Occurrence of *Meloidogyne fallax* in North America, and Molecular Characterization of *M. fallax* and *M. minor* from U.S. Golf Course Greens

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Abstract

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Several species of root-knot nematodes (*Meloidogyne* spp.) are known to have significant presence on turfgrass in golf course greens, particularly in the western United States. Nematodes isolated from a golf course in King County, WA were identified as *Meloidogyne minor* based on analysis of the large ribosomal subunit (LSU 28S D2-D3 expansion segment), the internal transcribed spacers 1 and 2 (ITS rDNA), the intergenic spacer region 2 (IGS2), and the nuclear protein-coding gene Hsp90. Sequence-characterized amplified region (SCAR) primers that were originally designed to be specific for *M. fallax* were found to cross-react with *M. minor*. A population from California was determined to be *M. fallax* based on juvenile tail morphology and analysis of the ribosomal markers and Hsp90, comprising the first report of this species in North America. Using trees based on Hsp90 genomic alignments, the phylogenetic relationships of these populations and known root-knot nematode species were congruent with previous trees based on ribosomal genes. Resolution of *M. fallax* and *M. chitwoodi* using Hsp90 was equivalent to species separation obtained with 28S or 18S rDNA alignments. The strengths and weaknesses of ribosomal and Hsp90 markers, and the use of SCAR polymerase chain reaction as diagnostic tools are discussed.

In recent years, root-knot nematodes (*Meloidogyne* spp.) have emerged as an important problem on golf course greens in the western United States. *Meloidogyne minor* Karssen et al., 2004 has been associated with yellow patch disease on creeping bent grass (*Agrostis stolonifera* var. *stolonifera* L.) (15). This nematode has been found in the U.K., the Netherlands, and Belgium, occurring in pastures, golf courses, football pitches, and dunes (17,27,30). Within the Western Hemisphere, *M. minor* was first discovered on golf course greens in the state of Washington in 2010 (19). In greenhouse tests, *M. minor* reproduced on several economically important crops, including potato (*Solanum tuberosum* L.), alfalfa (*Medicago sativa* L.), lettuce (*Lactuca sativa* L.), oat (*Avena sativa* L.), tomato (*Solanum lycopersicum* L.), vetch (*Vicia sativa* L.), Italian ryegrass (*Lolium multiflorum* Lamk.), and carrot (*Daucus carota* L.) (15). It failed to reproduce on marigold (*Tagetes patula* L.) and maize (*Zea mays* L.). A study in the Netherlands was conducted to evaluate the host range of several crops for *M. minor* and its damage potential in potato fields (26). Potato was the only crop that supported significant reproduction of *M. minor* in that final population densities exceeded that of initial population. However, annual ryegrass also supported population densities that were higher than the fallow treatment, although densities were low.

Meloidogyne fallax Karssen, 1996 (13) was initially detected in the Netherlands in 1992, first described as a variant population of *Meloidogyne chitwoodi* Golden et al., 1980 (28). It has since been found on potato in the Netherlands, France, New Zealand, Australia, and South Africa (7,11,18,21). *M. chitwoodi* is another nematode of importance on potato, particularly in the Pacific Northwest region of the United States, where it was first described in 1980 (8). This nematode has also been reported in Argentina, Belgium, Germany, the Netherlands, Portugal, Mexico, and South Africa (1,6,7,14,29,31). The damage caused by *M. minor* on potato is similar to that caused by the quarantine nematodes *M. chitwoodi* and *M. fallax*; therefore, it is also considered an organism of regulatory importance in the United States, Europe, and many other countries (1,5).

During a survey of golf course greens from 2008 to 2011, we previously identified five *Meloidogyne* species from seven western states: *M. chitwoodi*, *M. graminis*, *M. marylandi*, *M. minor*, and *M. naasi* (19). *M. fallax* was not found on the 238 golf courses included in that survey. Late in 2011, samples were received from golf courses in King County, WA and San Francisco County, CA containing infective juveniles (J2) that morphologically resembled *M. fallax*. Molecular analyses of those samples are described herein. The Washington isolate is here identified as *M. minor*. The California isolate is identified as *M. fallax*, representing the first report of this nematode within North America and the United States.

Materials and Methods

Nematodes were extracted from the soil under an intermittent mist for 72 h; J2 and males were collected and concentrated on a 500-mesh (25 μm) sieve. Males were included when present, since they are not frequently found and can be diagnostic for some species. Males and J2 were hand-picked for DNA sequencing and/or fixation for light microscopy. Juveniles for light microscopy were fixed in cold 4% formalin and 1% glutaraldehyde in 0.01 M phosphate buffer, pH 7.3, and stored at 4°C. Juveniles were then processed to anhydrous glycerin by the Seinhorst rapid method (23) and mounted on glass slides. Photomicrographs of J2 (Fig. 1) were taken with a Q-Imaging Color Digital Camera attached to a Leica Leitz DMRB compound microscope at ×1,000 magnification under an oil immersion objective, and measurements (Table 1) were made with an ocular micrometer on the same microscope.

Specimens for sequencing were placed in DESS (33) and stored at 4°C for up to a year. For molecular analysis, males and J2s were

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rinsed for 20 min in sterile distilled water, transferred individually into a 10-µl drop of sterile lysis buffer (10 mM Tris, pH 8.0, 0.25 M GuHCl, 0.25% Triton X-100, 0.25% Tween 20, 2.0 µl of Proteinase K: 934 units per milliliter) on a clean glass coverslip and cut open with a sharp scalpel blade (19). The lysis buffer with the two sections of the nematode was transferred to a sterile, 0.6-ml polymerase chain reaction (PCR) tube containing 30 µl of the same buffer. Lysis was completed by incubating the tubes at 60°C for 20 min followed by 10 min at 98°C to inactivate the Proteinase K.

Sequences of PCR primers used in this study are listed in Table 2. Sequence-characterized amplified region (SCAR)-PCR to distinguish *M. chitwoodi* from *M. fallax* included the *M. fallax*–specific primers Ff and Rf, or the *M. chitwoodi*–specific primers Fc and Rc, as described by Zijlstra (34). Additional molecular identification was based on analysis of four genes. Ribosomal (rRNA) markers examined were the large ribosomal subunit 28S D2-D3 expansion segment (LSU), amplified with primers D2A and D3B; the internal transcribed spacer region (ITS1-5.8S-ITS2), amplified with primers F195 and 5367; and the intergenic spacer (IGS2), amplified with primers 5S and 18S. A partial fragment of the gene encoding heat shock protein (Hsp90) was also analyzed. Amplification of the D2-D3 and ITS regions consisted of 5 µl 10× PCR buffer, 5 µl Q solution, 1 µl dNTPs (10 mM per nucleotide), 1 µl primer 1 (10 µM), 1 µl primer 2 (10 µM), 0.25 µl *Taq*, 31.75 µl nuclease-free water, and 5 µl of DNA in a reaction volume of 50 µl. For the 28S and ITS rRNA genes, cycling conditions were 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C

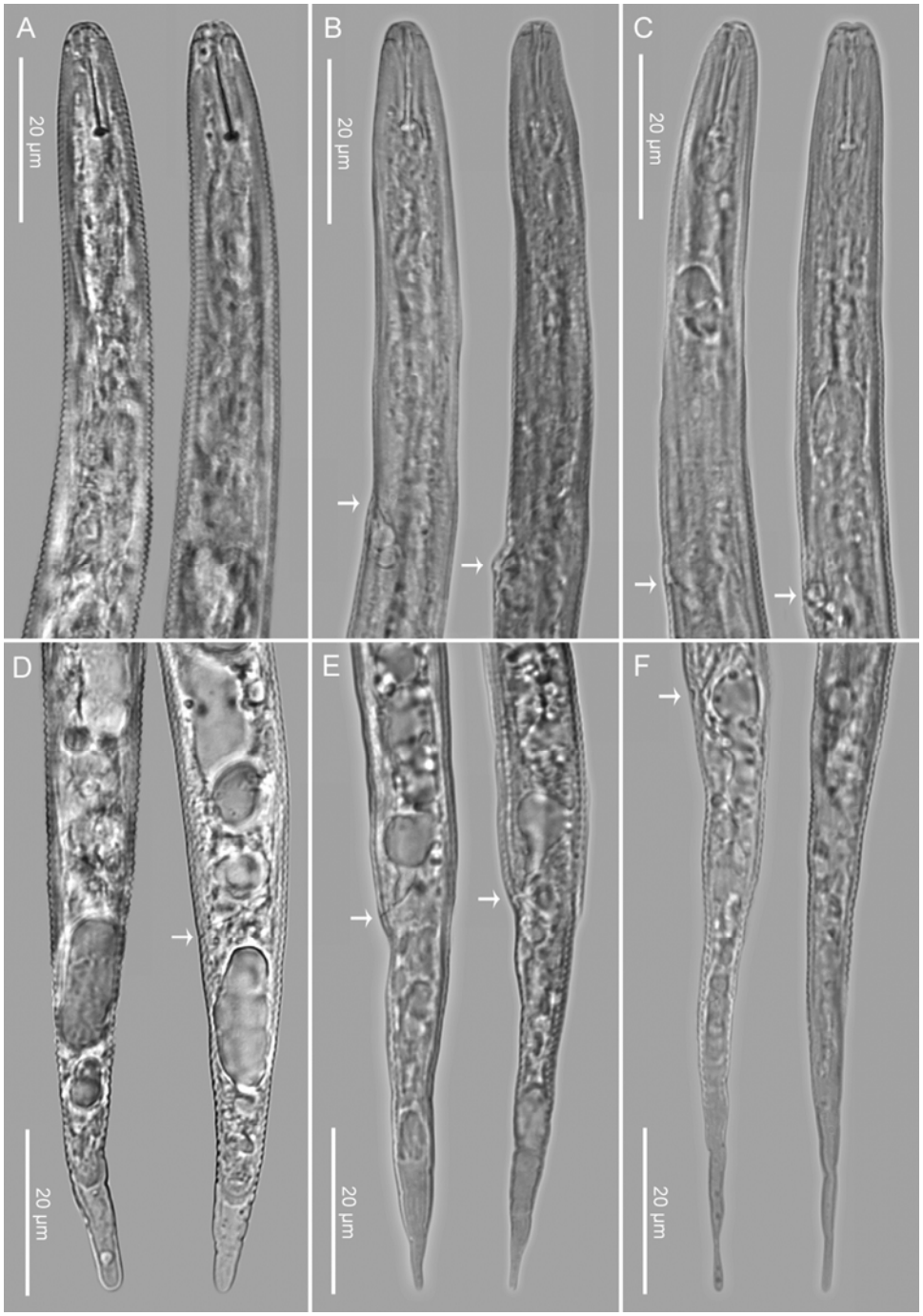

Fig. 1. Photomicrographs of second-stage juveniles of *Meloidogyne fallax, M. minor*, and *M. naasi*. **A,** *M. fallax* anterior regions showing stylet and part of esophagus. **D,** Tails with arrow showing anus. **B and E,** *M. minor* from Echo Falls, WA: **B,** anterior regions showing stylet and part of esophagus with arrows showing excretory pores; **E,** tails with arrows showing anal areas. **C and F,** *M. naasi* from Echo Falls, WA: **C,** anterior regions showing stylet and part of esophagus with arrows showing excretory pores; **F,** tails with arrow showing anus.

for 1 min, with a final extension at 72°C for 10 min. PCR products were visualized on a 1.0% agarose gel stained with ethidium bromide. PCR reactions for amplification of the IGS2 region consisted of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primer 5S, 0.5 µM primer 18S, 1 U Platinum *Taq*, 3 µl DNA extract, and 1× *Taq* enzyme buffer in a reaction volume of 25 µl. Cycling conditions were 94°C for 2 min, followed by 45 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 90 s, ending with a final extension at 72°C for 7 min (2). Amplification of Hsp90 consisted of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primer RKN-d1F, 0.5 µM primer RKN-5R, 1 U Platinum *Taq*, 3 µl DNA extract, and 1× *Taq* enzyme buffer in a reaction volume of 25 µl. Cycling conditions were 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 55°C for 20 s, and 68°C for 90 s with a final extension at 68°C for 5 min. PCR products for 28S and ITS were extracted from the agarose gels with the QiaQuick Gel extraction kit (Qiagen, Valencia, CA) following the manufacturer's protocol, and the samples were sequenced at the University of Arizona Genetics Core Facility. IGS2 and Hsp90 products were sequenced at the University of Maryland Center for Biosystems Research. New sequences were submitted to GenBank under the accession numbers KC262220–KC262264 (Table 3).

All sequences were compared with GenBank entries using BLASTn and aligned with sequences of authenticated specimens of *Meloidogyne* species (19). Multiple sequence alignments were performed in Geneious v. 6.0 (Biomatters Ltd., Auckland, NZ) using the MAFFT algorithm (16). Neighbor-joining phylogenetic trees for Hsp90 were made from the consensus alignment of 1,074 bp using the Tamura-Nei (TN) model for genetic distance, with *Meloidogyne hapla* (AY528416, AY528417) as the outgroup. Trees were resampled with 1,000 bootstrap replicates. Maximum parsimony trees were constructed in PAUP v 4.0d125, employing tree bisection-reconnection (TBR) branch swapping, and accelerated transformation (ACCTRAN) character state optimization with 1,000 bootstrap replicates.

Results

Molecular characterization of *M. minor* from King County, WA. Preliminary identification of the population from King County, WA as *M. fallax* was based on morphology of the infective J2. Subsequently, PCR of a SCAR was performed to confirm the morphological diagnosis (34). Using DNA extracted from J2 of the Washington isolate, amplification with *M. fallax*–specific SCAR primers Ff and Rf yielded a 530-bp product (Fig. 2A, lanes 2 to 7 and 2B, lanes 7 to 10), while no amplification occurred with the *M. chitwoodi*–specific primers Fc and Rc (not shown). Primers Ff and Rf were also tested in PCR with J2 from an authenticated population of *M. minor* (Fig. 2B, lanes 2 to 6); this reaction yielded the same sized product that was obtained from authenticated *M. fallax* (Fig. 2A, lane 9). As expected, SCAR PCR with the *M. fallax*–specific primers yielded no products when performed with J2 DNA from authenticated populations of *M. naasi, M. chitwoodi, M. graminicola, M. graminis,* and *M. marylandi* (data not shown).

Due to the unexpected results obtained from SCAR PCR, further molecular sequencing was undertaken to identify the Washington isolate. The 28S rRNA D2-D3 expansion segment was amplified from males and juveniles; these PCR products were subjected to direct sequencing and aligned with those from sequences from *M. minor, M. fallax,* and *M. chitwoodi.* The Washington isolate 28S was nearly identical to six other *M. minor* sequences (JN019322, JN019323, JN019324, JN628436, JN157846, and JN628437) differing at 0 to 2 bp out of 609 bp aligned (≥99.3% identical). The sequences were clearly distinct from *M. fallax* (JN157848 and FN429017), differing at 39 to 40 bp (6.4 to 6.5%), and from *M. chitwoodi* (JN019321 and AF435802), differing at 42 to 47 bp (6.9 to 7.4%). Sequence for the internal transcribed spacer region (ITS1 and 2) of rRNA from the Washington population was also consistent with a diagnosis of *M. minor*. A trimmed 557-bp alignment with other *M. minor* ITS sequences (GU432775, AY281855,

Table 1. Morphometrics of infective second-stage juveniles of *Meloidogyne fallax*, *M. minor*, and *M. naasi*[a]

Characters	*M. fallax* *n* = 11	*M. minor* *n* = 21	*M. naasi* *n* = 20
Linear (µm)			
Body length	363.7 ± 9.7 (350-381)	323.5 ± 18.8 (300-361)	372.4 ± 13.5 (340-396)
Maximum body width	15.3 ± 0.5 (15-16)	11.9 ± 0.8 (10-13)	12.5 ± 1.1 (10.5-14.0)
Stylet length	10.3 ± 0.5 (10-11)	9.7 ± 0.5 (9-10)	12.3 ± 0.4 (11-13)
Esophagus length[b]	122.4 ± 6.1 (110-135)	119.8 ± 10.7 (100-139)	126.9 ± 7.0 (115-140)
Body width at anus	10.6 ± 0.5 (10-11)	8.7 ± 0.8 (7.5-10)	8.9 ± 0.9 (7.5-10)
Tail length	47 ± 4.0 (42-55)	47.1 ± 3.7 (40-51.5)	68.8 ± 3.4 (61.5-75)
Hyaline tail terminus length	12.2 ± 1.3 (10.5-15)	14.8 ± 1.5 (12-17.5)	23.6 ± 2.0 (20-27)
Ratios			
a	23.6 ± 1.1 (21.8-25.4)	27.3 ± 2.0 (23.2-32.5)	30.1 ± 2.7 (25.4-35.7)
b	3 ± 0.2 (2.8-3.3)	2.7 ± 0.4 (2.2-3.3)	2.9 ± 0.2 (2.5-3.2)
c	7.7 ± 0.6 (6.5-8.6)	6.9 ± 0.4 (6.4-7.6)	5.4 ± 0.3 (5.1-6.2)
c′	4.4 ± 0.3 (4.1-5)	5.5 ± 0.7 (4.2-6.4)	7.8 ± 0.8 (6.2-9.3)

[a] Mean ± standard deviation (range). Measurements in µm were made with an ocular micrometer, taken with a Leica Leitz DMRB compound microscope at ×1,000 magnification under an oil immersion objective.
[b] From lips to base of glands.

Table 2. Primers used for polymerase chain reaction and DNA sequencing

Primer	Marker	Sequence (5′ to 3′)	Reference
Ff	SCAR	CCAAACTATCGTAATGCATTATT	Zijlstra (34)
Rf	SCAR	GGACACAGTAATTCATGAGCTAG	Zijlstra (34)
Fc	SCAR	TGGAGAGCAGCAGGAGAAAGA	Zijlstra (34)
Rc	SCAR	GGTCTGAGTGAGGACAAGAGTA	Zijlstra (34)
D2A	28S	ACAAGTACCGTGAGGGAAAGTTG	De Ley et al. (3)
D3B	28S	TCGGAAGGAACCAGCTACTA	De Ley et al. (3)
5367	ITS1-2	TTGATTACGTCCCTGCCCTTT	Schmitz et al. (22)
F195	ITS1-2	TCCTCCGCTAAATGATATG	Schmitz et al. (22)
5S	IGS2	TTAACTTGCCAGATCGGACG	Blok et al. (2)
18S	IGS2	TCTAATGAGCCGTACGC	Blok et al. (2)
RKN-d1F	Hsp90	GCYGATCTTGTYAACAACCYTGGAAC	This study
RKN-5R	Hsp90	TCGAACATGTCAAAAGGAGC	This study

JN157871, JN157867, EU881694, EU881695, EU881696, and EU878542) showed >99% identity (variation at 0 to 2 bp among all sequences), whereas the Washington population ITS sequence showed 41 to 43 bp divergence (7.7 to 9%) from *M. fallax* ITS (EU252015–EU252017, GU433344, JN157869).

Molecular characterization of *M. fallax* from San Francisco County, CA. Morphologically, J2 from the San Francisco County, CA golf course resembled *M. fallax*. Comparison of this population with *M. minor* revealed slight differences in the tails between isolates (Fig. 1 and Table 1). Seven individual J2s each from two separate samples were subjected to molecular analysis. Comparison of the 28S D2-D3 region with sequences from GenBank (noted above) revealed near identity to others from *M. fallax* (0 to 1 bp difference), 8 to 13 bp divergence from *M. chitwoodi* (1.3 to 1.9%), and 39 to 42 bp divergence from *M. minor* (6.4 to 7.1%). Alignment of eight ITS sequences obtained for this population showed >99% similarity with five others for *M. fallax* ITS, 17 to 18 bp differences from *M. chitwoodi* (3.3 to 3.9%) and 40 to 43 bp differences from *M. minor* (10.5 to 13.9%). Testing with the SCAR primers specific for *M. fallax* amplified the expected 515-bp band, but there was no amplification with the *M. chitwoodi*–specific primers (not shown). These molecular results confirm the diagnosis of *M. fallax* and comprise the first report of this species in North America.

Analysis of the ribosomal intergenic spacer region (IGS2) from *Meloidogyne* spp. Amplification of the IGS2 yielded fragments of 1,600 bp from the California isolate, 1,610 bp from authenticated *M. fallax*, 1,420 bp from *M. chitwoodi*, and 1,500 bp from *M. minor*. Alignment of four sequences from the California population showed greatest (98.9%) similarity to *M. fallax* GQ395584 and the sequence from the Netherlands population. Several other *M. fallax* sequences from GenBank were slightly less similar to the California isolate (98.7 to 98.8%). The major difference between the four sequences obtained from the California isolate and other *M. fallax* IGS was a 30-bp region near the 3′ end; the California sequences contained a 6-bp deletion and several base changes compared to *M. fallax* sequence GQ395575 and others. IGS sequences from *M. chitwoodi* reference populations included in this study were ≥99.6% identical to AF013992 and 25 others used for comparison. *M. minor* IGS showed the greatest amount of intraspecific and interspecific variation. Sequences were obtained from three populations from the U.K. and one from the Netherlands. Pairwise similarities ranged from 94.8 to 99.6%. Again, the major differences among sequences were limited to one area of the sequence; in this case, localized to a 75-bp region starting at base 1270. Sequence from the Lough Erne *M. minor* population had a deletion of 24 bp relative to those from Monkston and Dunmurry Springs; the Netherlands sequence had a 51-bp insertion. Obtaining a clear alignment of these *M. minor* sequences with those from *M. fallax* and *M. chitwoodi* was difficult, as there were many base changes and indels among them.

Molecular and phylogenetic analysis based on Hsp90 genomic DNA. A partial fragment of the protein-coding gene Hsp90 was amplified from the Washington *M. minor* and California *M. fallax* populations and from reference populations of *M. fallax, M.*

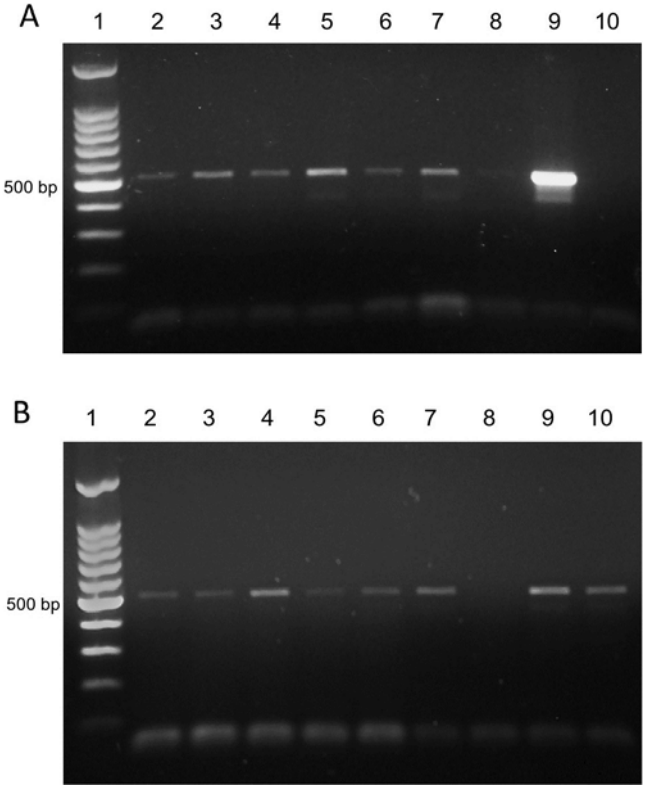

Fig. 2. Sequence-characterized amplified region polymerase chain reaction (SCAR PCR) with *Meloidogyne fallax*–specific primers with template from *M. minor*. **A,** Lane 1: DNA ladder; lanes 2–8: *M. minor* from Washington; lane 9: *M. fallax* control; lane 10: negative control. **B,** Lane 1: DNA ladder; lanes 2–6: *M. minor* control; lanes 7–10: *M. minor* from Washington.

Table 3. New molecular sequences obtained from *Meloidogyne minor* and *M. fallax* from golf course turf grass and from *Meloidogyne* spp. reference populations

			GenBank accession number			
Species	**Pop.**	**Origin**	**28S D2-D3 rDNA**	**ITS-rDNA**	**IGS2**	**Hsp90**
Meloidogyne chitwoodi	13B	Washington, USA	KC241980-KC241981		KC262254-KC262255	KC262220-KC262224
	10I6	Idaho, USA			KC262253	
M. fallax	HH	Netherlands				
	LW	Netherlands			KC262264	KC26225-KC262228
	824	California, USA	KC241962-KC241968	KC241954-KC241961		
	853	California, USA	KC241969-KC241975		KC262260-KC262263	KC262229-KC262233
M. minor	202	United Kingdom			KC262256	KC262242
	204	United Kingdom			KC262257-KC262258	KC262238-KC262241
	LW	Netherlands			KC262259	KC262234-KC262237
	820	Washington, USA	KC241976-KC241978	KC241953		KC262243-KC262246
M. naasi	12A5	Washington, USA	KC241979			KC262247-KC262252

minor, M. chitwoodi, and *M. naasi.* For *M. minor* Hsp90, intraspecific variation ranged from 1 to 4 bp (≥99.5% identity). Conversely, separation between *M. minor* and *M. fallax* was clear, with the pairwise divergences ranging from 12.5 to 13%. Four cloned Hps90 sequences representing a population of *M. chitwoodi* were ≥99.2% identical to each other, and the divergence between *M. fallax* and *M. chitwoodi* Hsp90 ranged from 3.5 to 3.9%. Four Hsp90 sequences obtained from the Washington population were closest to *M. minor,* diverging at 0 to 11 bp (0 to 1.3%) across all sequences. Four Hsp90 sequences from the California population were most similar to those from the Netherlands population of *M. fallax*, with variation ranging from 0 to 5 bp among all sequences. Neighbor-joining trees were constructed in Geneious from a genomic sequence alignment of 1,074 characters using the Tamura-Nei genetic distance model, with *M. hapla* as the outgroup and 1,000 bootstrap replicates (Fig. 3; support values shown above branches). Maximum parsimony analysis was performed for an alignment of 1,074 total characters, of which 320 were parsimony informative. Tree statistics generated in PAUP* were: tree length = 435; consistency index = 0.931034; retention index = 0.972248. Parsimony bootstrap support values are shown below the branches in the distance tree.

Discussion

Females of root-knot nematodes are seldom retrieved from soil samples from turfgrass, and none were available for this study. Initial diagnosis was based on morphology of the J2. Both *M. fallax* and *M. minor* J2 were found in greens, primarily *Poa annua* L. that contained *M. naasi* as the predominant species. *M. naasi* is easily distinguished from either *M. fallax* or *M. minor* on the basis of J2 morphology, its most distinctive characters being the shape and length of the tail (Fig. 1). Morphological differences between J2 of *M. fallax* and *M. minor* are more subtle and, in general, unsuitable for diagnostic purposes due to overlapping measurements of individual specimens (Table 1). Should *M. chitwoodi* also occur in mixed populations with *M. fallax*, diagnosis based on J2 morphology would be even more challenging.

While the Washington population resembled *M. fallax* based on juvenile tail morphology, clear discrimination from *M. minor* and *M. chitwoodi* often required molecular confirmation using either PCR or isozyme identification (11,12). The SCAR PCR assay designed by Zijlstra et al. (34) can distinguish *M. chitwoodi, M. fallax,* and *M. hapla* using species-specific pairs of primers that were based upon randomly amplified polymorphic DNA regions (RAPDs). This assay has become widely used, and is one of the molecular methods recommended by the European and Mediterranean Plant Protection Organization (EPPO) for identification of *M. fallax* and *M. chitwoodi* (1). The ability of *M. fallax*–specific primers to detect *M. minor* was not anticipated. However, the SCAR assay predates the description of *M. minor*, and there have been no published tests of the assay with this relatively new species. Therefore, the present results have important implications for diagnostic, research, and regulatory purposes. Sole reliance on the previously reported SCAR primers for the identification of *M. fallax*, a quar-

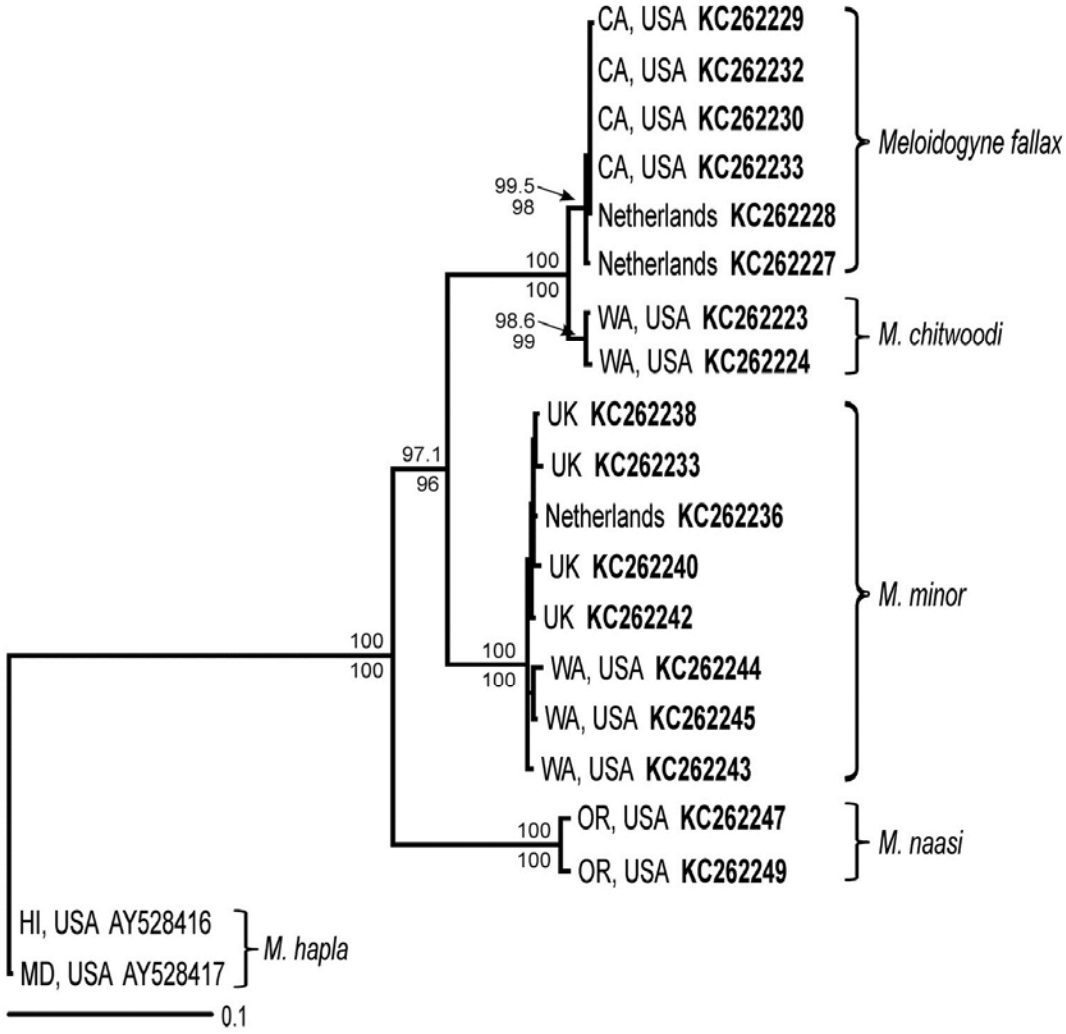

Fig. 3. Neighbor-joining 50% majority-rule consensus tree based on 1,074-bp alignment of Hsp90 genomic DNA sequences from selected *Meloidogyne* spp. Bootstrap values from 1,000 replicates are indicated above the branches; bootstrap support values from a maximum parsimony tree constructed for the same alignment (not shown) are indicated below the branches.

antined species in some countries, is not advisable if *M. minor* is suspected or known to be present.

Identification of the Washington isolate as *M. minor* was confirmed through analysis of the 28S D2-D3 expansion regions, which had a high degree of similarity with six known populations of *M. minor*, including others from Washington (U.S.), Belgium, and the U.K. Likewise, ITS rDNA showed near identity with sequences from populations originating from Washington, the Netherlands, and the U.K. Sequences previously reported for *M. minor* from Washington were also found on golf course turfgrass (19). The California population was identified as *M. fallax* based on 28S rDNA sequences, having near identity to populations of this species from Belgium and New Zealand. ITS rDNA sequences from the California population also show near identity to *M. fallax* sequences from Australia, Belgium, and New Zealand. The amount of intraspecific variation was always less than the interspecific variation, giving unambiguous distinction of *M. fallax* and *M. minor* based on either of these markers.

IGS2 sequences from the Washington and California populations were also consistent with the identification based on the other two ribosomal genes. However, a significant amount of intraspecific variation was detected in this marker, with indels occurring among sequences within both *M. fallax* and *M. minor*. IGS from the latter species was particularly variable among the populations examined from Washington, the Netherlands, and the U.K. The explanation for this variation is not clear, but it renders the IGS less reliable for identification of *M. minor* than either 28S or ITS.

Hsp90 has been increasingly included in phylogenetic studies of plant-parasitic nematodes (20,24,25) or used for species identification (9,25). Neighbor-joining and maximum parsimony trees based upon Hsp90 genomic DNA alignments from five species of root-knot nematodes agreed with the species relationships in more extensive 28S and 18S phylogenies. Moreover, the Hsp90 trees showed better separation of *M. chitwoodi* and *M. fallax* than those based on either 18S or mitochondrial DNA sequences (19). Variation within introns was higher than in coding regions, and first and second codon positions were generally more conserved than third codon positions. Phylogenies constructed from alignments of coding sequences only (introns removed) had branch support values that were the same or lower than the genomic alignment (not shown). The consistency of Hsp90 tree topologies with those based on ribosomal genes thus provides another option for phylogenetic analysis of the root-knot nematodes.

Many molecular diagnostic techniques have been developed for the identification of *M. fallax* and *M. chitwoodi*, including species-specific SCAR PCR (34), multiplex PCR based on ITS rDNA (7), size discrimination of IGS PCR products (32), and ITS-based real time TaqMan PCR (35). Most of these assays have not been re-examined using *M. minor*, so their specificity may or may not stand the test of time. More recently, Holterman et al. (10) employed high-resolution melting curve (HRMC) analysis based on IGS for identification of *M. chitwoodi*, *M. fallax,* and *M. hapla* and their distinction from tropical root-knot nematodes. These authors did not include *M. minor* in their study, and based upon the intraspecific variation we found in IGS from that species, HRMC of this species could result in false positives. The only test specifically developed for detection of *M. minor* was a real-time PCR assay based on ITS rDNA primers and a fluorescent TaqMan probe (4). The additional sequence information obtained from the U.S. and European populations of *M. minor* should facilitate the design of additional diagnostic tools or procedures for identifying this nematode. For instance, multiplex PCR for simultaneous discrimination of *M. minor*, *M. fallax,* and *M. chitwoodi* would be diagnostically useful for plant quarantine to limit the future spread of these nematodes within the United States.

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