiei*, *M. enterolobii* (N01-514-3B), *M. floridensis* (N03-01894), *M. graminis* (N06-100-31B), *M. graminicola* (N03-540-9B), *M. hapla* (Y11-22-4B), *M. incognita* (N04-635-2B), *M. javanica* (N05-1404-20B), and *M. partityla* (N08-1319) using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Protocols of polymerase chain reaction (PCR) for rRNA genes and sequencing were described by Tanha Maafi et al. (2003). The PCR reaction for amplification of mtDNA was performed in 25 µl containing two units of *Taq* PCR Master Mix kit (Qiagen), 1.5 µl of each primer (10 pmol), 6.5 µl of nuclease-free water (Sigma, St. Louis, MO), and 3 µl of DNA. Amplifications were performed in a PTC-100 Programmable Thermal Cycler (MJ Research, Watertown, MA) as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing for 1 min at 57°C, and extension at 72°C for 1.30 min.

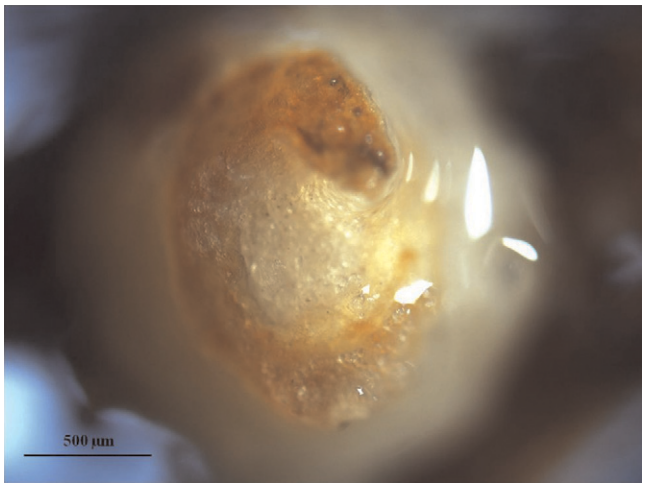

Fig. 2. A close-up view of the tubular and coiled egg mass produced by *Meloidogyne christiei* in Turkey oak (*Quercus laevies*).

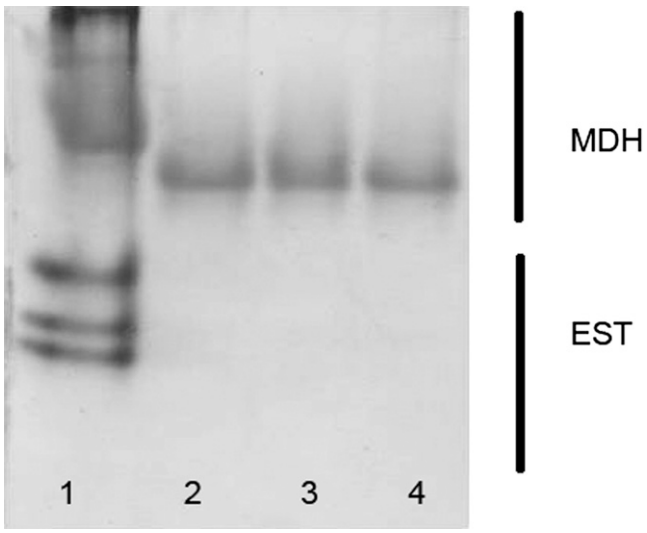

Fig. 3. Malate dehydrogenase (MDH) and esterase (EST) patterns. Lane1: macerate from single female of *Meloidogyne javanica* (MDH = N1 and EST = J3 phenotypes) (control); and lanes 2 to 4: macerate from single female of *M. christiei* (MDH = N1a phenotype).

The following primer sets were used for amplification: partial 18S rRNA gene with forward G18SU (5′-GCT TGT CTC AA GAT TAA GCC-3′) and reverse R18Tyl1 (5′-GGT CCA AGA ATT TCA CCT CTC-3′) primers (Chizhov et al., 2006); D2-D3 expansion segments of 28S rRNA gene with forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and reverse D3B (5′ -TCG GAA GGA ACC AGC TAC TA-3′) primers (Castillo et al., 2003); ITS of rRNA with forward F194 (5′-CGT AAC AAG GTA GCT GTA G-3′) (Ferris et al., 1993) and reverse 5368 (5′-TTT CAC TCG CCG TTA CTA AGG-3′) primers (Vrain, 1993); and fragment between mitochondrial COII gene and the large (16S) rRNA gene with forward C2F3 (5′-GGT CAA TGT TCA

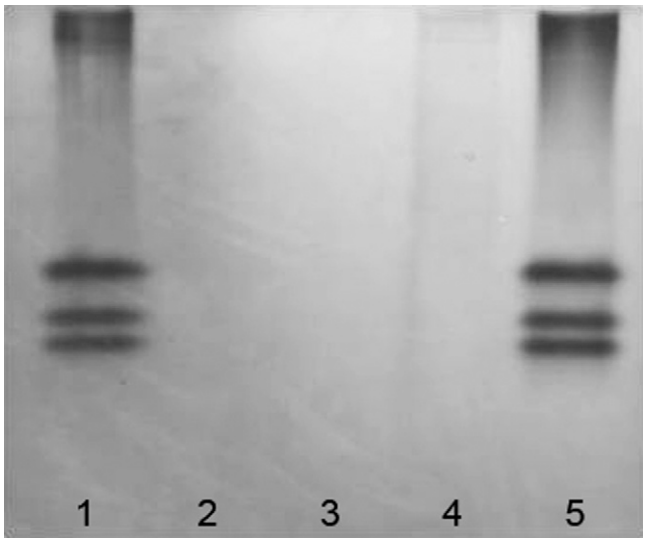

Fig. 4. Esterase analysis using macerate from 20 females of *Melodiogyen christiei*. Lanes 1 and 5 = single females of *M. javanica* (control) per well; lanes 2 to 4 = macerate of 20 females of *M. christiei* per well.

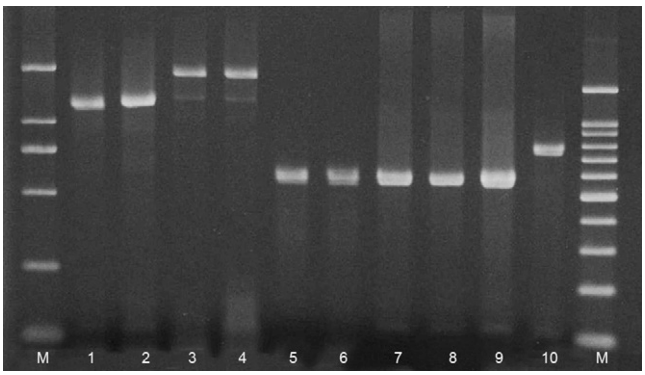

Fig. 5. Amplification products generated from individual females with primer set 1108 and C2F3. Lane 1 = *M. arenaria*; lane 2 = *M. floridensis*; lane 3 = *M. incognita*; lane 4 = *M. javanica*; lane 5 = *M. hapla*; lane 6 = *M. partityla*; lane 7 = *M. graminis*; lane 8 = *M. graminicola*; lane 9 = *M. christiei*; lane 10 = *M. enterolobii*; lane M = 100-bp ladders.

GAA ATT TGT GG-3′) and reverse 1108 (5-TAC CTT TGA CCA ATC ACG CT-3) primers (Powers and Harris, 1993). PCR products of D2-D3 of 28S rRNA and ITS rRNA genes were purified using QIAquick (Qiagen) gel extraction kits and then cloned using pGEM-T Vector System II kit (Promega, Madison, WI). Sequences of one or two clones for these fragments were obtained. New sequences of *M. christiei* were deposited in GenBank under the following accession numbers: KR082316 to KR082320.

The new sequences of 18S rRNA, D2-D3 of 28S rRNA, ITS rRNA, and mtDNA genes were aligned using ClustalX 1.83 (gap opening – 5; gap extension – 3) with their corresponding published gene sequences of *Meloidogyne* species (De Ley et al., 2002; Castillo et al., 2003, 2009; Tenente et al., 2004; Powers et al., 2005; Tigano et al., 2005; Holterman et al., 2009) and others (Table 1). Outgroup taxa for each data set were chosen according to the results of previously published data (Castillo et al., 2003; Holterman et al., 2009; Kiewnick et al., 2014). Alignments for each gene fragment and an alignment containing all genes were separately analyzed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) and maximum likelihood using Garli 2.01 (Zwickl, 2006). The best fit model of DNA evolution for each gene fragments was obtained using the program jModeltest 0.1.1 (Posada, 2008) under the Akaike information criterion. BI analysis was initiated with a random starting tree under the GTR + G model, which was considered as the best one for all datasets, and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities are given on appropriate clades.

Results

Isozyme analysis: The phenotype N1a detected from a single egg-laying female of *M. christiei* extracted from infected Turkey oak roots, exhibiting both sign and typical root symptoms induced by this nematode species (Figs. 1,2) showed one very strong band of MDH activity (Rm: 28.1) (Fig. 3); however, no EST activity was detected using macerate of one (Fig. 3) or even up to 20 females per well (Fig. 4). The species-specific EST phenotype J3 (Rm: 38.37, 40.69, and 44.18) and MDH phenotype N1 (Rm: 20.2) were found in the control, *M. javanica*, used in this study.

Molecular analysis: The PCR products of COII-16S rRNA were 1.2 kb for both *M. floridensis* and *M. arenaria*, 1.7 kb for *M. javanica*, and *M. incognita*. *M. hapla, M. partityla*, *M. graminis*, *M. graminicola*, and *M. christiei* produced a fragment of ca. 530 bp. *M. enterolobii* produced a fragment of ca. 705 bp (Fig. 5). The following sequence lengths of four gene fragments for *M. christiei* were obtained in this study: partial 18S rRNA = 753 bp; D2-D3 of 28S rRNA = 672 bp, 680 bp; ITS rRNA = 598 bp; COII-16S rRNA = 477 bp. Two sequences of D2-D3 of 28S rRNA gene clones were different in 36 bp (5.3%) from each other.

The combined sequence alignment contained 48 *Meloidogyne* species, two *Pratylenchus* species used as outgroups, which was 2,685 bp in length and consisted of the following gene fragments: partial 18S rRNA gene (36 *Meloidogyne* species, 812 bp), D2-D3 of 28S rRNA gene (33 species, 745 bp), ITS1 with 5.8S rRNA gene (27 species, 608 bp), and tRNA-His gene with partial 16S rRNA gene of mtDNA (26 species, 520 bp). Phylogenetic relationships within the genus *Meloidogyne* as inferred from BI analysis is given in Figure 6. Topologies of BI and ML trees were congruent. In both trees *M. christiei* formed a separate lineage within the crown group of *Meloidogyne*, and its relationships with any of three *Meloidogyne* clades are not resolved. Unresolved relationships of *M. christiei* with other *Meloidogyne* belonging to crown clades or any other *Meloidogyne* were observed in the separate analyses of partial 18S rRNA gene or tRNA-His gene with partial 16S rRNA gene of mtDNA.

Discussion

Numerous biochemical studies have demonstrated that most *Meloidogyne* species can be differentiated by nonspecific EST and malate enzyme phenotypes (Esbenshade and Triantaphyllou, 1985; Blok and Powers, 2009). However, no EST activity was observed in extracts from one single or even 20 egg-laying females of *M. christiei* in this study. Very low EST activity has been observed in *M. haplanaria* (Eisenback et al., 2003), three weak EST bands in *M. floridensis* (Brito et al., 2008) and only a "smear" of EST activity has been detected in *M. graminicola* (Esbenshade and Triantaphyllou,

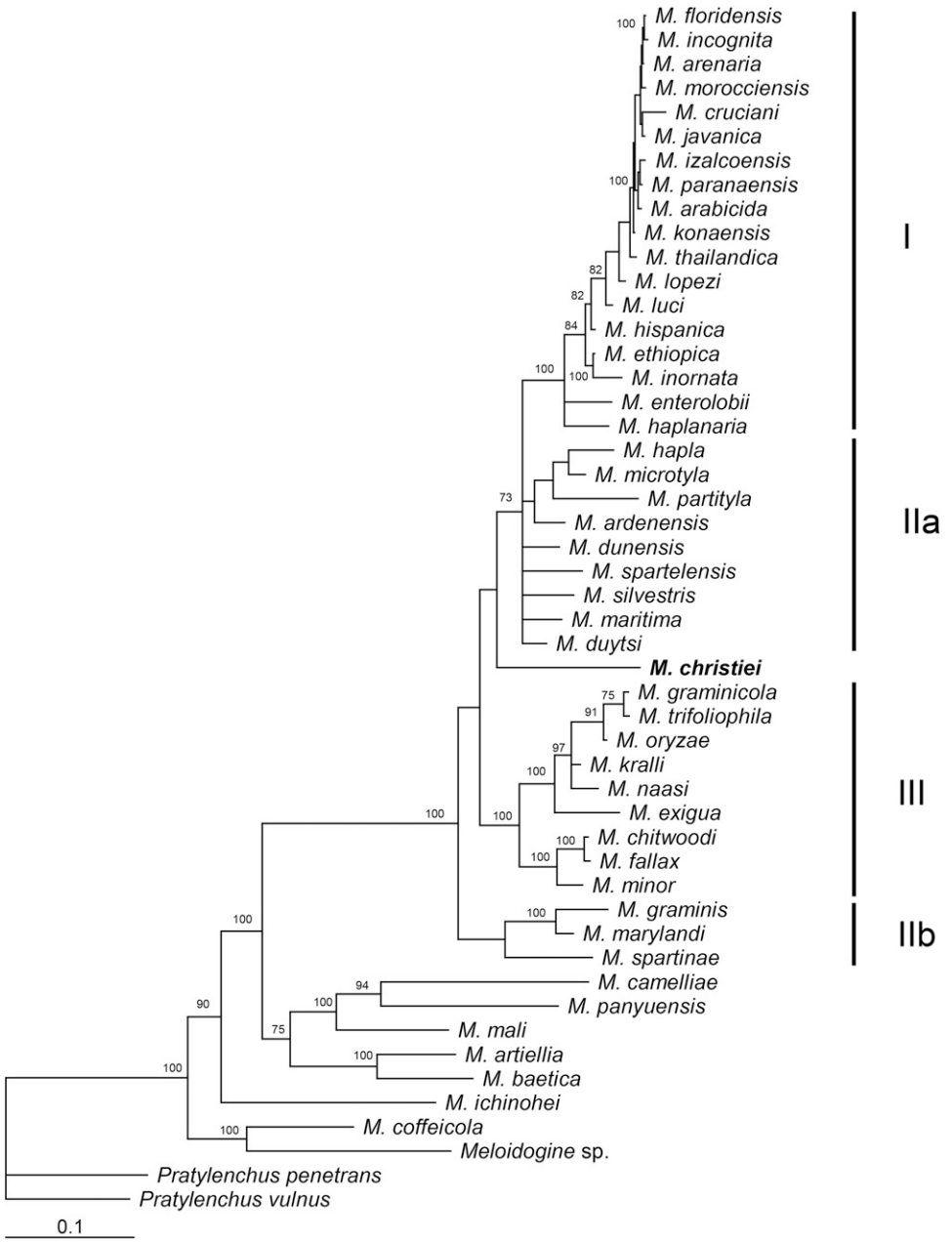

FIG. 6. Phylogenetic relationships between *Meloidogyne christiei* and other *Meloidogyne* species: Bayesian majority rule consensus tree reconstructed under the GTR + G model and inferred from the combined sequence alignment (partial 18S rRNA gene, D2-D3 of 28S rRNA gene, ITS1 with 5.8S rRNA gene, and tRNA-His gene with partial 16S rRNA gene of mtDNA). Posterior probabilities more than 70% are given on appropriate clades. Root-knot nematode grouping of the crown *Meloidogyne* clade are given according to Holterman et al. (2009).

1985; Brito et al., 2008). Despite of being used for root-knot nematode identifications in many laboratories and countries around the world, very little is known about the gene(s) coding for EST in *Meloidogyne* spp., and the lack of EST activity detection in *M. christiei* even when using 20 females in the current study, needs to be further investigated. Nonetheless, the *ges-1* gene, which codes for nonspecific EST in *Caenorhabditis elegans* and known to be expressed in its gut has been cloned and characterized (Kennedy et al., 1993). This findings could serve as a paradigm to study homolog gene(s), if any present in *M. christiei*.

One strong band of MDH activity, N1a phenotype, was detected from a single egg-laying female of *M. chritiei* and one strong band N1 as expected in the control, *M. javanica*. This same MDH phenotype, N1a, found in the population of *M. christiei* has been reported also in other *Meloidogyne* spp. occurring in Florida and other parts of the world, including several populations of *M. enterolobii* from Africa (Fargette et al., 1996), Brazil (Carneiro et al., 2001), China (Yang and Eisenback, 1983), Florida (Brito et al., 2004; 2008), and Martinique (Carneiro et al., 2000). This phenotype also was reported in four populations of *M. chitwoodi* and

one population each of *M. enterolobii*, *M. graminicola*, *M. naasi*, *M. oryzae*, and *M. platani* (Esbenshade and Triantaphyllou, 1985). These findings indicate that MDH phenotype has low specificity and of limited diagnostic value to distinguish *M. christiei* from those nematode species.

The amplification of mtDNA with the C2F3 and 1108 primer set yielded a fragment of approximately 530 bp, which makes *M. christiei* undistinguishable using a PCR product size from several other *Meloidogyne* species, including *M. partityla* known to infect oaks in Florida (Brito et al., 2013), *M. graminis*, and *M. graminicola*. However, the sequence of COII-16S rRNA is quite different from any other presently sequenced *Meloidogyne* species. The phylogenetic tree obtained in the results of analysis of the combined alignment containing four gene fragments and more species numbers, than any previous publications, is generally congruent with several other published *Meloidogyne* phylogenies (De Ley et al., 2002; Castillo et al., 2009; Holterman et al., 2009; Kienwnick et al., 2014). The analysis places *M. christiei* in a separate lineage within the crown *Meloidogyne* group or the group of the clades I, II, and III according to De Ley et al. (2002), Holterman et al. (2009), and Kiewnick et al. (2014) and shows that this species has a unique evolutionary history.

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