Root-Knot Nematodes in Golf Course Greens of the Western United States

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Abstract

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A survey of 238 golf courses in 10 states of the western United States found root-knot nematodes (*Meloidogyne* spp.) in 60% of the putting greens sampled. Sequence and phylogenetic analyses of 18S rRNA, D2-D3 of 28S rRNA, internal transcribed spacer-rRNA, and mitochondrial DNA gene sequences were used to identify specimens from 110 golf courses. The most common species, *Meloidogyne naasi*, was found in 58 golf courses distributed from Southern California to Washington in the coastal or cooler areas of those states. In the warmer regions of the Southwest, *M. marylandi* was recovered from 38 golf courses and *M. graminis* from 11 golf courses. This constitutes the first report of *M. marylandi* in Arizona, California, Hawaii, Nevada, and Utah, and the first report of *M. graminis* in Arizona, Hawaii, and Nevada. Two golf courses in Washington were infested with *M. minor*, the first record of this nematode in the Western Hemisphere. Columbia root-knot nematode, *M. chitwoodi*, was found in a single golf course in California. Polymerase chain reaction restriction fragment length polymorphism of the intergenic region between the cytochrome oxidase and *16S* rRNA genes in the mitochondrial genome with restriction enzyme *Ssp*I was able to distinguish populations of *M. graminis* from *M. marylandi*, providing a fast and inexpensive method for future diagnosis of these nematodes from turf.

Turfgrass, in all its aspects, is a major industry in the United States. Considered as a "crop" and including commercial, residential, and recreational components, turf is the largest irrigated crop in America. Three times more irrigated turf than irrigated corn is grown, covering a surface area of more than 128,000 km^2 (21). Golf is a primary consumer and producer of turf. The 16,000 golf courses in the United States utilize an estimated 908,000 ha of land, including 609,000 ha of maintained turfgrass (19). The economic impact of the golf course industry is significant, estimated in 2005 to have an annual value of $195 billion (1).

Plant-parasitic nematodes are an important factor affecting the health, quality, production, and maintenance of turfgrass on golf courses. Nematodes frequently associated with turfgrass in the western United States include root-knot nematodes, *Meloidogyne* spp.; cyst nematodes, *Heterodera* spp.; the Pacific shoot gall nematode, *Anguina pacificae*; ring nematodes, *Criconemella* spp.; spiral nematodes, *Helicotylenchus* spp.; and stubby root nematodes, *Trichodorus* spp. Lance nematodes, *Hoplolaimus* spp., are seldom a problem in western states, and the sting nematode, *Belonolaimus longicaudatus*, considered the most damaging plant-parasitic nematode on turfgrasses in the Southeast (7), is on only a few isolated golf courses in the Coachella Valley of California. Recently, root-knot nematodes have gained attention as a serious threat to both cool- and warm-season turfgrasses (Fig. 1). In most cases, the species of root-knot nematodes found in western golf greens have not been identified. In 2002, 22 Southern California golf courses were surveyed to determine the range of genera and populations of plant-parasitic nematodes, including *Meloidogyne* spp. (34). Nematode counts were used in an attempt to determine the relationship between green quality and nematode populations but no significant correlation was found. More recently, a preliminary survey of nematodes in western golf greens included 17 courses in Arizona and 2 in California. In all, 12 of the 17 courses were infested with unidentified species of *Meloidogyne* and 3 of the 12 showed nematode counts above the "damage threshold", based on threshold calculations from Florida (13). A survey of 14 golf courses in Northern California found root-knot nematodes in 64% of the greens sampled but no attempt was made to determine the nematode species or to relate population levels to turf quality (40). A recent study examined the effect of root-knot nematode numbers on green performance for 18 bentgrass greens at the La Jolla Country Club in San Diego County, CA. No correlation was found between "good"- and "poor"-performing greens and nematode population levels, including those of an unidentified *Meloidogyne* sp. (33). Species identification is important for selection of appropriate turf grasses having resistance or tolerance to certain nematode species in new and renovated greens and for application of control measures that may be species specific, such as the introduction of *Pasteuria penetrans* for biological control.

The utility of molecular methods for identification of *Meloidogyne* spp. was demonstrated in a large-scale regional survey of potato acreage in the central United States (27). DNA markers that have aided identification of *Meloidogyne* spp. include the small subunit (SSU) 18S ribosomal RNA (rRNA) gene (25); the large subunit (LSU) 28S D2-D3 expansion segments of the rRNA gene (4,23); the internal transcribed spacer (ITS) of rRNA gene (5); and the mitochondrial (mt)DNA marker, which spans the region between genes encoding cytochrome oxidase subunit II (*COII*) and *16S* rRNA (3,26,32,41). In this article, we document the occurrence and distribution of root-knot nematodes in golf course greens in 10 western states, with identifications based on morphological examination and analysis of the D2-D3 expansion segments of 28S, partial 18S, ITS rRNA, and mtDNA gene sequences. A new diagnostic assay for *Meloidogyne graminis* and *M. marylandi*, based on restriction fragment length polymorphism (RFLP) of amplified mtDNA, is presented.

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Materials and Methods

Sampling. Samples consisting of soil and sod were collected from one or more putting greens of 238 golf courses. A large number of samples were provided by Frank P. Wong and Naveen Hyder at the Turf Pathology Diagnostic Laboratory, University of California, Riverside. Samples submitted to their laboratory were examined for other turf diseases and then forwarded to the University of Arizona for nematode assays. Additional samples were sent directly to the Arizona laboratory by golf course superintendents and pest control advisors. Samples from Utah and southern Arizona were collected by the authors. In all, 776 samples were processed. Samples sizes included 8- or 10-cm-diameter cup plugs and 8 to 12 bulked, 1.9-cm-diameter plugs taken to a depth of 7.5 cm. Soil and roots were separated from the shoots and thatch. *Meloidogyne* infective juveniles (J2) were extracted from the soil and roots under an intermittent mist (2) for 72 h and the nematodes were collected from the tubes on a 500-mesh (25-µm) sieve. Numbers of J2 per cubic centimeter of soil were determined by counting an aliquot of the extract, and individual specimens were hand picked for sequencing or fixation for light microscopy. Nematodes for sequencing were placed in DESS (43) and stored at 4°C for up to a year. 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.

Species identification. Topotypes of *M. graminis* were collected from the St. Augustine grass (*Stenotaphrum secundatum*) lawn around the Division of Plant Industry Laboratory in Winter Haven, FL, by Larry W. Duncan, University of Florida. Authenticated *M. minor* and *M. naasi* from several locations in the United Kingdom were provided by Colin Fleming, Agri-Food and Biosciences Institute, Belfast, Northern Ireland, and *M. graminicol*a was supplied by Teodora Cabasan at the International Rice Research Institute in the Philippines. James Starr, Texas A&M University, contributed *M. graminis* and *M. marylandi* from Texas, and an Israeli population of *M. marylandi* came from the Division of Nematology, Institute of Plant Protection, ARO, the Volcani Center, Bet Dagan, Israel, courtesy of Yuji Oka. Topotypes of *M. marylandi* were collected by M. A. McClure from the collar surrounding the number 9 green at the University of Maryland Golf Course, College Park, MD (Table 1). Population number 500 from Brazos County, TX contained a mixture of *M. graminis* (GenBank JN241869 and JN241870) and *M. marylandi* (GenBank JN241838 and JN241868). The mixture was maintained on 'Jackpot' wheat in the greenhouse. Preliminary morphological identification of nematodes was made using J2 and female perineal patterns. Final species delimitation and identification was based on an integrated approach that considered morphological evaluation combined with molecular-based phylogenetic inference (tree-based methods) and sequence analyses (genetic distance methods) (30).

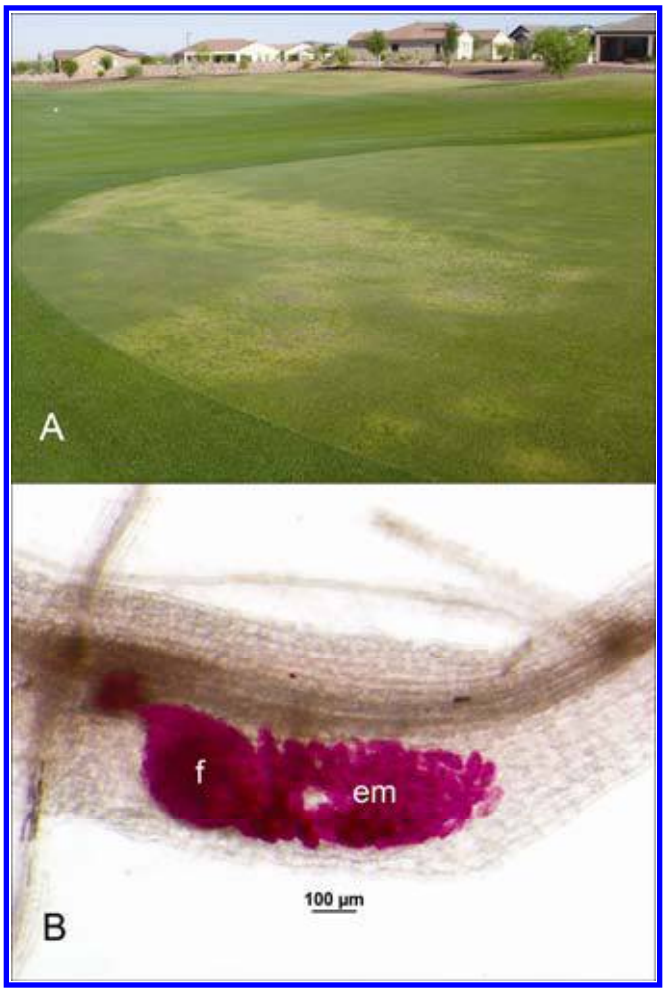

Fig. 1. Root-knot nematodes in turf grass. **A,** Damage caused by *Meloidogyne marylandi* to a bermudagrass (*Cynodon dactylon)* green; **B,** *M. naasi* female (f) with egg mass (em) in an annual bluegrass (*Poa annua)* root stained with acid fuchsin.

DNA extraction, polymerase chain reaction, cloning, and sequencing. Nematodes preserved in DESS were prepared for molecular analysis using two slightly different protocols. In protocol 1 (University of Arizona), nematodes were rinsed for 20 min in sterile distilled water and then transferred, individually, into a 10-µl drop of sterile lysis buffer (10 mM Tris [pH 8.0], 0.25 M GuHCl, 0.25.0% Triton X-100, 0.25% Tween 20, and 2.0 µl of Proteinase K; 934 units/ml) (11) on a clean glass cover slip and cut in half with a sharp scalpel blade. Scalpel blades were decontaminated prior to each cut by immersion in DNase Displace (Fisher Scientific, Pittsburgh) for several minutes, followed by three rinses in distilled water. The two halves of the nematode, and as much of the lysis buffer as possible, were 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. When available, a minimum of eight J2 from each sample selected for analysis were lysed and at least three were sequenced. Lysed nematodes were stored at –20°C for up to 2 weeks prior to PCR.

A Taq PCR Core kit (Qiagen, Valencia, CA) was used for PCR amplification of the D2-D3 region of the 28S gene and the ITS. Total reaction volume of 50 µl contained 5 µl of 10× PCR buffer, 5 µl of Q solution, 1 µl of dNTPs, 1 µl of primer F (10 µmol), 1 µl of primer R (10 µmol), 0.25 µl of Taq, 31.75 µl of nuclease-free water, and 5 µl of DNA. Primers D2A (5′-ACAAGTACCGTGAGG GAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′) (18) were used for the D2-D3 region. For the ITS region, we used 5367 (5′-TTGATTACGTCCCTGCCCTTT-3′) and F195 (5′-TCCTCCGCTAAATGATATG-3′) (18). The thermocycler was programmed as follows: 94°C for 3 min; followed by 40 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min; with a final extension at 72°C for 10 min. PCR products were separated on a 1.0% agarose gel, stained with ethidium bromide, and viewed under UV light. SCAR-PCR, using the primer sets and protocols described by Zijlstra (45), was used to identify *M. chitwoodi* and distinguish it from *M. fallax*. For sequencing, the PCR product bands were cut from the gel and purified using a QIAquick Gel Extraction Kit Gel (Qiagen). Purified bands were sequenced at the Genetics Core Facility at The University of Arizona. The sequences obtained were submitted for a search in GenBank using the BLASTN algorithm and compared with our sequences from authenticated populations.

In protocol 2 (United States Department of Agriculture, Beltsville, MD), specimens were mechanically disrupted in 20 µl of extraction buffer (37), then stored in PCR tubes at –80°C until needed. Extracts were prepared by incubating the tubes at 60°C for 60 min followed by 95°C for 15 min to deactivate the proteinase K, and centrifuged briefly prior to use in PCR. Each 25-µl PCR reaction contained 1 unit of Platinum Taq (Invitrogen, Carlsbad, CA),

1× reaction buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, and 2.5 mM $MgCl_2$), 0.2 mM dNTP mix, 0.3 µM each primer, and 2 µl of nematode extract. Partial (3′ end) 18S sequence was amplified from selected survey populations using primer 18s1.2 (5′-GGCGATCAGATACCGCCCTAGTT-3′) with 18sr2b (5′-TAC AAAGGGCAGGGACGTAAT-3′). Cycling conditions were 1 cycle of 94°C for 2 min; followed by 40 cycles of 94°C for 20 s, 59°C for 30 s, and 72°C for 30 s; and finishing with 1 cycle of 72°C for 5 min. A longer 18S sequence was obtained from reference populations by generating two additional overlapping PCR fragments. Reactions with primers 988F (5′-CTCAAAGAT TAAGCCATGC-3′) and 1912R (5′-TTTACGGTCAGAACT AGGG-3′) were amplified as above, substituting annealing at 65°C; reactions with primers 550F (5′-GGCAAGTCTGGTGCC AGCAGCC-3′) and 1108R (5′-CCACTCCTGGTGGTGCCC TTCC-3′) were amplified as described by Holterman et al. (12). For some populations, the ITS 1 and 2 rDNA region was amplified with primers TW81 (5′-GGTCAATGTTCAGAAATTTGTGG-3′) and AB28 (5′-GGTCAATGTTCAGAAATTTGTGG-3′) according to Skantar et al. (31); 28S D2-D3 rDNA was amplified as described previously (9,42). Amplification of the mtDNA region between the *COII* and *16S* rRNA genes included primers 1RNAF (5′-TACCTTTGACCAATCACGCT-3′) and COIIR (5′-GGTCAATGT TCAGAAATTTGTGG-3′). Cycling conditions included 1 cycle at 94°C for 2 min; followed by 45 cycles of 94°C for 30 s, 48°C for 30 s, and 68°C for 2 min; ending with 1 cycle of 68°C for 5 min. PCR products were analyzed by electrophoresis on 1% agarose and 1× sodium borate-EDTA (SB). Gels were stained with ethidium bromide and visualized using the U:Genius gel documentation system (Syngene, Frederick, MD). DNA was excised from the gels and purified as described in protocol 1. PCR products were quantified using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh) and sequenced at the University of Maryland Center for Biosystems Research. Selected amplicons were cloned with the Strataclone PCR Cloning Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Plasmid DNA was prepared with the QiaPrep Spin Miniprep Kit and digested with *Eco*RI to verify the presence of the insert. Two or more clones per amplicon were sequenced. DNA sequences were assembled using Sequencher 4.10.1 (Genecodes, Ann Arbor, MI) and analyzed using the BLASTN megablast program optimized for highly similar sequences (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Selected sequences were submitted to GenBank (Tables 1 and 2).

Mitochondrial PCR products from selected reference and golf course populations were used for analysis of restriction fragment polymorphisms. PCR product (8 to 10 µl, approximately 600 ng) was digested overnight in 20-µl reactions with restriction enzymes *Dra*I or *Ssp*I in the supplied reaction buffers (New England Biolabs, Ipswich, MA). Digested DNA was run on a 2.5% agarose gel buffered with SB, stained, and photographed as described above. The length of each restriction fragment was determined by virtual digestion of mitochondrial sequences in Sequencher.

Sequence and phylogenetic analysis. The newly obtained sequences for each gene were aligned using ClustalX 1.83 (16) with default parameters with corresponding published gene sequences (8,24,27,36,38). Outgroup taxa for each dataset were chosen according to the results of previously published data (8,27,36). Phylogenetic analyses of the dataset were performed with Bayesian inference (BI) using MrBayes 3.1.2 (14). BI analysis under the GTR + I + G model was initiated with a random starting tree 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. The log-likelihood values of the sample points stabilized after approximately 10^3 generations. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PPs) are given on appropriate clades.

Table 1. Reference populations used for identification of *Meloidogyne* spp. in a survey of golf course greens in the western United States

Species	Source	Origin	GenBank accession number			
			D2-D3 of 28S	ITS-rRNA[a]	18S	MtDNA
Meloidogyne chitwoodi	Russell Ingham: Oregon State University, Corvallis	Oregon	…	JN157868	…	…
	Kathy Merrifield: Oregon State University, Corvallis	Washington	…	…	…	JN241945–JN241949
	Saad Hafez: University of Idaho, Parma	Idaho	…	JN241864, JN241865	…	JN241902
M. fallax	Hans Helder: Wageningen University, The Netherlands	The Netherlands	JN157869	…	JN389789	JN241954
	Lieven Waeyenberge: Instituut voor Landbouw-en Visserijonderzoek, Belgium	The Netherlands	JN157848	…	JN389788	JN241950–JN241953
M. graminicola	Teodora Cabasan: International Rice Research Institute, Los Banos, The Philippines	The Philippines	JN157844	…	…	…
	George Abawi: Cornell University, Ithaca, NY	Bangladesh	…	JN241866, JN241867	…	JN241927, JN241929
	George Abawi: Cornell University, Ithaca, NY	India	…	…	…	JN241926, JN241939
M. graminis	Larry Duncan, University of Florida, Lake Alfred	Type locality, Florida	JN157849	…	JN389786	JN241922–JN241925
	Nicholas Sekora: University of Florida, Gainesville	Florida	JN157850	…	…	…
M. marylandi	Michael McClure: University of Arizona, Tucson	Type locality, Maryland	JN157851	…	…	JN241917, JN241955
	Yuji Oka: The Volcani Institute, Gilat Research Center, Negev, Israel	Israel	JN157852	…	JN241856	JN241918–JN241921
M. minor	Colin Fleming: Agri-Food and Biosciences Institute, Belfast, UK	United Kingdom	JN157846	JN157871	JN389787, JN241839, JN241840	…
	Lieven Waeyenberge: Instituut voor Landbouw-en Visserijonderzoek, Belgium	Belgium	JN628436, JN628437	…	…	…
M. naasi	Colin Fleming: Agri-Food and Biosciences Institute, Belfast, UK	United Kingdom	JN157847	…	JN241841	JN241944
	Kathy Merrifield: Oregon State University, Corvallis	…	…	…	…	…
	Washington	…	…	…	JN241909	…

[a] ITS = internal transcribed spacer.

Sequence differences between samples were calculated with PAUP* 4b10 (35) as an absolute distance matrix and the percentage was adjusted for missing data.

Results

Root-knot nematode distribution. Of the 238 golf courses sampled in this survey, 60% were infested with root-knot nematodes. Numbers of J2 ranged from 0.08 to 53.3 per cubic centimeter of soil (*data not shown*). In all, 112 of the infested samples were selected for sequencing of DNA markers amplified from J2 DNA: 24 from Arizona, 58 from California, 5 from Hawaii, 4 from Nevada, 4 from Oregon, 4 from Utah, and 13 from Washington (Table 2). The most common species was *M. naasi*, (found in 52% of the samples), which was distributed from San Diego County in Southern California to King County in Washington (Fig. 2). One sample from Washoe County, NV also contained *M. naasi*. None

Table 2. *Meloidogyne* spp. collected in a survey of golf course greens in the western United States

		Origin		GenBank accession number			
Species	**Pop.[a]**	**County**	**State**	**D2-D3 of 28S**	**ITS-rRNA[b]**	**18S**	**MtDNA**
Meloidogyne chitwoodi	186	San Luis Obispo	California	JN019321	…	JN632480	…
M. graminis	027	Maricopa	Arizona	…	JN157864; N241882–JN241885	JN241851	JN241901
	268	Pinal	Arizona	JN019326	…	JN241837	JN241907
	182	Kern	California	JN019327	…	JN241854	JN241915
	428	Orange	California	JN019328	…	…	…
	090	Riverside	California	JN019329	JN241860–N241863	JN241843	JN241900
	699	Riverside	California	…	JN157865	…	…
	108	San Diego	California	JN019330		…	…
	724	San Diego	California	…	JN241857–N241859	JN241834	JN241898
	730	San Diego	California	JN019331	…	…	…
	474	Kona	Hawaii	JN019339	…	…	…
	281	Clark	Nevada	JN019332	JN157866		
M. marylandi	001	Maricopa	Arizona	JN019333	JN157853; JN241874–N241877	JN241848	JN241916
	017	Maricopa	Arizona	JN019334	JN157854	JN241844	JN241904
	019	Maricopa	Arizona	JN019335	…	…	…
	021	Maricopa	Arizona	JN019336	…	…	…
	034	Maricopa	Arizona	JN019337	…	JN241852	JN241905
	389	Maricopa	Arizona	…	JN241878–N241881	JN241850	JN241906
	472	Maricopa	Arizona	JN019338	…	…	…
	623	Maricopa	Arizona	JN019340	JN157855	…	…
	333	Pima	Arizona	JN019341	JN157856; JN241890–N241896	JN241833	JN241908
	494	Pima	Arizona	JN019342	…	…	…
	603	Pima	Arizona	JN019343	…	…	…
	618	Pima	Arizona	JN019344	JN157857	…	…
	625	Pima	Arizona	JN019345	…	…	…
	627	Pima	Arizona	JN019346	…	…	…
	637	Pima	Arizona	JN019347	…	…	…
	641	Pima	Arizona	JN019348	…	…	…
	643	Pima	Arizona	JN019325	…	…	…
	656	Pima	Arizona	JN019349	JN157858	…	…
	658	Pima	Arizona	JN019350	…	…	…
	721	Pima	Arizona	JN019351	…	…	…
	047	Pinal	Arizona	JN019352	…	…	…
	330	Pinal	Arizona	JN019353	…	…	…
	650	Inyo	California	JN019354	…	…	…
	256	Los Angeles	California	JN019355	…	…	…
	427	Orange	California	JN019356	…	…	…
	480	Orange	California	JN019357	…	…	…
	481	Orange	California	JN019358	…	…	…
	359	Riverside	California	JN019359	…	…	…
	373	Riverside	California	JN019360	…	…	…
	434	Riverside	California	JN019361	…	…	…
	071	San Bernardino	California	JN019362	…	…	…
	436	Hawaii	Hawaii	JN019363	…	…	…
	447	Kona	Hawaii	JN019364	…	…	…
	474	Kona	Hawaii	JN019365	…	…	…
	463	Maui	Hawaii	JN019366	…	…	…
	386	Clark	Nevada	JN019367	…	…	…
	473	Clark	Nevada	JN019368	…	…	…
	737	Washington	Utah	JN019369	…	…	…
	763	Washington	Utah	JN157845	…	…	…

(*continued on next page*)

[a] Population number.
[b] ITS = internal transcribed spacer.

were found in samples from Arizona, Colorado, or Hawaii. In all, 38 of the samples (34%) contained J2 whose sequences matched those of *M. marylandi* and 11 samples matched the sequences of *M. graminis*. Three samples from Washington contained *M. minor*, a species previously not known in North America. *M. chitwoodi* was recovered from a golf course in San Luis Obispo County, CA. *M. graminicola* was not found in any of the samples sequenced. Seven samples from Colorado, five from Montana, and three from Idaho were examined but none contained root-knot nematodes.

Species identification using molecular approaches. Phylogenetic relationships among the root-knot nematodes, as inferred from BI analysis of the 28S D2-D3 expansion segments, ITS rRNA, partial 18S rRNA, and mtDNA gene sequences, are given in Figures 3, 4, 5, and 6, respectively. Using traditional morphologi-

Table 2. (*continued from preceding page*)

		Origin		GenBank accession number			
Species	**Pop.[a]**	**County**	**State**	**D2-D3 of 28S**	**ITS-rRNA[b]**	**18S**	**MtDNA**
M. minor	383	Snohomish	Washington	JN019322	JN157867	JN389792	…
	437	King	Washington	JN019323	…	JN389791	…
	438	King	Washington	JN019324	…	…	…
M. naasi	179	Alameda	California	JN019265	…	…	…
	209	Alameda	California	JN019266	…	…	…
	452	Alameda	California	JN019267	…	…	…
	151	Contra Costa	California	JN019268	…	…	…
	068	Los Angeles	California	JN019269	…	…	…
	115	Los Angeles	California	JN019270	…	…	…
	248	Los Angeles	California	JN019271	…	…	…
	267	Los Angeles	California	JN019272	…	…	…
	195	Marin	California	JN019273	…	…	…
	009	Monterey	California	JN019274	…	…	…
	010	Monterey	California	JN019275	JN157859; JN241871–N241873	JN241842	JN241897
	023	Monterey	California	JN019276	…	…	…
	025	Monterey	California	JN019277	…	…	…
	311	Monterey	California	JN019278	JN157860	…	…
	713	Monterey	California	JN019279	…	…	…
	734	Monterey	California	JN019280	JN157861	…	…
	191	Orange	California	JN019281	…	…	…
	264	Orange	California	JN019282	…	…	…
	342	Orange	California	JN019283	…	…	…
	138	Riverside	California	JN019284	…	…	…
	098	Sacramento	California	JN019285	…	…	…
	277	San Bernardino	California	JN019286	…	…	…
	726	San Bernardino	California	JN019287	…	…	…
	163	San Diego	California	JN019288	…	JN241847	JN241913
	255	San Diego	California	JN019289	…	…	…
	730	San Diego	California	JN019290	…	…	…
	670	San Francisco	California	JN019291	JN157862	…	…
	263	San Luis Obispo	California	JN019292	…	JN241846	JN241910
	162	San Mateo	California	JN019293	…	…	…
	663	San Mateo	California	JN019294	…	…	…
	074	Santa Barbara	California	JN019295	…	…	…
	599	Santa Barbara	California	JN019296	…	…	…
	101	Santa Clara	California	JN019297	…	…	…
	147	Santa Clara	California	JN019298	…	…	…
	218	Santa Clara	California	JN019299	…	…	…
	245	Santa Clara	California	JN019300	…	JN241836	JN241912
	422	Santa Clara	California	JN019301	…	…	…
	057	Stanislaus	California	JN019302	…	…	…
	707	Stanislaus	California		JN241886–N241889	JN241853	JN241899
	282	Ventura	California		…	JN241849	JN241911
	041	Ventura	California	JN019303	…	…	…
	013	Washoe	Nevada	JN019304	…	…	…
	410	Jackson	Oregon	JN019305	…	…	…
	054	Lane	Oregon	JN019306	…	JN241845	JN241903
	512	Multnomah	Oregon	JN019307	…	…	…
	275	Washington	Oregon	JN019308	…	…	…
	547	Washington	Utah	JN019309	…	…	…
	591	Utah	Utah	JN019310	JN157863	…	…
	530	Clackamas	Washington	JN019311	…	…	…
	194	King	Washington	JN019312	…	JN241855	JN241914
	437	King	Washington	JN019313	…	…	…
	438	King	Washington	JN019314	…	…	…
	524	King	Washington	JN019315	…	…	…
	351	Kitsap	Washington	JN019316	…	…	…
	361	Pierce	Washington	JN019317	…	…	…
	578	Pierce	Washington	JN019318	…	…	…
	150	Snohomish	Washington	JN019319	…	…	…
	424	Snohomish	Washington	JN019320	…	…	…

cal taxonomic characteristics and molecular criteria (apomorphies and genetic distances), we distinguished the following species within studied samples from our survey: *M. naasi, M. minor, M. chitwoodi, M. marylandi*, and *M. graminis*.

In all, 148 sequences, 115 of which were new, were included in the analysis of D2-D3 expansion segments of the 28S rRNA gene. The 28S rRNA alignment was 721 bp in length. Several moderate and highly supported major clades were distinguished in the majority consensus BI tree (Fig. 3): (i) *M. naasi* + *M. graminicola* (PP = 100), (ii) *M. exigua* (PP = 100), (iii) *M. minor* + *M. chitwoodi* + *M. fallax* (PP = 100), (iv) *M. hapla* + *M. dunensis* + *M. silvestris* + *M. hispanica* + *Meloidogyne* sp. from the tropical group (PP = 94), and (v) *M. marylandi* + *M. graminis* (PP = 94). Sequences of *M. marylandi* formed two subclades. Intraspecific sequence variation for species reached the following percentages: *M. naasi*, 0.7% (5 bp); *M. minor*, 0.5% (3 bp); *M. chitwoodi,* 0.3% (2 bp); *M. fallax,* 0.4% (3 bp); *M. marylandi,* 1.7% (9 bp); *M. graminis*, 1.0% (7 bp); and *M. graminicola,* 0.1% (1 bp). Interspecific sequence variation between some species pairs was 4.6% (27 bp) for *M. marylandi* and *M. graminis* and 0.4% (3 bp) *M. chitwoodi* and *M. fallax*.

The ITS rRNA alignment was 679 bp in length and included 99 sequences, 57 of which were newly obtained in this study. The following moderate and highly supported major clades were distinguished in the ITS tree (Fig. 4): (i) *M. naasi* + *M. graminicola* (PP = 100), (ii) *M. chitwoodi* and *M. fallax* (PP = 97), (iii) *M. minor* (PP = 100), (iv) *M. marylandi* + *M. graminis* (PP = 100), (v) *M. silvestris*, (vi) *M. hapla*, (vii) *M. dunensis*, (viii) *M. hispanica* + *M. enterolobii* + *Meloidogyne* sp. from the tropical group, and (ix) *M. panyuensis*. Intraspecific sequence variation for some species was as follows: *M. naasi*, 0 to 1.5% (0 to 9 bp), *M. minor*, 0 to 1% (0 to 3 bp), *M. chitwoodi,* 0 to 0.4% (0 to 2 bp); *M. fallax,* 0 to 0.2% (0 to 2 nucleotides); *M. marylandi,* 0 to 6.5% (0 to 35 bp), *M. graminis,* 0 to 4.3% (0 to 23 bp), and *M. graminicola,* 0 to 0.9% (0 to 5 bp). Interspecific sequence variation between *M. chitwoodi* and *M. fallax* was 1.7 to 2.0% (8 to 11 bp).

In all, 102 sequences, 30 of which were new, were included in the 18S rRNA alignment with a length of 656 bp. *M. graminis* and *M. marylandi* clustered together (Fig. 5) and were not distinguishable. *M. chitwoodi* and *M. fallax* also had similar sequences, and they differed from closely related *M. naasi* by one nucleotide.

The mtDNA alignment included 71 sequences, was 462 bp long, and included 45 novel mtDNA sequences. The BI tree (Fig. 6) contained several highly supported major clades (PP = 99 to 100): (i) *M. chitwoodi* + *M. fallax*, (ii) *M. naasi* + *M. graminicola*, (iii) *M. marylandi* + *M. graminis*, (iv) *M. hapla* + *M. partityla*, and (v) *M. enterolobii*. Sequences for *M. graminis, M. graminicola*, and *M. naasi* each formed two subclades. Intraspecific sequence divergence for some species were varied: across the whole *M. graminis clade*, divergence was 0 to 5% (0 to 22 bp) whereas, within same subclade, it was 0 to 0.6% (0 to 3 bp); *M. marylandi* variation was 0 to 2.0% (0 to 9 bp), with a maximum differences between Maryland and other isolates; *M. naasi* variation was 0 to 0.9% (0 to 4 bp). *M. chitwoodi* and *M. fallax* differed by one nucleotide deletion or insertion.

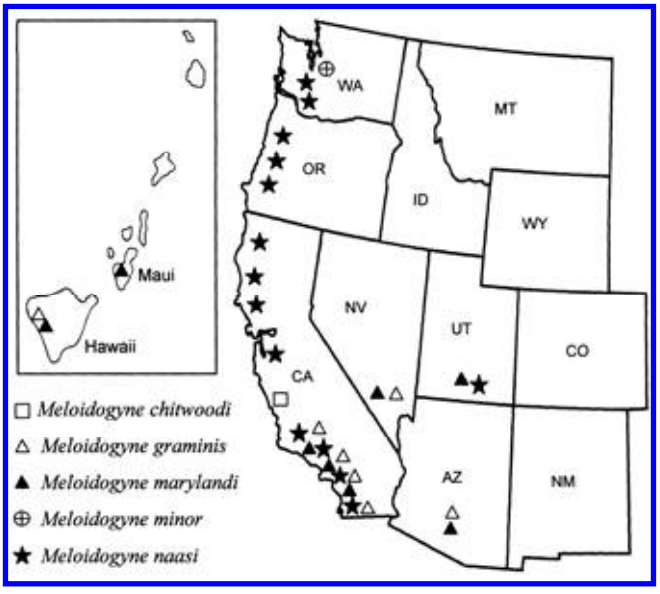

Fig. 2. Distribution of root-knot nematodes in golf course greens in the Western United States. Seven samples from Colorado, five from Montana, and three from Idaho were negative for *Meloidogyne* spp. Wyoming and New Mexico were not included in the survey. Symbols represent distribution of the species.

Mitochondrial PCR products from the root-knot nematodes included in the study ranged in size from 487 to 539 bp. Sequences were extremely AT-rich (>78%), limiting the restriction enzymes predicted to generate diagnostically informative polymorphisms. Digestion of the mitochondrial PCR products with restriction enzyme *Ssp*I allowed discrimination among several of the species found in the survey (Table 3; Fig. 7). Identical restriction patterns of *M. chitwoodi* and *M. fallax* were distinct from *M. naasi* and the other species but not each other. The pattern of *M. graminis* differed from that of *M. marylandi*. A unique restriction pattern was also found for *M. graminicola*.

To further test discrimination between *M. graminis* and *M. marylandi*, RFLP analysis was conducted on multiple individuals from reference populations from Florida and Israel, respectively, and from the type locality of *M. marylandi*, a golf course in College Park, MD. Distinct restriction patterns for each species were observed with enzymes *Dra*I and *Ssp*I, with all J2 tested from a population giving the same pattern (Fig. 8A and B, respectively). Digestion of PCR products generated from J2 of selected golf course isolates gave mixed results with these enzymes. *Dra*I digestion of mtDNA products amplified from one or more J2 from *M. graminis* survey population numbers 027, 090, 182, and 724 revealed the presence of a 227-bp fragment, similar to the largest fragment from *M. marylandi*, rather than the 306-bp band observed for Florida *M. graminis* (Fig. 9A). Two of four J2 tested from Texas population number 500 (Fig 9B, lanes 1 and 3) also showed patterns that matched *M. marylandi* (lane 15). *Ssp*I digests of the PCR products from the same specimens clearly separated *M. graminis* from *M. marylandi* (Fig. 9B). The *Ssp*I profiles of juveniles from survey population numbers 027, 090, 182, 724, and 500 conformed to the Florida *M. graminis* pattern.

In silico analysis of mtDNA sequences from the reference populations, golf course isolates, and sequences available from GenBank confirmed the restriction site variability seen in the RFLP gel patterns (Table 3). Sequence types corresponding to different *Dra*I restriction profiles were found for *M. graminis* populations; the addition of two *Dra*I sites changed a 307-bp fragment to 227 + 78 bp, and changed a 74-bp fragment to 57 + 16 bp. Two sequence types that could lead to *Dra*I and *Ssp*I polymorphisms were also found for *M. graminicola*. No differences affecting these enzymes were found in *M. naasi*, *M. chitwoodi*, or *M. fallax* sequences.

M. graminis and M. marylandi sequences were further examined *in silico* for the utility of *Alu*I, *Dde*I, and *Pac*I restriction sites. Two *Alu*I restriction sites were found in mtDNA sequences of *M. graminis* survey populations but not in the reference population from Florida. The same two sites were found in sequences from the *M. marylandi* type locality (Maryland) but were not present in the population from Israel; nor were they found in any of the survey populations identified as *M. marylandi*. Due to the intraspecific variation affecting these sites, *Alu*I cannot discriminate *M. graminis* from *M. marylandi.* The enzyme *Dde*I cleaves *M. graminis* mtDNA into two fragments but does not cut *M. marylandi*, although the fragment length differences may be too slight to be practically useful. A single *Pac*I site was found in all *M. graminis* and *M. marylandi* sequences. Thus, this enzyme cannot distinguish these species but may be useful for setting them apart from others.

Discussion

Molecular identification of root-knot nematodes. During the present survey, we identified five species of root-knot nematodes: *M. naasi, M. minor, M. chitwoodi, M. marylandi*, and *M. graminis*. With the exception of *M. naasi*, which has a distinctively attenuated tail, the four other species of *Meloidogyne* encountered in this survey are not easily diagnosed by morphology of the J2, the stage

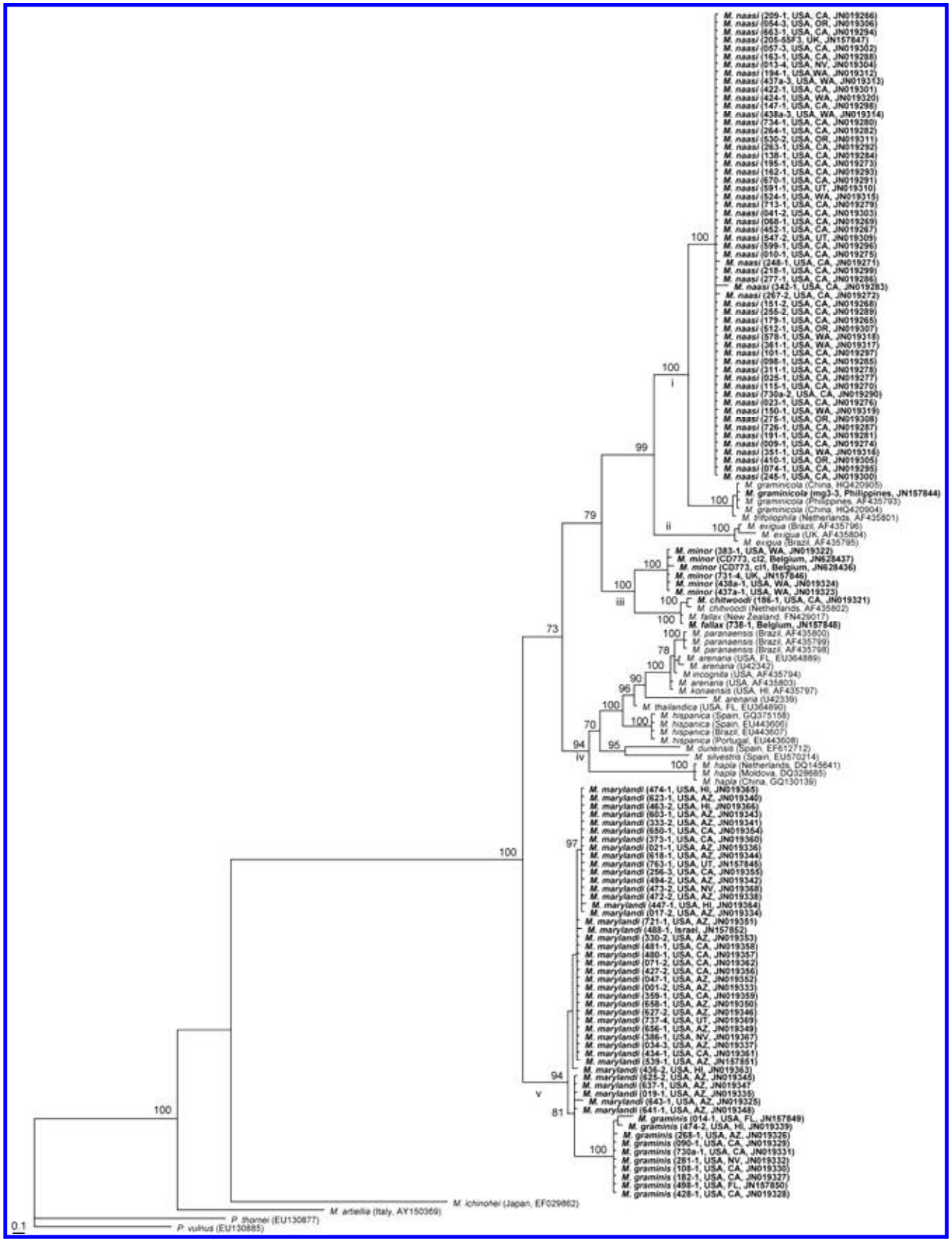

Fig. 3. Phylogenetic relationships within root-knot nematodes as inferred from Bayesian analysis of the D2-D3 of 28S rRNA gene sequences. Posterior probability values more than 70% are given on appropriate clades. Newly obtained sequences are indicated in bold.

routinely found in soil samples. In recent years, analysis of DNA sequences has been increasingly used for identification of *Meloidogyne* spp. (25). In the present study, we used sequence information from three fragments of rRNA genes and one fragment of mtDNA for sample identification. For the first time, sequences are reported here for D2-D3 of 28S rRNA from *M. naasi, M. minor,* and *M. marylandi*; for mtDNA from *M. marylandi* and *M. naasi*; for ITS rRNA from *M. graminis* and *M. marylandi*; and for partial

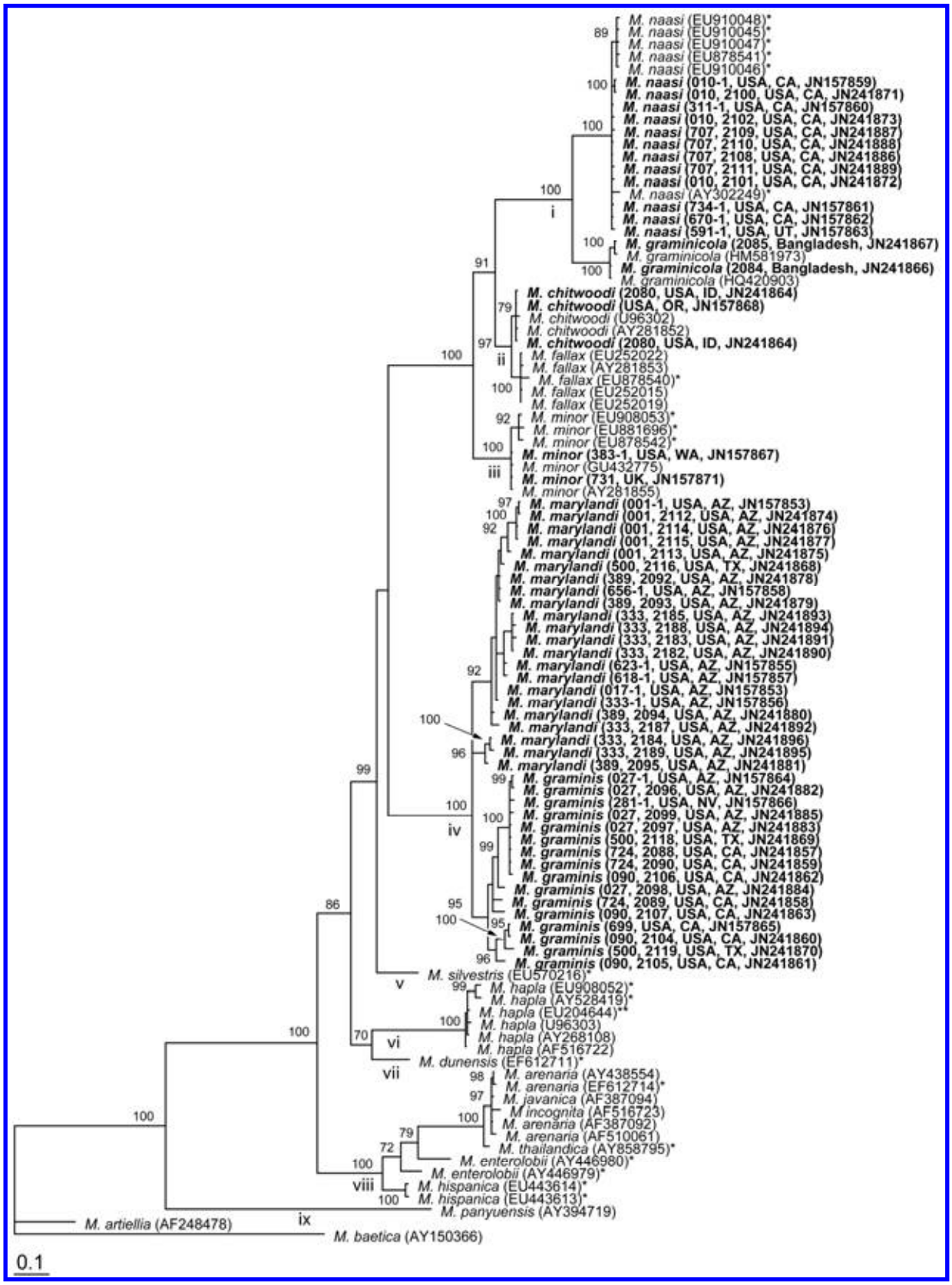

Fig. 4. Phylogenetic relationships within root-knot nematodes as inferred from Bayesian analysis of the internal transcribed spacer (ITS)1-5.8S rRNA-ITS2 gene sequences. Posterior probability values more than 70% are given on appropriate clades. Newly obtained sequences are indicated in bold; * indicates only ITS1 used for the analysis; ** indicates originally identified as *Meloidogyne ethiopica.*

18S rRNA gene from *M. marylandi.* These gene fragments varied with respect to their usefulness for species diagnostics. The D2-D3 expansion segments of 28S rRNA and the mtDNA sequences showed the best discrimination power. Although the ITS rRNA gene sequences distinguished most root-knot nematode species from each other, heterogeneity in this gene fragment did not allow unambiguous diagnosis of *M. graminis* and *M. marylandi.* Conversely, the partial 3′ end of 18S rRNA was relatively conservative and did not contain enough nucleotide differences to separate *M. graminis* from *M. marylandi* or *M. chitwoodi* from *M. fallax.*

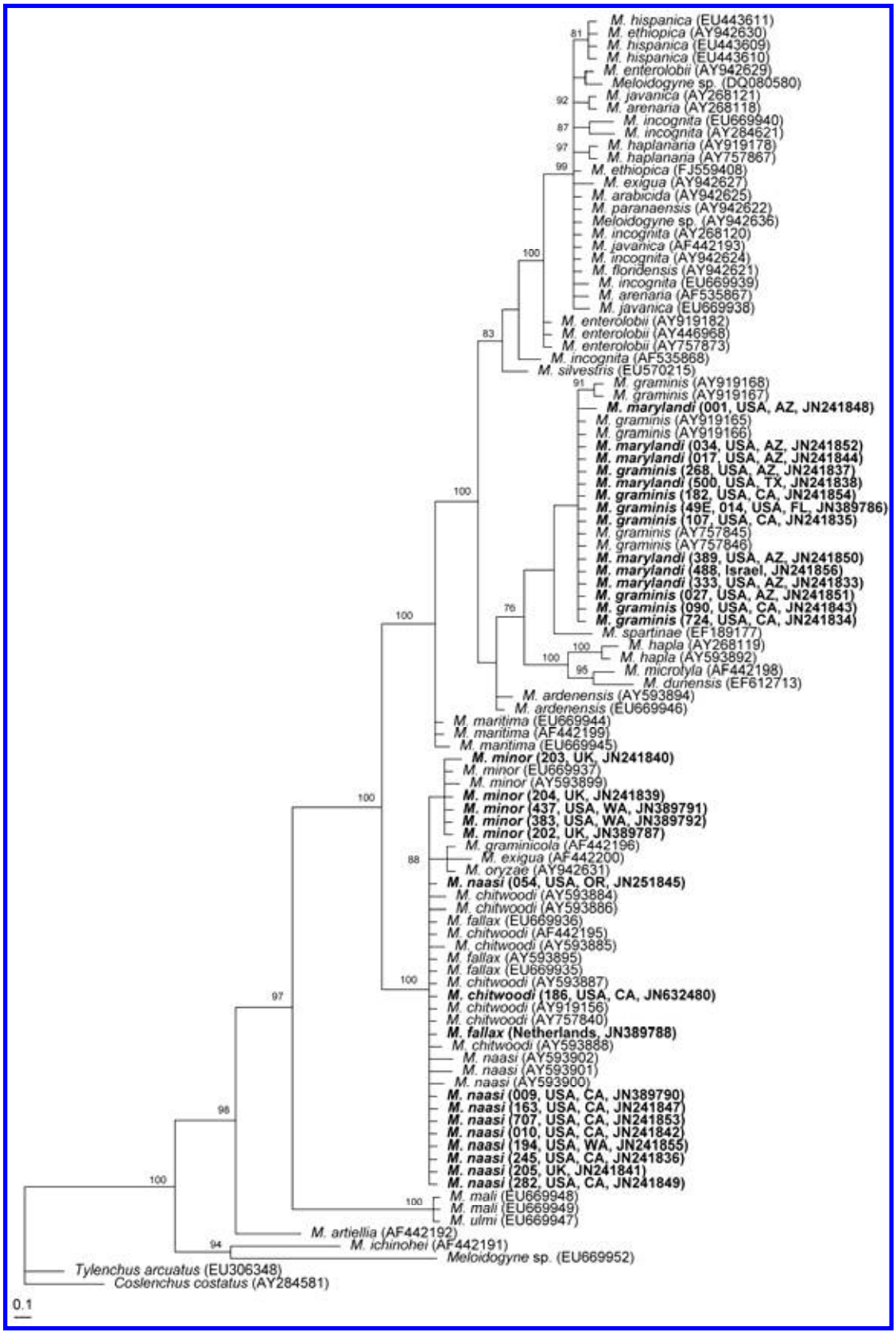

Fig. 5. Phylogenetic relationships within root-knot nematodes as inferred from Bayesian analysis of the partial 18S rRNA gene sequences. Posterior probability values more than 70% are given on appropriate clades. Newly obtained sequences are indicated in bold.

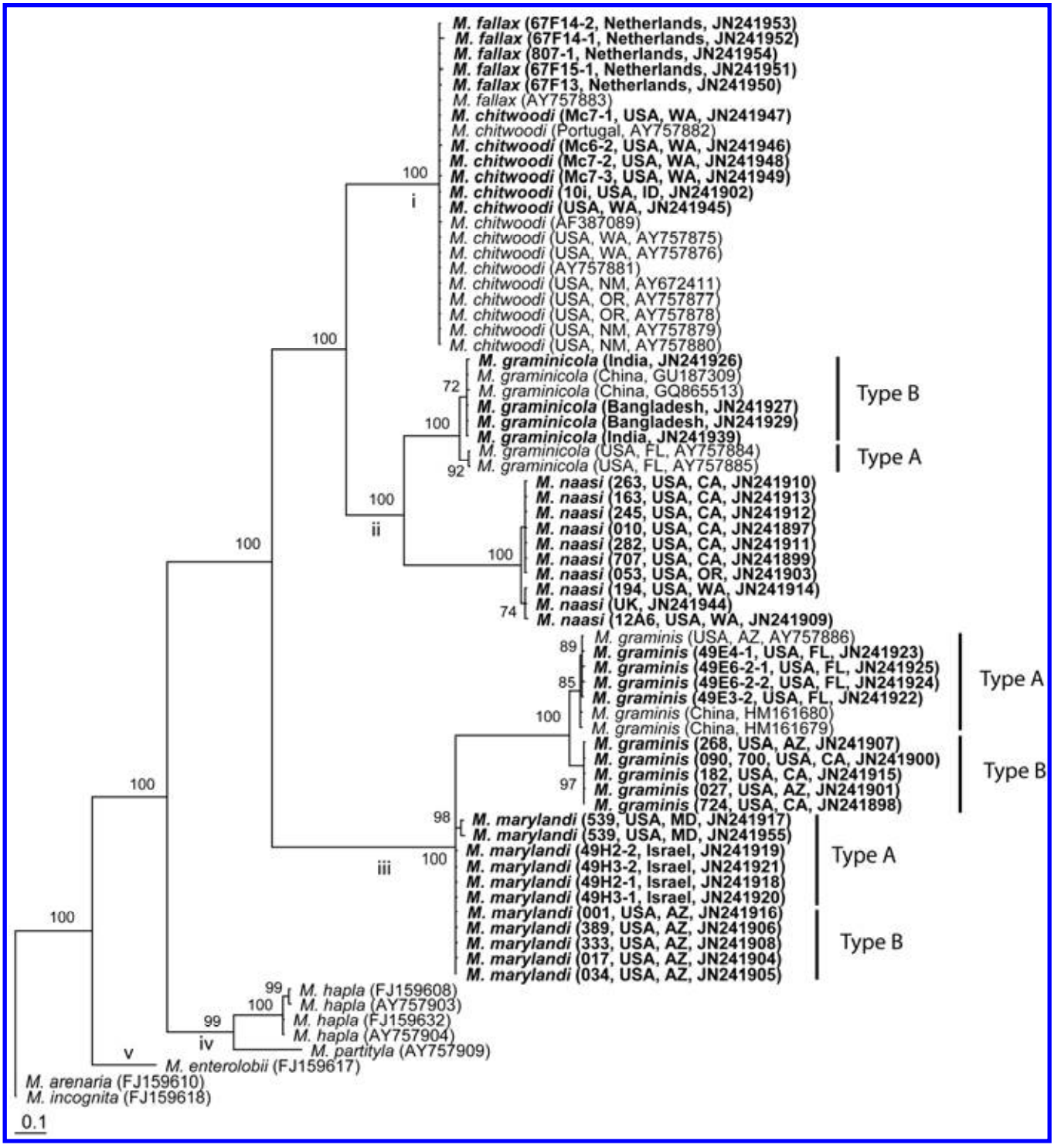

Fig. 6. Phylogenetic relationships within root-knot nematodes as inferred from Bayesian analysis of the partial mitochondrial DNA sequences. Posterior probability values more than 70% are given on appropriate clades. Newly obtained sequences are indicated in bold.

Particular attention has been given to the region of mtDNA including partial *COII* and *16S* rRNA genes, owing to the rapid evolution of this molecule relative to nuclear rRNA genes (15). This marker was originally targeted as a potential means for differentiating the five common *Meloidogyne* spp. by giving different-sized amplified PCR products (3,18,22,25,28). A large group of species, including *M. hapla, M. chitwoodi*, *M. fallax*, *M. graminicola*, *M. graminis*, *M. mali*, *M. marylandi*, *M. microtyla*, *M. naasi*, *M. oryzae*, *M. suginamiensis*, and *M. trifoliophila,* fell into the smallest size class, those lacking an AT-rich region in the amplified product (2). Our analysis also revealed higher interspecific sequence variation in mtDNA compared with nuclear rRNA genes in all studied root-knot nematodes, except for *M. chitwoodi* and *M. fallax*, whose mtDNA fragment surprisingly differed by a single deletion or insertion.

Our sequence analysis of mtDNA also showed the presence of different haplotypes for some species (Table 3), although only for *M. graminicola* did there appear to be any association of sequence type with geographic origin (Asia versus the United States). Powers et al. (26) previously reported three mtDNA haplotypes for *M. chitwoodi*. Sequences obtained for *M. chitwoodi* populations in our study conformed to those designated type A, including populations from Washington, Idaho, and Portugal, but distinct from type C populations from Oregon and New Mexico. *M. graminis* sequences

Table 3. Approximate sizes of restriction fragments generated by two diagnostic enzymes after digestion of mitochondrial DNA polymerase chain reaction products from root-knot nematodes, *Meloidogyne* spp.

Species	Origin	Size (bp)		
		Length	*Dra*I	*Ssp*I
Meloidogyne graminicola type A	Florida	531	311, 220	114, 111, 102, 92, 65, 47
M. graminicola type B	Bangladesh, China, India	531	313, 156, 62	167, 113, 112, 92, 47
M. graminis type A	Arizona, Florida, China	540	307, 74, 42, 33, 32, 30, 22	187, 148, 115, 61, 28
M. graminis type B	Arizona, California, Texas	537	227, 78, 57, 41, 33, 33, 30, 22, 16	187, 147, 114, 60, 28
M. marylandi type A	Arizona, Maryland, Israel	534	228, 80, 78, 62, 39, 33, 14	148, 122, 115, 89, 60
M. marylandi type B	Arizona	534	228, 78, 62, 50, 39, 33, 30, 14	148, 122, 115, 89, 60
M. fallax	The Netherlands	520	258, 118, 86, 40, 18	239, 234, 47
M. chitwoodi	Oregon	519	257, 118, 86, 40, 18	238, 234, 47
M. naasi	United Kingdom	530	312, 127, 91	225, 93, 77, 50, 39, 30, 16

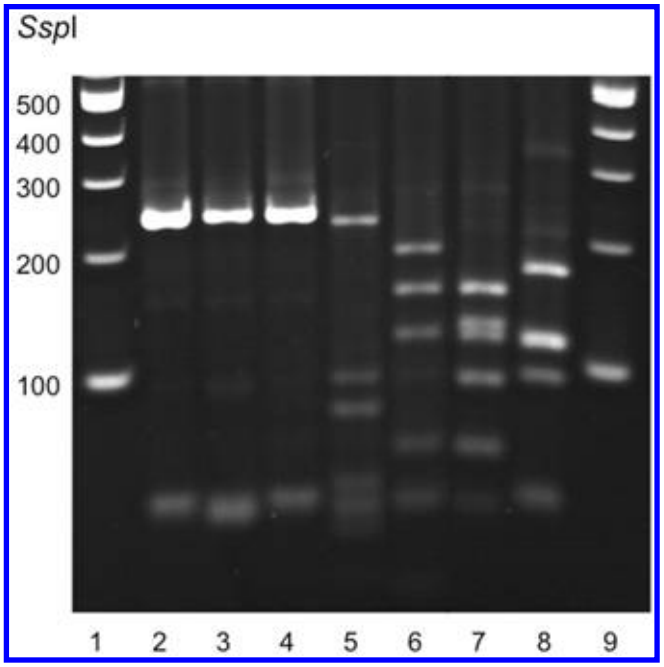

Fig. 7. Polymerase chain reaction restriction fragment length polymorphism profile of mitochondrial DNA for root-knot nematode species. Lanes 1 and 9: 100-bp DNA ladder; lane 2: *Meloidogyne chitwoodi*, Washington, United States; lane 3: *M. fallax*, The Netherlands; lane 4: *M. fallax*, The Netherlands; lane 5: *M. naasi*, (205), United Kingdom; lane 6: *M. graminis*, (014), Florida, United States; lane 7: *M. marylandi*, (488), Israel; lane 8: *M. graminicola*, India.

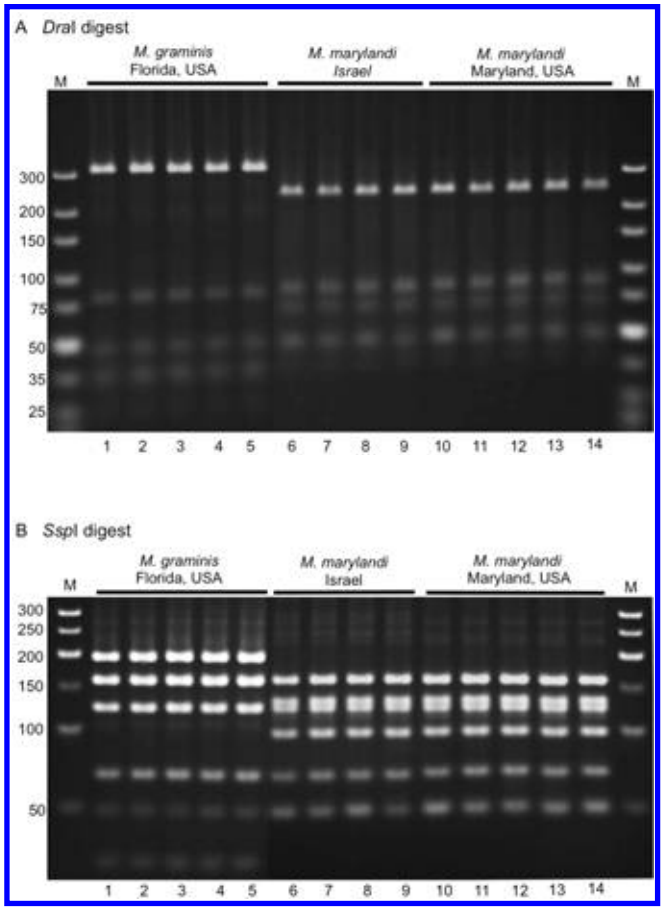

Fig. 8. Restriction enzyme profiles of mitochondrial DNA polymerase chain reaction products generated from selected root-knot nematode reference populations and digested with enzymes **A,** *Dra*I or **B,** *Ssp*I. Infective juveniles from reference populations served as template in A and B. Lanes 1–5, *Meloidogyne graminis*, Lake Alfred, FL; lanes 6–9: *M. marylandi*, Israel; lanes 10–14: *M. marylandi*, College Park, MD; lanes M = 100-bp ladder.

from the Florida reference population corresponded to type A sequences reported from Arizona (AY757886) and China (HM161679 and HM161680), whereas those from California (numbers 090, 182, and 724) and Arizona (numbers 027 and 268) constituted a novel type B pattern not reported previously. No other *M. marylandi* mtDNA sequences were available for comparison; therefore, there is need for additional sampling to determine the extent of sequence types present in other geographic areas.

PCR-RFLP of mtDNA has been a reliable and rapid method of diagnostics for the root-knot nematodes (25,26). We found that amplification of the mtDNA fragment followed by digestion with *Ssp*I consistently discriminated populations of *M. graminis* from *M. marylandi* and was not subject to the heterogeneity that can confound *Dra*I RFLPs. To our knowledge, this is the first diagnostic assay designed to separate these two species, providing a simple, inexpensive assay that can be applied to DNA derived from single juveniles. However, due to the low complexity and high AT content of mtDNA in root-knot nematodes, further confirmation by sequencing multiple DNA markers may be necessary for identification of new or unusual populations.

Root-knot nematodes from golf courses. Except for Florida, where the incidence is 89% (6), the percentage of golf courses in this survey that were infested with root-knot nematodes (60%) was considerably higher than that reported from elsewhere in North America: Alabama, 36.8% (29); Ontario, Canada, 6.6% (44); and Kansas, 0.0% (39). These differences could be due to a variety of reasons, including edaphic, climatic, and procedural factors, such as sampling methods. One explanation for an increase in the incidence of root-knot nematodes in recent years is the trend for golf course greens to be constructed or renovated according to standards set by the United States Golf Association, which specifies a sand content of 92% or greater in the top 12 inches. The sand content of most greens is further augmented by frequent top dressings with sand, (up to 15 kg of sand per square meter annually), providing an ideal substrate for root-knot nematodes to infect susceptible hosts. In general, the distribution of *Meloidogyne* spp. on Western golf courses was correlated with average annual temperatures: *M. naasi, M. minor,* and *M. chitwoodi* in the cooler regions and *M. marylandi* and *M. graminis* in the warmer regions. *M. marylandi*

was found in golf course greens in Death Valley, CA, at 65 m below sea level, the lowest elevation in the United States, and one of the hottest and driest. The type of turf grown in these regions may also influence distribution of the species. Golf greens in cooler climates are commonly annual bluegrass (*Poa annua*) or bentgrass (*Agrostis* spp.), whereas those in the warmer climates are either bentgrass or bermudagrass (*Cynodon dactylon*). Fescue greens (*Festuca* spp.) and others such as *Zoysia* spp. are not widely used in the Western states. Precise determination of the host was difficult, primarily because many of the greens sampled consisted of mixed turf. Bentgrass greens in coastal California, Oregon, Utah, and Washington frequently are invaded by *P. annua*, which may constitute 50% or more of the turf, and bermudagrass is a common invader of bentgrass greens in parts of Southern California and Arizona.

M. graminis is one of the most common root-knot nematodes on turf grasses in the southern United States, especially Florida (20). It is less common in the Southwest, where the principal species in golf course greens is *M. marylandi*. In the current survey, both species were found cohabiting a single green on a golf course in San Diego County, CA. Whether this resulted from a coincidence of preferred climatic and edaphic factors was not resolved but, clearly, the opportunity exists for *M. graminis* to be more widely distributed than presently found. Morphologically, the J2 are very similar and, where *M. marylandi* predominates, mixed populations would be very difficult to detect by microscopic examination alone. However, RFLP analysis of multiple individuals using *Ssp*I could be used to screen for the presence of both species.

M. chitwoodi is widely distributed on potato and other crops in the Pacific Northwest, including Northern California, but its occurrence on a mature golf course green near the central coast of California was unexpected. The host range of *M. chitwoodi* includes both monocots and dicots (10,22,28) but, to our knowledge, this is the first report of a turfgrass host in a golf course green, in this case, a mixture of *P. annua* and creeping bentgrass (*A. palustris*).

M. minor is a relatively new species that was first found on potato in The Netherlands in 2004 and, subsequently, on turfgrass from a score of golf courses and sports fields in Ireland and the United Kingdom, where it causes symptoms of a yellow patch disease (17). Recently it has been found in Portugal (C. Fleming, *personal communication*) and Chile (G. Karssen, *personal communication*), raising interesting questions regarding its likely origin. The two golf courses in Washington where *M. minor* was detected in our survey are within 50 km of each other. Other courses nearby may also be infested but a dedicated survey would be required to determine the distribution of *M. minor* in the Pacific Northwest. In the United Kingdom, mixed populations of *M. minor* and *M. naasi* are common. In the Washington golf courses, these species occurred together in a ratio of approximately 1:4. The potential exists for *M. minor* to spread from these two golf courses to agricultural crops but the risk has not been assessed. Both of the golf courses in Washington where *M. minor* was found are relatively isolated from commercial agriculture. Spread to other golf courses in the region is more likely. Golf clubs and golf shoes, contaminated with soil containing *M. minor* eggs or J2, could carry the pathogen from course to course, and even from region to region, where conditions favor establishment of the nematode.

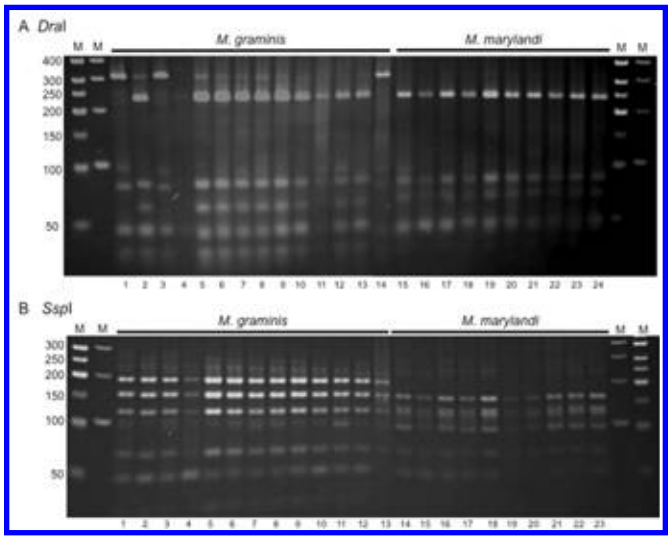

Fig. 9. Restriction enzyme profiles of mitochondrial DNA polymerase chain reaction products generated from selected root-knot nematode golf course populations and digested with enzymes **A,** *Dra*I or **B,** *Ssp*I. **A,** *Meloidogyne graminis* survey populations include lanes 1–4: number 500; lanes 5 and 6: number 724; lanes 7 and 8: number 027; lanes 9 and 10: number 090; lane 11: number 268; lanes 12 and 13: number 182: lane 14: reference population, Lake Alfred, FL. *M. marylandi* populations include lane 15: reference population, Israel; lanes 16 and 17: number 333; lanes 18 and 19: number 389; lane 20: number 001; lanes 21 and 22: number 017; lanes 23 and 24: number 034. **B,** *M. graminis* survey populations include lanes 1–4: number 500; lane 5: number 724; lanes 6 and 7: number 027; lanes 8 and 9: number 090; lane 10: number 268; lanes 11 and 12: number 182: lane 13: reference population, Lake Alfred, FL. *M. marylandi* populations include lane 14: reference population, Israel; lanes 15 and 16: number 333; lanes 17 and 18: number 389; lane 19: number 001; lanes 20 and 21: number 017; lanes 22 and 23: number 034. Lanes M = 100-bp ladder. Further details for populations can be found in Tables 1 and 2.

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