Mitochondrial Haplotype-Based Identification of Ethanol-Preserved Root-Knot Nematodes from Africa

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ABSTRACT

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The asexual root-knot nematodes (RKNs) (*Meloidogyne* spp.) exemplified by *Meloidogyne incognita* are widespread and damaging pests in tropical and subtropical regions worldwide. Comparison of amplification products of two adjacent polymorphic regions of the mitochondrial genome using DNA extracts of characterized RKN strains, including 15 different species, indicate that several species are derived from the same or closely related female lineages. Nevertheless, *M. javanica*, *M. enterolobii*, *M. incognita*, and other key species could each be assigned unique

mitochondrial haplotypes based on polymerase chain reaction fragment size and restriction cleavage patterns. *M. arenaria* isolates did not group as a single haplotype, consistent with other reports of diversity within this species. To test the utility of this assay, we characterized ethanol-preserved samples from 103 single-species isolates from four countries in sub-Saharan Africa (Benin, Nigeria, Kenya, and Tanzania). Mitochondrial haplotypes corresponding to *M. javanica* and *M. incognita* were the most prevalent. Samples from western Africa included several instances of *M. enterolobii* but this species was not detected in samples from East Africa. This protocol provides progress toward a standardized strategy for identification of RKN species from small, preserved samples and a rational starting point for classifying species present in regions where previous knowledge has been limited.

Root-knot nematodes (RKNs) (*Meloidogyne* spp.) are globally distributed pests that cause substantial economic damage to crops worldwide (21,35,49). Most species associated with crops in tropical agriculture belong to a closely related, asexually reproducing (apomictic) species complex including *Meloidogyne incognita*, *M. javanica*, and *M. arenaria*, which we refer to collectively as the *M. incognita* group (MIG) (30,41). Although the host range of these species is exceptionally broad, there are differences between species. Therefore, accurate characterization of prevailing nematode populations is essential to efficiently employ management strategies such as host plant resistance, biological control, and crop rotation, which may be dependent on the nematode taxa present. Similarly, for quarantine determination, accurate species diagnosis is essential. Notably, reports of the highly pathogenic and invasive species *M. enterolobii* have increased recently due, in part, to its pathogenicity on hosts resistant to MIG species (15).

Although host range tests provide the most directly useful information for agricultural applications, these require a prohibitive amount of time, resources, and well-characterized host material. Species identification can also be based on morphological differences (43) and isozyme polymorphisms (22). However, these approaches are time consuming and labor intensive and require living or well-preserved specimens. DNA-based identification, mostly utilizing polymerase chain reaction (PCR)-

based amplification, has improved our ability to identify and diagnose RKNs (2,4). Specific primers that direct amplification of a genomic fragment only from a target species have been developed for several parthenogenetic RKN lineages. This approach can be useful but suffers from the limitation that a negative result could indicate either a failed reaction or that the nematode is a different species or variant.

The DNA sequence of ribosomal RNA (rDNA) genes has been useful for determining phylogenetic relationships and as a diagnostic tool for many organisms. Comparison of rDNA sequences has divided *Meloidogyne* spp. into three well-supported major clades, with clade I containing the polyphagous tropical species and clades II and III containing nematodes from more temperate climates (26,28,48). However, whereas clade II and clade III species were well resolved, those in clade I, particularly the MIG species, remained unresolved because sequence differences of rDNA copies within an individual RKN showed greater diversity than between species. Comparisons of sequences of single-copy nuclear genes also fail to form phylogenetic lineages concordant with recognized species, leading to speculation that MIG species have reticulate origins; that is, they arose by hybridization of two sexual species, perhaps with a common parent, followed by loss of ability to reproduce sexually (33). In support of this, whole-genome sequence analysis of *M. incognita* has revealed that it carries two or three diverged copies of many genomic sequences (1,34).

The mitochondrial genome, due to its uniparental inheritance, can circumvent some of the difficulties of differentiating hybrid, parthenogenetic lineages (25). In fact, the mitochondrial genome of apomictic RKNs has revealed a useful source of diagnostic markers. The mitochondrial DNA (mtDNA) between the cyto-

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chrome oxidase II (COII) and large subunit of rDNA carries a noncoding intergenic spacer in MIG RKN species (40). Length differences of the intergenic spacer and restriction fragment length polymorphisms (RFLPs) of this region were used to develop a PCR-based assay that could distinguish five major RKN species (39), and this assay has been extended to other species (4). Hugall et al. (27) also examined mitochondrial RFLP patterns and, using over 50 independent isolates from Australia, observed perfect correspondence of mtDNA polymorphisms with esterase phenotypes. A diagnostic strategy based on amplification of two mtDNA regions that, together, span the spacer and part of the adjacent large subunit (16S) rRNA (lrDNA) gene was developed (46) (Fig. 1). The size of the intergenic spacer and sequence polymorphisms in the lrDNA that were revealed following digestion with the restriction enzymes *HinfI* and *MnII* were used to assign haplotypes.

In practice, RKNs are often identified only to genus, especially from locations where resources for morphological or molecular analysis are limited or inaccessible. Plant yield reductions due to RKNs are a principal production constraint for small-holder farmers in tropical countries and directly responsible for widespread misuse of pesticides (32). RKNs also undermine staple crop production, causing significant losses to staple food crops such as maize and cassava across sub-Saharan Africa (SSA) (6,20). Knowledge of the species present is limited and is needed for planning management strategies (38). Here, we present a protocol to extract DNA from single egg masses of ethanol-preserved samples. We demonstrate that this DNA can be routinely amplified using the two sets of mitochondrial primers and used to assign a mitochondrial haplotype. We test this strategy using unknown samples from several countries in SSA as a step toward improving our ability to routinely characterize RKN species, especially from areas where our knowledge is less complete.

MATERIALS AND METHODS

Nematode strains. Characterized strains of RKN species used in this work are listed in Table 1. Strains with the designation BR following species name were from Brazil and have been previously identified or described using esterase phenotypes (8), sequence-characterized amplified region markers (18,19,42,47, 53) or morphological approaches (7,10,13). DNA of *M. arenaria* F was provided by L. Perfus-Barbeoch, INRA, Sophia, France and *M. arenaria* 03-1 was provided by J. Starr, Texas A&M. DNA was extracted from these samples as previously described (31,42).

Samples from Africa. Sampling of field-cultivated crops was undertaken in Benin, Kenya, Nigeria, and Tanzania between 2009 and 2012. Infected roots were observed for egg masses, which were individually removed, placed in individual wells, and allowed to hatch. When egg masses could not be easily observed, the roots were coarsely chopped and used to infect 3-week-old ‘Moneymaker’ tomato seedlings, grown in steam-sterilized soil in 1-liter pots. Upon maturity of the nematodes, egg masses were individually removed as above. To initiate pure species cultures, the juveniles (J2) from each single well were inoculated onto 3-week-old tomato Moneymaker seedlings grown in steam-sterilized soil in 1-liter pots in the screenhouse in each respective country. Upon maturity of the nematodes, 5 to 10 egg masses were removed from tomato roots of each single-egg mass culture and placed separately in 95% ethanol (1 ml) in a durable micro-fuge tube. Samples were shipped at ambient temperature then stored, upon receipt, at University of California, Davis at -20°C .

DNA extraction from ethanol-preserved samples. A single egg mass was transferred from ethanol to 5 ml of Tris-EDTA buffer in a 60-mm petri dish to hydrate for at least 15 min. The egg mass was teased apart to release eggs and emerged J2s. For each extract, 5 to 10 J2s or 10 disrupted eggs from a single egg mass were transferred into 10 μl of extraction buffer (10 mM Tris, 1 mM EDTA, 0.1% triton X, and proteinase K [PCR grade, Fermentas] at 1 mg/ml) and the nematodes were disrupted using a probe. Samples were frozen in 0.2-ml PCR tubes at -20°C overnight or longer. For extraction, samples were incubated at 56°C for 1 h followed by 95°C for 10 min, then used immediately for PCR or stored at -20°C .

mtDNA analysis. mtDNA fragments were amplified using primer sets TRNAH (TGAATTTTTTATTGTGATTAA) and MRH106 (AATTTCTAAAGACTTTTCTTAGT) or MORF (ATC GGGGTTTAATAATGGG) and MTHIS (AAATTCAATTGAA ATTAATAGC) developed by Stanton et al. (46). DNA amplification by PCR was carried out using one unit per reaction of Amplitaq DNA polymerase (Applied Biosystems) with Buffer II supplied by the manufacturer. Amplification was carried out in a volume of 25 μl with 0.5 μM each primer; 0.2 mM each dATP, dCTP, dGTP and dTTP; 3 mM MgCl_2 , and 1.5 μl of DNA extract. Amplification conditions consisted of an initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 60 s; and a final extension at 68°C for 7 min. DNA fragments were separated by electrophoresis in Tris-borate-EDTA buffer and 1.5% agarose gels containing GelRed (Biotium). Restriction enzymes *HinfI* and *MnII* were obtained from New England Biolabs and used as recommended by the manufacturer.

Fig. 1. Diagnostic region of root-knot nematode mitochondrial genome. The top bar represents the region of the mitochondrial genome spanning the gene encoding cytochrome oxidase II (COII), the intergenic spacer, tRNA^{His}, and the large subunit of the ribosomal RNA gene (l-rRNA). Position of the diagnostic DNA fragment amplified with primers C2F2 and 1108 (39) is shown below the bar. Below this are positions of fragments amplified with primer pairs MORF and MTHIS, and TRNAH and MRH106 (46). Numbers below fragments refer to positions in *Meloidogyne javanica* reference sequence (accession number L76261).

Prior to sequencing, PCR products were treated using an EXoSAP-IT for PCR product Cleanup kit (Affymetrix). Sequencing was carried out by the DNA Sequencing Facility, College of Biological Sciences, University of California, Davis, with the same primers as used to generate the fragment.

RESULTS

Mitochondrial haplotype determination of characterized RKN. Primers TRNAH and MRH106 were used to amplify a 557-bp region of the mitochondrial genome, including part of the large subunit rRNA gene (Fig. 1). We amplified DNA from characterized RKN strains, including multiple isolates of the most common MIG species and additional species listed in Table 1. A fragment of ≈ 557 bp was amplified from all tested strains except for *M. enterolobii*, which produced a fragment of ≈ 722 bp (Fig. 2A; Table 1). DNA sequence of the amplified fragments from *M. incognita*, *M. javanica*, *M. arenaria*, and the other clade I species, except for *M. enterolobii*, showed high sequence similarity (>99%) compared with the reference *M. javanica* sequence (L76261.1). *M. enterolobii* showed 87% similarity to the reference sequence, and examination of the sequence revealed that the increased length was due to an insertion of 166 bp adjacent to primer TRNAH. This same insertion was previously noted when *M. enterolobii* was amplified with primers C2F2/1108 and has been annotated as a tRNA^{his} gene (5). *M. hapla*, *M. chitwoodi*, and *M. exigua* sequence divergence from the *M. javanica* reference sequence was even greater (87, 80, 72, and 71% identity, respectively).

Comparison of the sequences of the amplified TRNAH/MRH106 fragment from the MIG species (all clade I species in Table 1 except for *M. enterolobii*) revealed minor differences in length due to single-nucleotide insertions or deletions in a 150-bp tract of 100% AT in the large subunit of the ribosomal RNA gene (1-rRNA) gene; however, these length differences did not correlate with species. The remaining sequence differences correlated strongly with species and are summarized in Figure 3. Several of the sequence polymorphisms between MIG species affected cleavage sites for *HinfI* or *MnII*, and cleavage of the amplification

product with these enzymes allowed *M. incognita*, *M. enterolobii*, *M. javanica*, and *M. hapla* to be easily differentiated (Fig. 2B and C; Table 1). For example, an A residue is present at position 1,182 in the reference *M. javanica* sequence and in both strains of *M. javanica* that we examined as well as in the reference sequence L76261.1. In all other MIG species, a G residue exists in this position, resulting in a cleavage site for the restriction enzyme *HinfI*. Due to an A residue at position 1,086, the *M. izalcoensis* fragment lacked an *MnII* site and produced a novel digestion pattern. However, *M. arabicida*, *M. ethiopica*, *M. hispanica*, *M. inornata*, *M. moroccensis*, *M. petunia*, and *M. paranaensis* produced the same TRNAH/MRH106 cleavage pattern as *M. arenaria* and this group of species was not differentiated.

In an attempt to further differentiate MIG species, we amplified DNA extracts with primers MORF and MTHIS, which direct amplification of the intergenic spacer region (Fig. 1). These primers amplified a fragment from all the MIG species but not from other RKNs, including *M. enterolobii*. A fragment of ≈ 743 bp was amplified from both strains of *M. javanica* tested as well as from *M. incognita* strains VW6, BR4, and BR6 (Fig. 2D; Table 1). However, *M. incognita* strain BR5 produced a shorter product (≈ 636 bp). The amplification product from *M. arenaria* strain BR3 was 743 bp whereas those from *M. arenaria* strains F and 03-1 were only 214 bp in length. DNA sequences of the amplified products from the two *M. javanica* strains were identical to each other and to the published *M. javanica* sequence (L76261.1, nucleotides 54 to 660). The corresponding regions from *M. incognita* strains VW6, BR4, and BR6 were identical to each other in sequence and differed by 8 nucleotides (nt) from L76261.1. These differences included the 6-nt changes reported by Stanton et al. (46) for Australian *M. incognita* isolates. The shorter length of the amplification product from *M. incognita* BR5 compared with the other *M. incognita* strains was due to two small deletions in the intergenic spacer (Fig. 4). *M. arenaria* strain BR3 amplified a 743-bp fragment that differed in 5 nt from L76261.1, whereas the 214-bp amplification products from *M. arenaria* strains F and 03-1 were identical to each other in sequence and showed a deletion of 530 nt within the intergenic region (Fig. 4). The MORF/MTHIS amplification product from

TABLE 1. Root knot nematode strains and their mitochondrial amplification products

Clade	Species, strain	Est ^b	TRNAH/MRH106 fragment (bp) ^a			MORF/MTHIS Fragment (bp)	Host; geographical origin; source or reference
			Frag	<i>HinfI</i> dig	<i>MnII</i> dig		
I	<i>Meloidogyne arenaria</i> G	–	557	445, 112	340, 140, 77	214	Tomato; Guadeloupe; L. Perfus-Barbeoch, INRA
I	<i>M. arenaria</i> 03-1	–	557	445, 112	340, 140, 77	214	Peanut; Texas; J. Starr, Texas A&M
I	<i>M. arenaria</i> BR3	A2	557	445, 112	340, 140, 77	743	Soybean; Ibituba, RS, Brazil; 16
I	<i>M. enterolobii</i> BR17	E4	723	723	583, 140	NP	Guava; Petrolina, PR, Brazil; 48
I	<i>M. enterolobii</i> CR	–	722	722	582, 140	NP	Guava; Costa Rica; 29
I	<i>M. incognita</i> BR4	I1	557	396, 112, 49	340, 217	742	Soybean; Londrina, PR, Brazil; 16
I	<i>M. incognita</i> BR5	I2	557	396, 112, 49	340, 217	636	Cotton; Umuarama, PR, Brazil; 44
I	<i>M. incognita</i> BR6	I2	557	396, 112, 49	340, 217	742	Cotton; Campo Verde, MT, Brazil; 44
I	<i>M. incognita</i> VW6	–	557	396, 112, 49	340, 217	742	Cotton; California; 50
I	<i>M. javanica</i> VW4	J2	558	558	341, 140, 77	743	Tomato; California; 52
I	<i>M. javanica</i> BR1	J2	558	558	341, 140, 77	743	Tomato; Brasília, DF, Brazil; 45
II	<i>M. hapla</i> VW9	H1	556	446, 110	556	NP	Tomato; California; 31
II	<i>M. hapla</i> LM	–	556	446, 110	556	NP	Unknown; La Mole, France; 50
II	<i>M. hapla</i> BR19	H1	556	446, 110	556	NP	Strawberry; Botucatu, SP, Brazil; 11
I	<i>M. arabicida</i> BR14	AR2	556	444, 112	339, 140, 77	789	Coffee; Turialba, Costa Rica; 7
I	<i>M. ethiopica</i> BR18	E3	557	445, 112	340, 140, 77	742	Kiwi; Faropilha, RS, Brazil; 14
I	<i>M. hispanica</i> BR8	Hi3	559	447, 112	342, 140, 77	744	Squash; Jacupe, BA, Brazil; 9
I	<i>M. inornata</i> BR9	I3	557	445, 112	340, 140, 77	742	Yacon; Capão Bonito, SP, Brazil; 13
I	<i>M. izalcoensis</i> BR15	I4	558	446, 112	418, 140	758	Coffee; Izalco, El Salvador; 10
I	<i>M. moroccensis</i> BR7	A3	558	446, 112	341, 140, 77	214	Soybean; Santa Rosa, RS, Brazil; 16
I	<i>M. paranaensis</i> BR11	P1	557	445, 112	340, 140, 77	425	Coffee; Londrina, PR, Brazil; 7
I	<i>M. paranaensis</i> BR12	P2	557	445, 112	340, 140, 77	425	Coffee; Herculândia, SP, Brazil; 7
I	<i>M. petunia</i> BR16	Pe2	557	445, 112	340, 140, 77	742	Petunia; Riacho Fundo, DF, Brazil; 17
III	<i>M. chitwoodi</i> WAMC	–	547	547	547	NP	Potato; Washington; 36
III	<i>M. exigua</i> BR13	E1	556	556	556	NP	Coffee; Lavras, MG, Brazil; 37

^a Fragment sizes predicted from partial sequence and band sizes after gel electrophoresis; NP = no product or weak, nonspecific bands.

^b Est = esterase isozyme; – = not available.

M. morocciensis was 214 bp in length and identical in sequence to that of *M. arenaria* strains F and 03-1. *M. paranaensis* also carried a deletion in the intergenic spacer compared with L76261.1. The MORF/MTHIS fragment of *M. hispanica* BR8 differed from L76261.1 by 15 nt and was identical in sequence to the corresponding region in a previously reported sequence from *M. hispanica* (JN673274). Fragments amplified from *M. inornata* BR9, *M. petunia* BR16, and *M. ethiopica* BR18 were identical in sequence and differed by 5 nt from L76261.1. MORF/MTHIS fragments amplified from *M. izalcoensis* and *M. arabicida* differed in length from 743 bp due to various insertions or deletions compared with *M. javanica* (Fig. 4).

Mitochondrial haplotype analysis of samples from Africa. Comparison of mtDNA fragments amplified in our study with those previously found (46) supported a robust key that separates the major tropical RKN species into mitochondrial haplotypes (Table 2). All *M. javanica* strains, and only strains of this species, produced a match for haplotype D. All *M. incognita* strains tested, with the exception of *M. incognita* BR5, and only *M. incognita* strains produced the haplotype B pattern. We refer to *M. incognita* BR5, which produced a shorter MORF/MTHIS fragment, as *M. incognita* variant 1 (V1) and the haplotype pattern as B'. Two of the three *M. arenaria* strains that we tested matched haplotype A and the third corresponded to haplotype G. Several additional

MIG species produced patterns corresponding to haplotype G and were not differentiated by this assay. The TRNAH/MRH106 pattern produced by *M. enterolobii*, which was not previously examined, was assigned haplotype K (Table 2).

To test the utility of the key in Table 2 for categorizing new isolates of RKNs, we prepared DNA extracts, each derived from a single egg mass, from 103 ethanol-preserved samples obtained from four countries in SSA. With primer set TRNAH/MRH106, an amplification product was obtained for all extracts tested. For 86 extracts, the product size was ≈ 556 nt and, for the remaining extracts, the product was ≈ 723 bp, as expected for *M. enterolobii*. From some extracts small, less-intense bands were seen at <100 bp following amplification; therefore, haplotype assignments following digestion were based on consideration only of fragments >100 bp. Based on the sizes of the *MnII* and *HinfI* digestion products of the TRNAH/MRH106 fragments and the presence and size of the MORF/MTHIS fragment, we were able to assign a haplotype to all but three isolates (Table 3). Two isolates, both from coastal region of Kenya (Mtwapa) on tomato, produced a haplotype B pattern for the TRNAH/MRH106 fragment following digestion with the *MnII* but a haplotype G pattern following *HinfI* digestion. DNA sequence of the amplified fragment showed that it differed from that of *M. incognita* by a single nucleotide, resulting in the loss of an *HinfI* site (H-790 in Fig. 2). One sample

Fig. 2. Diagnostic amplification products from mitochondrial genomes of *Meloidogyne* spp. **A**, Agarose gel showing sizes of amplification products from characterized root-knot nematode strains obtained using primers TRNAH and MRH106. Fragments obtained after cleavage of the same products with *HinfI* (**B**) or *MnII* (**C**). **D**, Amplification products obtained with primers MORF/MTHIS. Species and strain of DNA source are indicated below each lane: Mi = *Meloidogyne incognita*, Mj = *M. javanica*, Ma = *M. arenaria*, Me = *M. enterolobii*, and Mh = *M. hapla*. Lanes labeled M contain 100-bp marker ladder (Fermentas), with the position of the 500-bp band indicated by an arrow.

from Benin produced the TRNAH/MRH106 cleavage patterns of haplotype D but the MORF/MTHIS appeared slightly shorter. Sequence revealed a 35-nt deletion compared with L7261.1 (nucleotides 311 to 345).

Eight extracts from Benin and nine from Nigeria were assigned to haplotype K (*M. enterolobii*) based on the size and digestion pattern of the TRNA/MRH106 fragment and the lack of an amplification product using the MORF/MTHIS primers. Sequence

Fig. 3. Sequence polymorphism comparisons of TRNAH/MRH106 amplification products from *Meloidogyne incognita* group species. The diagram shows the sites of restriction site polymorphisms used to distinguish species. Numbers below fragments refer to positions in *M. javanica* reference sequence (accession number L76261.1). H and M indicate positions of diagnostic cleavage sites for enzymes *HinfI* and *MnlI*, respectively. The gray shading shows the position of a stretch of 152-nucleotide sequence that lacks any G or C nucleotides. This region was excluded from the sequence comparison. Sites in parentheses are missing in *M. javanica* but present in other species. Nucleotides that are identical with those of L76261 are indicated by a dot (•); those that differ are indicated by a letter corresponding to the nucleotide; - indicates that a single nucleotide is missing.

Fig. 4. Comparison of sizes of amplification products spanning the mitochondrial intergenic spacer region of *Meloidogyne incognita* group root-knot nematode species. The top line represents the reference fragment size amplified from *M. javanica* reference sequence L76261 using primers MORF/MTHIS. Brackets indicate positions of deletions compared with L76261 and triangles below lines indicate positions and size of insertions.

analysis of the nine fragments from Nigeria revealed that six were identical and three were 99% identical to BR17, supporting their identification as *M. enterolobii*.

DISCUSSION

This work was stimulated by the need for a simple diagnostic method to identify agriculturally damaging RKNs, especially for tropical regions with limited resources. An important consideration was the ability to utilize DNA extracted from preserved samples and, in this work, we used samples preserved in ethanol to test the assay. We also used primers that would produce a product from a spectrum of species rather than species-specific primers, which we considered important, because prior knowledge of the species present is often lacking. In preliminary experiments, we compared the ability of the mitochondrial primer sets developed by Powers and Harris (39) and by Stanton et al. (46) to amplify samples prepared in various ways and found that the latter primer sets had a higher success rate and better resolved MIG species. Optimization of the assay conditions, most notably lowering the PCR extension temperature to 68°C, further increased this success rate. The current protocol works well with J2 and eggs from ethanol-preserved egg masses and produced informative amplification products from all 103 samples from SSA. In other tests, we found that single females dissected from ethanol-preserved root pieces, males, or fresh J2 produced extracts that amplified well (results not shown). However, Baermann funnel extracts stored in ethanol were not useful because nematode morphology was poorly preserved and RKN J2 could not readily be distinguished from other nematodes in the extract.

Previous work (46) assigned haplotypes to the most common species of RKN found in Australian agriculture. To test and extend the utility of the assay, we amplified DNA from a wide range of species from diverse geographical locations, prepared from well-characterized RKN strains, including several strains of the most common species (Table 1). The product produced by the TRNAH/MRH106 primers, which spans part of the mitochondrial l-rRNA gene and has not been widely used for species identification, was particularly useful. These primers amplified products from all RKN species tested, including more distantly related species *M. hapla*, *M. chitwoodi*, and *M. exigua*. In addition, *M. enterolobii*, which was not previously assessed, was easily dis-

tinguished from MIG species and all the other RKN species tested by the distinct fragment size. In agreement with the previous results, *M. incognita* and *M. javanica* gave consistent fragment sizes and cleavage patterns that uniquely defined these two species. However, most other MIG species were not resolved with this primer pair.

Amplification with primers MORF/MTHIS, which flank the nontranscribed spacer, produced a product only for MIG species. Comparison of product sizes resolved some species but also revealed within-species variability for the length of the intergenic spacer, as has been previously noted (5). One isolate of *M. incognita* amplified a slightly smaller MORF/MTHIS fragment (listed as haplotype B' in Table 2). More notably, strains identified as *M. arenaria* fell into two groups based on the size of amplification product with MORF/MTHIS primers. In Table 2, we refer to the strains that amplify a 214-bp fragment (haplotype A) as *M. arenaria* because these appear to have the widest geographic distribution (5,39,51). The remaining strains amplify a 743-bp MORF/MTHIS fragment and can be further subdivided based on the TRNAH/MRH106 pattern. A group of strains from Australia identified as *M. arenaria* produced a TRNAH/MRH106 cleavage pattern not seen for any of the known samples in this study and were classified as haplotype C/H (46). We refer to these isolates as *M. arenaria* V1 and to the isolate described in our study that falls into haplotype G as *M. arenaria* V2 (Table 2). Within-species differences have previously been noted for isolates of *M. arenaria* in host range and using isozymes, RFLP analysis, PCR markers, and morphological characters (12,24,27), together indicating that this species, as currently defined, encompasses a wider range of diversity than other MIG species.

In spite of intraspecies differences in length, the intergenic region may be useful for resolving some of the species grouped into haplotype G. For example, the MORF/MTHIS fragment of *M. hispanica* BR8 differed in sequence at several positions from *M. javanica* but was identical to the corresponding region in a previous report for *M. hispanica*. MORF/MTHIS fragments amplified from *M. paranaensis*, *M. izalcoensis*, and *M. arabicida* differed in length from 743 bp due to various insertions or deletions compared with *M. javanica* (Fig. 3), and these differences may be diagnostic. However, we examined only one or two strains of each of these species. Additional isolates should be examined to better determine whether these differences could be

TABLE 2. Mitochondrial haplotype patterns of root-knot nematodes

Haplotype	TRNAH/MRH106 ^a				Species ^b	
	Uncut	HinfI cut	MnlI cut ^c	MORF/MTHIS ^d	Stanton et al.	This work
A	557	445, 112	340, 140, 77	214	<i>Meloidogyne arenaria</i>	<i>M. arenaria</i> , <i>M. moroccensis</i>
B	557	396, 112, 49	340, 217	743	<i>M. incognita</i>	<i>M. incognita</i>
B'	557	396, 112, 49	340, 217	636	–	<i>M. incognita</i> V1
C/H	557	445, 112	417, 140	743	<i>M. arenaria</i> V1	–
D	557	557	340, 140, 77	743	<i>M. javanica</i>	<i>M. javanica</i>
G	557	445, 112	340, 140, 77	743	<i>M. hispanica</i>	<i>M. arenaria</i> V2, <i>M. ethiopica</i> , <i>M. hispanica</i> , <i>M. inornata</i> , <i>M. petunia</i>
K	723	723	583, 140	NP	–	<i>M. enterolobii</i>

^a Fragment sizes vary slightly due to lengths of AT tracts.
^b V1 or V2 indicate strains with mitochondrial haplotypes different from the most prevalent for that species.
^c MnlI cleaves DNA at 7 to 8 nucleotides from its recognition site; to reflect this, we have adjusted fragment sizes from those in Stanton et al. (46).
^d NP = no product or weak, nonspecific bands.

TABLE 3. Mitochondrial haplotypes of Africa samples

Country	Number of samples	Mitochondrial haplotype						
		A	B	C/H	D	G	K	Novel
Tanzania	15	1	3	0	11	0	0	0
Kenya	15	1	7	0	5	0	0	2
Nigeria	50	0	36	0	5	0	9	0
Benin	23	0	7	0	4	3	8	1

used as a diagnostic. Although a considerable resource of sequences spanning the intergenic spacer of various MIG species is available in the public domain, because this region is internal to that amplified by primers developed by Powers and Harris (39), for many of these entries, a species designation is not provided. In cases where species are specified, the criteria used for species assignment are not usually provided, limiting their value in assigning diagnostic characters. Additional diagnostic criteria may be necessary to assign species for some haplotype G samples. For example, species-specific primers have been developed for several haplotype G species and may be useful for resolution within this group (4).

Our goal here was to identify MIG species and other clade I species such as *M. enterolobii* because these groups are predominant in tropical agriculture. However, three isolates of *M. hapla*, the most prevalent clade II species, and two clade III species, *M. chitwoodi* and *M. exigua*, were also included in this work. All of these species produce an amplification product with TRNAH/MRH106 primers but none of the products were digested with *MnII*, distinguishing them from MIG species and *M. enterolobii* (Table 1). For identification of species outside of the MIG, or for determining the relationship of new species to those that have been characterized, a large resource of 18S rDNA sequences is available (26).

Origin and evolution of MIG species. MIG species have been defined by morphological and molecular characters as well as by host range (30). However, because these species reproduce asexually and molecular evidence indicates that they are derived from interspecific hybrids, determining species boundaries is a complex issue. The limited differences between mitochondrial genomes of MIG species supports the theory of a recent separation from a common (or highly related) ancestral mother. Nonetheless, these small differences, especially for the TRNAH/MRH106 fragment, align with species as determined by other criteria, most notably esterase phenotype, and are of diagnostic value. The uniformity of sequence of the mitochondrial region in isolates of the most common species from Australia, Brazil, the United States, and Africa indicates that global dissemination is recent and likely occurred through agricultural practices. Within-species differences in length of the intergenic region indicate that these changes may be more recent and may define separately evolving lineages (Fig. 4). The presence of many species in haplotype G may imply that this group is evolving more rapidly. Alternatively, if, as suggested by Fargette et al. (23) and Hugall et al. (28), diversity is due to ancestral crosses before parthenogenesis, haplotype G species may represent lineages derived from closely related females mating with more diverse males.

What we learned about distribution of RKN in Africa. Each of the 103 samples from SSA that we examined produced mitochondrial haplotypes consistent with MIG species or *M. enterolobii*, with matches to haplotypes B and D, corresponding to *M. incognita* and *M. javanica*, respectively, highly represented in isolates from all countries. Several West African isolates, based on the TRNAH/MRH106 fragment size, were identified as haplotype K (*M. enterolobii*) but this haplotype was not observed from East Africa. Two samples from East Africa were of haplotype A (*M. arenaria*), which was not identified in West Africa. Only three samples, all from Benin, were a match for haplotype G. These haplotype G samples and the three novel haplotypes identified from Kenya and Benin will require further attention to identify.

Because we have examined only one egg mass per isolate from a limited range of locations and hosts, our work should be considered a proof of concept rather than a comprehensive description of species present in these countries. Greater depth of assessment, including using several egg masses from each locale to detect mixed populations, would provide a more thorough picture. However, our findings are consistent with the range of species presented in other reports from SSA (3,5,23). Altogether, our

study indicates that the mitochondrial haplotype approach is a useful and straightforward first step to assess the range and variability of clade I RKN species in areas that have not yet been well surveyed.

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Erratum

The labeling on Figure 2A, B, and C has been corrected to read Mj VW4 and Mi VW6. Changes to this article were made on 4 March 2015.