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Diversity of Root-knot Nematodes Associated with Tubers of Yam (*Dioscorea* spp.) Established Using Isozyme Analysis and Mitochondrial DNA-based Identification

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Abstract: The root-knot nematodes (RKN), *Meloidogyne* spp., represent an important threat to yam (*Dioscorea* spp.) production in West Africa. With the aim to establish the diversity of RKN species affecting yam tubers, for control and resistance screening purposes, surveys were conducted in the main yam producing areas of Nigeria. Galled tubers (N = 48) were collected from farmers' stores and markets in nine states in Nigeria and in one district in Ghana. RKN isolated from yam tubers were identified using enzyme phenotyping (esterase and malate dehydrogenase) and mitochondrial DNA (mtDNA) NADH dehydrogenase subunit 5 (Nad5) barcoding. Examination of 48 populations revealed that yam tubers were infested by *Meloidogyne incognita* (69%), followed by *M. javanica* (13%), *M. enterolobii* (2%), and *M. arenaria* (2%). Most of the tubers sampled (86%) were infected by a single species, and multiple species of RKN were detected in 14% of the samples. Results of both identification methods revealed the same species, confirming their accuracy for the identification of these tropical RKN species. In addition to *M. incognita*, *M. javanica*, and *M. enterolobii*, we report for the first time *M. arenaria* infecting yam tubers in Nigeria. This finding extends the list of yam pests and calls for caution when developing practices for yam pest management.

Key words: detection, esterase, isozymes, *M. arenaria*, *M. enterolobii*, *M. incognita*, *M. javanica*, malate dehydrogenase, *Meloidogyne*, Nad5, sequences, yam.

Yam (*Dioscorea* spp.) is the second most important tuber crop after cassava (*Manihot esculenta*) in sub–Saharan Africa. It provides a valuable source of carbohydrates for more than 60 million people from an estimated annual production of 44 million MT (Nweke et al., 1991; Orkwor 1998; FAO, 2014). More than 90% of the total world yam production is produced in West Africa (FAO, 2014), primarily by smallholder farmers. Of the various constraints affecting yam production, plant-parasitic nematodes are particularly problematic (Ayensu and Coursey, 1972; Bridge et al., 2005; Arnau et al., 2010). Root-knot nematodes (RKN) (*Meloidogyne* spp.) are the most economically important nematode group across crop production systems (Sasser, 1980; Jones et al., 2013) and are among the most important pests of yam. In West Africa, and especially in Nigeria, *Meloidogyne incognita* and *M. javanica* are commonly reported from yam tubers (Unny and Jerath, 1965; Adesiyan and Odihirin, 1978; Nwauzor and Fawole, 1981; Bridge et al., 2005; Onkendi et al., 2014). Caveness (1967) also recovered *M. arenaria* from yam fields but only from the soil. Root-knot nematode infection of yam can inflict substantial losses during production and storage, causing serious galling and crazy root symptoms on tubers, affecting their marketable value or rendering them unmarketable even (Ekundayo and Naqvi, 1972; Fawole, 1988; Bridge and Starr, 2007). Synthetic chemical treatment can reduce the problem, but is in general not commonly used, due in part to their cost and also due to the removal from the market of the most noxious products for environmental reasons (Castagnone-Sereno, 1988; Haydock et al., 2006; Nyczepir and Thomas, 2009). In light of increasingly intensified yam cropping systems and a seemingly corresponding rise in nematode problems on yam (Akinola and Owombo, 2012), there is urgency to identify and develop nematode management options, including the breeding of resistant cultivars and promoting use of nematode-free seed material (Aighewi et al., 2015). Although tropical RKN are known for their high pathogenicity and their wide host range (Jepson, 1987; Moens et al., 2009; Onkendi et al., 2014), their accurate identification is an important step to achieving appropriate management strategies. Identification of RKN species, especially tropical RKN, continues to pose an obstacle, given their morphological similarity and that multiple *Meloidogyne* species regularly occur together (Karssen et al., 2013). Traditionally, morphometrics, perennial patterns (Hunt and Handoo, 2009), and the host range test (Hartman and Sasser, 1985), have been relied on for species identification. However, these methods have serious limitations. DNA-based techniques, such as the use of Restriction Fragment Length Polymorphism (Curran et al., 1986; Powers et al., 2005) or species-specific primers (Zijlstra et al., 2000; Qui et al., 2006; Adam et al., 2007; Kiewnick et al., 2013), have been developed and successfully used to identify the tropical RKN species. Using the species-specific primers, which amplify Sequence-Characterized Amplified Regions, is simple, life-stage

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TABLE 1. Details on the samples of galled yam tubers collected in Nigeria and in Ghana from 2012 to 2014 and used for identification of root-knot nematode species.

State	Locality	Latitude (°)	Longitude (°)	Altitude (m)	Yam variety[a]	Source[b]	Year	Sample code[c]	Pure-population
Abia	Isiala-ahala	5.38346	7.54485	137	TDr-Onitsha	F	2012	**Isiala-ahala 1**	
	Isiala Ngwa South	5.28921	7.33037	97	TDr-Ugu	M	2012	**Isiala Ngwa South 1**	
Abuja	Gwagwalada	8.95105	7.10912	188	TDr-Makakusa	M	2013	**Gwagwalada 1**	
					TDr-Makakusa	M		**Gwagwalada 2**	
	Ijah	8.7981	7.08173	244	TDr-Gwagwa	F	2013	**Ijah 1**	
	Kutunku	8.92875	7.05503	204	TDr-Makakusa	F	2013	**Kutunku 1**	
					TDr-Makakusa	F	2013	**Kutunku 2**	
					TDr-Hembakwase	F	2013	**Kutunku 3**	
					TDr-Gwari	F	2013	**Kutunku 4**	
					TDr-Gwari	F	2013	**Kutunku 5**	
					TDr-Makakusa	F	2013	**Kutunku 6**	
	Kwali	8.87588	7.12596	260	TDr-Gwari	F	2012	**Kwali 1**	Kwali 1_1
					TDr-Gwari	F	2012	**Kwali 2**	Kwali 2_2
									Kwali 2_6
								Kwali 3	Kwali 3_2
Anambra	Igbariam	6.30112	6.96508	69	TDr-Obiaoturugo	F	2013	**Igbariam 1**	
					TDr-Obiaoturugo	F	2013	Igbariam 2	
Benue	Otukpo	7.19181	8.13369	137	TDr-Opeke	M	2012	Otukpo 1	
					TDr-Ame	M	2012	**Otukpo 2**	Otukpo 2_1
		7.04758	8.05616	159	TDr-Ame	M	2012	**Otukpo 3**	Otukpo 3_4
		7.19212	8.13327	196	TDr-Amula	M	2013	Otukpo 4	
					TDa-Matches	M	2013	**Otukpo 5**	
					TDr-Chenke	M	2013	Otukpo 6	
					TDr-Pepa	M	2013	Otukpo 7	
	Tsiabie 1	7.26453	8.2509	108	TDr-Ame	F	2013	**Tsiabie 1**	
Ekiti	Ikole	7.80343	5.52085	587	TDr-Idere	M	2013	**Ikole 1**	
Kogi	Abekpe	7.8143	5.86995	504	TDr-Agbakumo	F	2013	**Abekpe 1**	
					TDr-Okumodu	F	2013	**Abekpe 2**	
	Ega	7.10123	6.72912	29	TDr-Ame	M	2013	Ega 1	
	Idah	7.11558	6.74378	93	TDr-Akpaji	F	2013	**Idah 1**	
					TDr-Abudokie	F	2013	Idah 2	
	Okene check point	7.527	6.25557	326	TDr-Idere	M	2013	**Okene check point 1**	
	Oke-Ola Iyakaba	7.80582	6.07788	424	TDr-Chukuchuku	M	2013	Oke-Ola Iyakaba 1	
Nasarawa	Eggon	8.71445	8.5409	271	TDr-Aloshi	F	2013	**Eggon 1**	
	Kadaroko	8.22377	8.57468	271	TDr-Ogoja	F	2013	**Kadaroko 1**	
	Kokona	8.84788	8.01392	314	TDr-Gwari	M	2012	**Kokona 1**	Kokona 1_2
									Kokona 1_7
					TDr-Amula	M	2012	**Kokona 2**	Kokona 2_1
									Kokona 2_2
					TDr-Oda	M	2012	**Kokona 3**	Kokona 3_1
									Kokona 3_3
									Kokona 3_5
									Kokona 3_6
					TDr-Aloshi	M	2012	**Kokona 4**	Kokona 4_1
	Rimi Uka	8.49365	8.51598	175	TDr-Pepa	M	2012	**Rimi Uka 1**	Rimi Uka 1_1
									Rimi Uka 1_2
Niger	Kpaki	9.29105	5.2696	124	TDr-Hembakwase	F	2013	**Kpaki 1**	
		9.291	5.27133	121	TDr-Hembakwase	F	2013	**Kpaki 2**	
	Lambata	9.28007	6.99692	280	TDr-Hembakwase	M	2013	**Lambata 1**	
	Tufakampani	9.24145	6.91663	256	TDr-Gwagwa	F	2013	Tufakampani 1	
					TDr-Hembakwase	F	2013	**Tufakampani 2**	
					TDr-Hembakwase	F	2013	**Tufakampani 3**	
Oyo	Akobo	7.43258	3.94331	235	Celosia	F		Akobo 1	
	Saki	8.67718	3.39945	505	TDr-Amula	M	2013	Saki 1	
East Gonja	Akarma	8.57755	-0.52029	162	TDr-Puna	M	2014	Akarma 1	

[a] TDa = Tropical *Dioscorea alata*; TDr = Tropical *Dioscorea rotundata*.
[b] Source: F = farmer's store, M = market.
[c] All samples were collected in Nigeria except for Akarma 1 collected in the district of East Gonja, Ghana. Samples in bold were analyzed with the isozymes phenotyping.

TABLE 2. Root-knot nematode species identified from yam tubers in Nigeria using isozyme analysis and mtDNA-based technique and enzyme analysis.

		PhastSystem						mtDNA-based technique				
		Enzyme profiles[b]		RKN[c]				RKN[d]				Sequences (Nad5)
Sample code[a]	Pure-population	Est	Mdh	Ma	Me	Mi	Mj	Ma	Me	Mi	Mj	Accession numbers
Isiala-ahala 1		I2	N1			✓						
Isiala Ngwa South 1		I2	N1			✓						
Gwagwalada 1		I, I1, I2	N1, N1			✓						
Gwagwalada 2		I, I2	N1, N1, N1			✓						
Ijah 1		M2, I, I2	N1a, N1		✓	✓						
Kutunku 1		I1, I2	N1			✓						
Kutunku 2		I2	N1			✓						
Kutunku 3		J3	N1				✓					
Kutunku 4		E3, M2	N1a		✓						✓	KY522787
Kutunku 5											✓	KY522788, KY522789
Kutunku 6		I1, I2	N, N1			✓						
Kwali 1	Kwali 1_1	I1, I2	N1			✓				✓		KY522782, KY522783
Kwali 2	Kwali 2_2	I1, I2	N1			✓				✓		KY522753
	Kwali 2_6	I2	N1			✓						
Kwali 3	Kwali 3_2	I1	N1, N1			✓						
Igbariam 1		M2, I1, I2	N1a, N1		✓	✓			✓	✓		KY522747, KY522759, KY522760
Igbariam 2									✓			KY522748
Otukpo 1										✓		KY522773, KY522774, KY522775
Otukpo 2	Otukpo 2_1	I1, I2	N1			✓				✓		KY522754, KY522776, KY522777
Otukpo 3	Otukpo 3_4	I1	N1			✓						
Otukpo 4										✓		KY522770
Otukpo 5		A2	N3	✓				✓				KY522743, KY522744, KY522745, KU372355
Otukpo 6										✓		KY522771
Otukpo 7										✓		KY522772
Tsiabie 1		J3	N1, N1				✓				✓	KY522786, KU372416
Ikole 1		I1, I2	N1, N1			✓						
Abekpe 1		I1, I2	N1, N1			✓						
Abekpe 2		I1, I2	N1, N1			✓				✓		KY522752

		PhastSystem						mtDNA-based technique				
		Enzyme profiles[a]		RKN[b]				RKN[c]				Sequences (Nad5)
Sample code	Pure-Pop	Est	Mdh	Ma	Me	Mi	Mj	Ma	Me	Mi	Mj	Accession numbers
Ega 1		I2	N1			✓				✓		KY522769
Idah 1		I2	N1			✓				✓		KY522756, KY522757, KY522758, KU372362
Idah 2											✓	KY522784, KY522785
Okene check point 1		I1	N1			✓						
Oke-Ola Iyakaba 1										✓		KY522768
Eggon 1		M2, I2	N1a, N1		✓	✓			✓			KY522746
Kadaroko 1		J3	N1				✓					
Kokona1	Kokona 1_7	I1	N1			✓				✓		KY522780, KY522781
Kokona 2	Kokona 2_1	I1	N1			✓				✓		KY522778, KY522779
	Kokona 2_2	I2	N1			✓						
Kokona 3	Kokona 3_1	J3	N1				✓				✓	KY522790
	Kokona 3_3	J3	N1				✓					
	Kokona 3_5										✓	KY522791, KY522792, KY522793, KY522794
	Kokona 3_6	J3	N1				✓					
Kokona 4	Kokona 4_1	I2	N1			✓						
Rimi Uka 1	Rimi Uka 1_1									✓	✓	KY522755, KY522795
	Rimi Uka 1_2	I1, I2	N1			✓						
Kpaki 1		I	N1			✓						
Kpaki 2		I1, I2	N1			✓						
Lambata 1		M2, N1, J3	N1a , N1		✓	✓	✓					
Tufakampani 1										✓		KY522765, KY522766, KY522767, KY522761, KY522762
Tufakampani 2		I2	N1			✓				✓		
Tufakampani 3		A2, I2	N3, N1	✓		✓		✓		✓		KY522742, KY522763, KY522764, KU372353
Akobo 1		M2, N1, J3	N1, N1a		✓	✓	✓			✓		KU372374
Saki 1		I2	N1			✓						
Akarma 1[¥]										✓		KY522749, KY522750, KY522751

[a] All samples were collected in Nigeria except for Akarma 1 collected in the district of East Gonja, Ghana.
[b] Est = Esterase, Mdh = Malate dehydrogenase. Enzyme patterns are given following the alphabetical order of root-knot nematodes species and a comma is used to list multiple patterns. Rows filled in dark grey represent samples identified using both method. Samples in rows filled in grey are identified using both techniques.
[c,d] Ma = *Meloidogyne arenaria*, Me = *M. enterolobii*, Mi = *M. incognita*, Mj = *M. javanica*.
[e] Not original sequence.

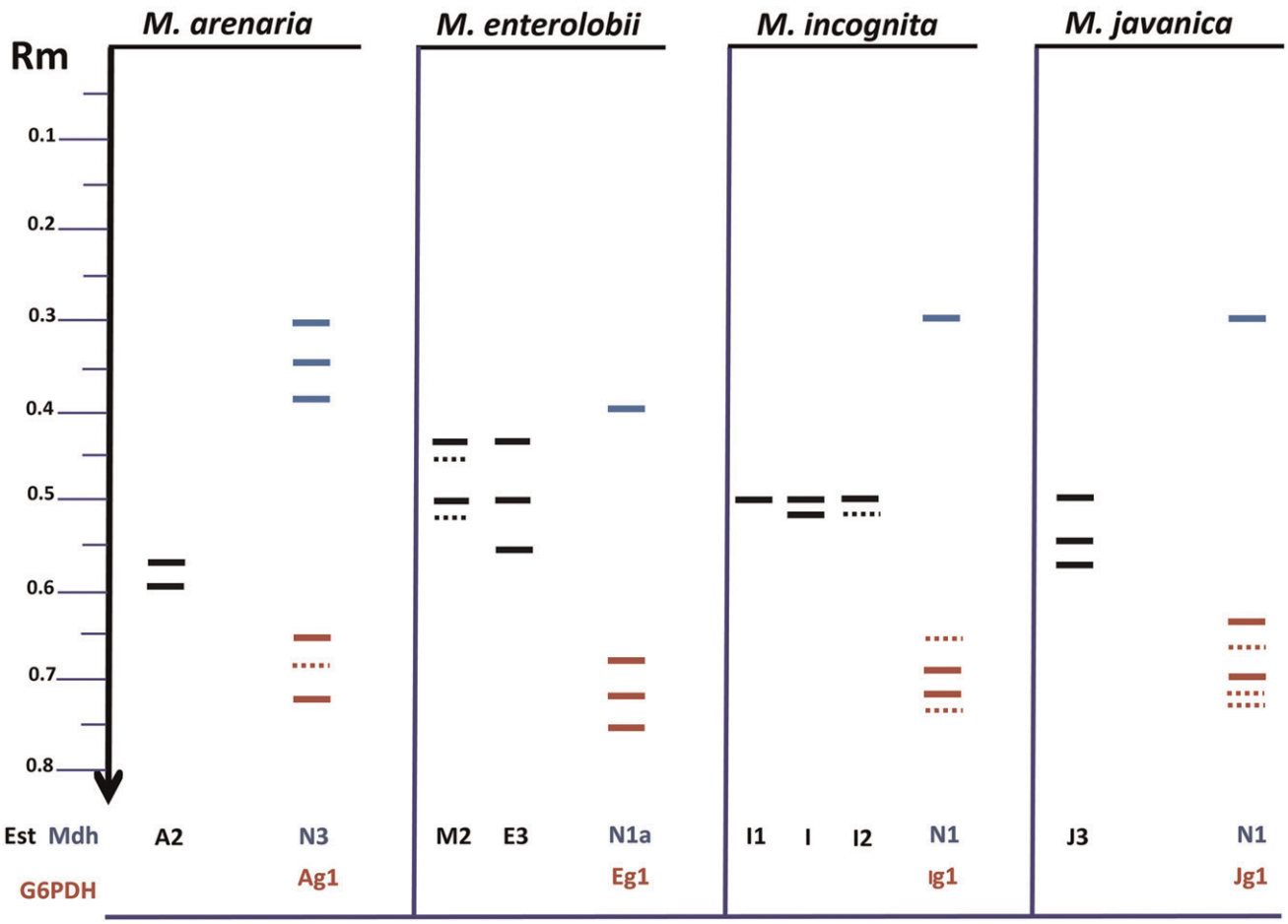

Fig. 1. Enzyme patterns observed for *Meloidogyne arenaria*, *M. enterolobii*, *M. incognita*, and *M. javanica* on yam tubers from Nigeria. Est = Esterase; Mdh = Malate dehydrogenase; G6PDH: Glucose-6-phosphate dehydrogenase G6PDH (This enzyme pattern is always associated to the Mdh staining).

independent, cost-efficient, and permits numerous samples to be run within a reasonable amount of time. However, some challenges include ambiguous results, low sensitivity, poor band visibility, and lack of reproducibility between laboratories (Adam et al., 2007; Blok and Powers, 2009; Onkendi et al., 2014). The biochemical-based diagnostic technique, reliant on variations in esterase and malate dehydrogenase (Mdh) isozyme profiles, remains one of the most reliable and widely used diagnostic techniques for *Meloidogyne* species (Esbenshade and Triantaphyllou, 1985; Karssen et al., 1995; Carneiro et al., 2000), even though this technique is less important for identification of other plant pathogens. However, the technique is not without its drawbacks, such as (i) it is only applicable to young adult females and (ii) difficulty in interpreting profile variants between and within species (Blok and Powers, 2009).

Building on the work of Pagan et al. (2015), Janssen et al. (2016) used mitochondrial haplotypes that are strongly linked and consistent with traditional esterase isozyme patterns, indicating that the barcode region Nad5 can reliably identify the major lineages of tropical RKN.

The current study was aimed at determining the range of RKN species affecting yam and their distribution across the main yam growing area in Nigeria, using Nad5 barcoding, and comparing the results with isozyme identification.

Materials and Methods

Yam tuber collection and nematode culturing: Tubers infected with RKN, showing clear symptoms of galling, were collected from vendors in markets and farmers' stores in major yam growing areas during surveys. Surveys were carried out from 2012 to 2014 in Nigeria (Table 1) covering three agroecological zones viz. the Humid Forest, the Derived Savanna, and the Southern Guinea Savanna. Nematodes isolated from 48 samples (Table 1) were reared on seedlings of tomato (*Solanum lycopersicum* cv. Marmande) and plumed cockscomb (*Celosia argentea*) (Caveness and Wilson, 1977) in pots of steam-sterilized soil in the greenhouse (24–32°C), following the addition of chopped peels of galled yam tubers. One sample from galled tuber from Ghana (Table 1) was also included in the study. From 8 weeks

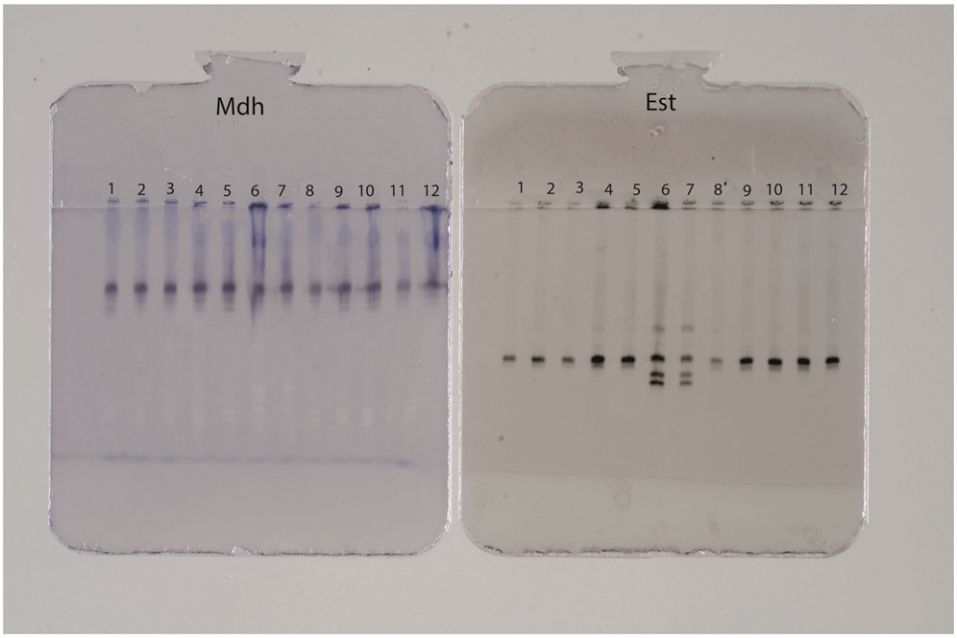

FIG. 2. Malate dehydrogenase (Mdh) and esterase (Est) profiles of *Meloidogyne incognita* (Lanes 1–5 and 8–12) from yam tubers in Nigeria. Reference sample *M. javanica* (Lanes 6 and 7).

after inoculation, plants were checked regularly for nematode development and 10 young egg-laying females removed for species identification using isozyme analysis. Additionally, individual egg masses were removed and single-egg mass cultures were established on tomato to generate pure, single species cultures.

Isozyme analysis: Ten females from each sample were isolated in isotonic (0.9% NaCl) solution based on esterase (Est) and Mdh isozymes (Karssen et al., 1995; Carneiro et al., 2000). Individual females, after desalting in reagent-grade water on ice for 5 min, were transferred into wells of sample-well stamp and stored at −80°C for future use. Samples were prepared for electrophoresis by transferring each female into sample wells, each containing 0.6 µl extraction buffer (20% sucrose, 2% Triton X-100, 0.01% Bromophenol Blue). Each female was then squeezed, macerated, and homogenized using a glass rod. Protein extractions were loaded onto a (8%–25%) polyacrylamide gradient gel and electrophoretically fractioned using a PhastSystem device (Pharmacia Ltd, Uppsala, Sweden). For reliable identification of enzyme phenotypes, females of a reference population of *M. javanica* (Karssen et al., 1995) were included in lanes 6 and 7 in each electrophoresis gel for direct comparison. After electrophoresis, gels were stained for 5 and 45 min to examine for Mdh and Est activity, respectively, rinsed with distilled water, and fixed using a 10% glycerol, 10% acetic acid, and distilled water solution. Gels were left to dry in the laminar flow cabinet and used for photography prior patterns examination and species identification using reference patterns (Esbenshade and Triantaphyllou, 1985; Carneiro et al., 1996; Karssen et al., 1995; Carneiro et al., 2000; Hernandez et al., 2004). For the analyses of pure, single egg-mass cultures, five females were used, which allowed for two samples per gel.

Molecular analysis: Genomic DNA was extracted from a single nematode (juvenile, male or female) using a quick alkaline lysis protocol (Stanton et al., 1998). Individual nematodes were transferred to 10 µl 0.05N NaOH, with 1 µl of 4.5% Tween added. The mixture was heated to 95°C for 15 min, and after cooling to room temperature 40 µl of double-distilled water was added and stored at −18°C for future use.

Polymerase chain reaction (PCR) amplification of the mitochondrial Nad5 was carried out in a total volume of 25 µl containing 2 µl genomic DNA, 0.25 µl of each primer (10 µM; Invitrogen) NAD5F2 (5′-TATTTTTTGTTTGAGATATATTAG-3′) and NAD5R1 (5′-CGTGAATCTTGATTTTCCATTTTT-3′), 2.0 µl PCR buffer (10×; Qiagen), 2.0 µl $MgCl_2$ (25 mM; Invitrogen), 0.5 µl deoxynucleotide triphosphate (dNTP; 10 mM; Qiagen), and 0.05 µl Toptaq DNA polymerase (5 U/µl; Qiagen). The PCR amplification was performed using a T100 Thermal Cycler (Bio-Rad) programmed for an initial denaturation for 2 min at 94°C, followed by 40 cycles of 60 sec at 94°C, 60 sec at 45°C, 90 sec at 72°C, and finally an extension for 10 min at 72°C. PCR products were electrophoretically fractioned on a 1% agarose gel in TAE buffer at 100 V for 30 min and visualized with ethidium bromide staining on a UV transilluminator. Successful reactions were purified and sequenced commercially by Macrogen Inc. (Europe) in forward and reverse direction. Consensus sequences were assembled using GENEIOUS 9.15 (Biomatters; http://www.geneious.com). De novo sequences were compared with online available

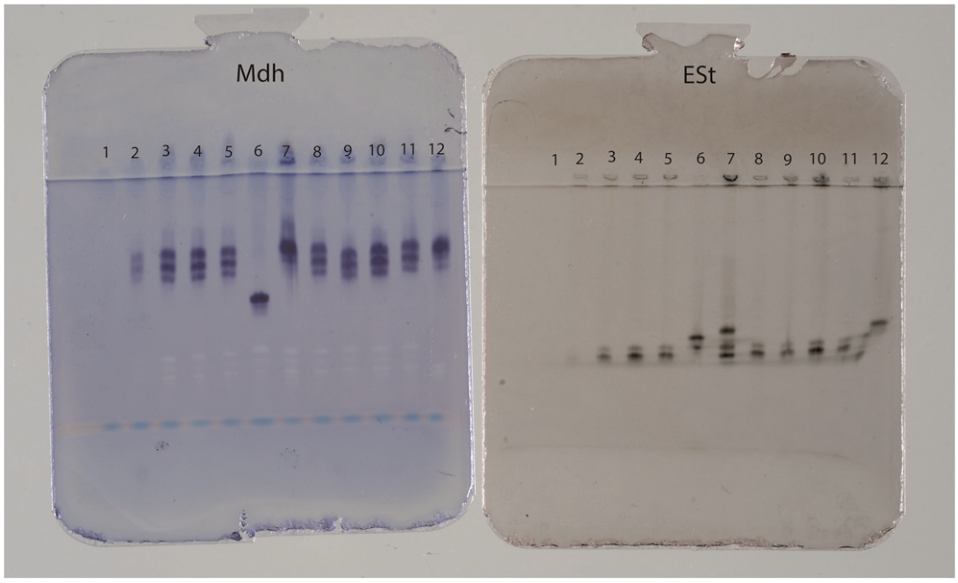

FIG. 3. Malate dehydrogenase (Mdh) and esterase (Est) profiles of *Meloidogyne arenaria* (Lanes 2–5 and 8–11) and *M. incognita* (Lane 12) from yam tubers in Nigeria. Reference sample *M. javanica* (Lanes 6 and 7).

sequences and deposited in GenBank (Table 1). Species identification was undertaken following species-specific sites after alignment using MAFFT 7.222 (Katoh and Standley, 2013) with reference sequences (Janssen et al., 2016). Identification using the DNA-based method was first conducted to confirm the result of isozyme analysis, based on four different individuals for the nonpure populations and based on a single individual (as single DNA template) for the samples with a single species based on the isozyme analyses. Second, samples not identified with the isozyme analysis were molecularly identified based on four individuals whenever possible.

RESULTS

Root-knot nematode identification: Four RKN species *M. arenaria*, *M. enterolobii*, *M. incognita*, and *M. javanica* were identified from the 48 samples studied using the isozyme and the mtDNA-based analysis (Table 2; Figs. 1–5). They were identified as *M. incognita* in 69% of the samples or *M. javanica* (13%) exclusively (Fig. 6). Two other

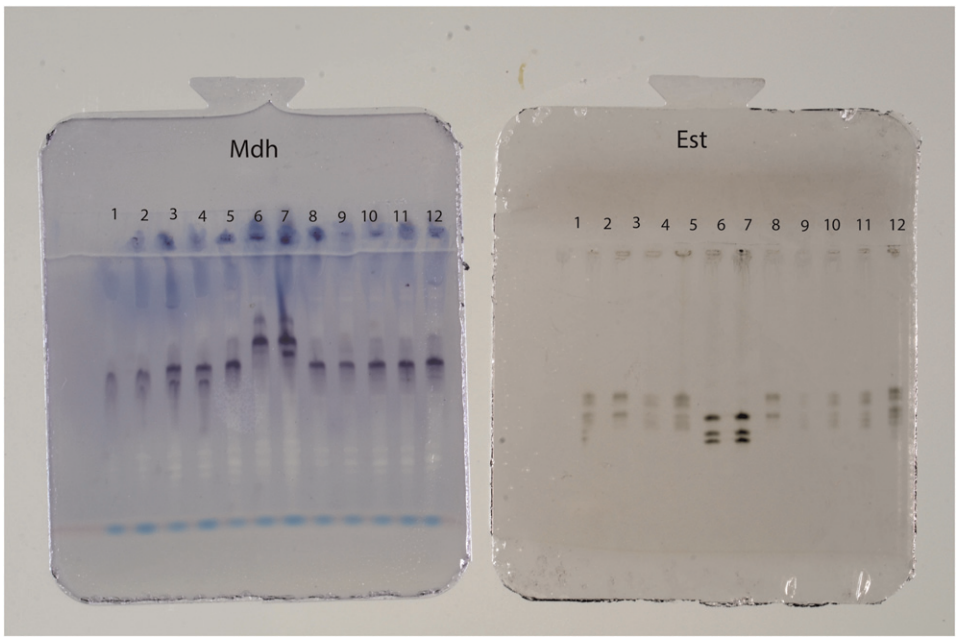

FIG. 4. Malate dehydrogenase (Mdh) and esterase (Est) profiles of *Meloidogyne enterolobii* (Lanes 1–5 and 8–12) from yam tubers in Nigeria. Reference sample *M. javanica* (Lanes 6 and 7).

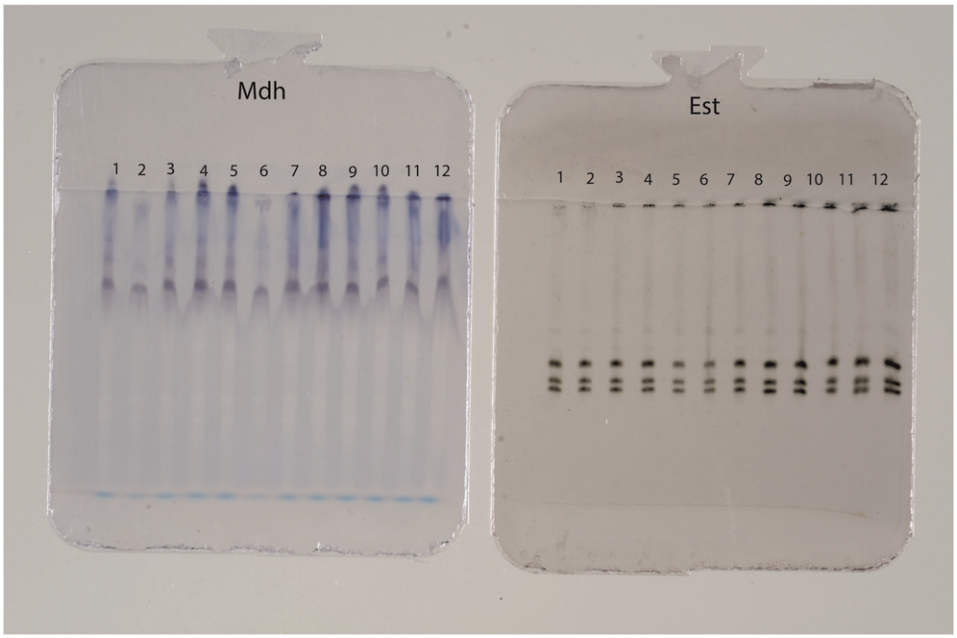

Fig. 5. Malate dehydrogenase (Mdh) and esterase (Est) profiles of *Meloidogyne javanica* (Lanes 1–5 and 8–12) from yam tubers in Nigeria. Reference sample *M. javanica* (Lanes 6 and 7).

species, *M. arenaria* or *M. enterolobii* were each identified exclusively in 2% of the samples. The concurrence of multiple species were found in 14% of the samples: *M. incognita* and *M. enterolobii* (6%); *M. incognita* and *M. arenaria* (2%); *M. enterolobii* and *M. javanica* (2%); and *M. enterolobii*, *M. incognita*, and *M. javanica* (4%) (Fig. 6).

Three patterns, N1 (89%), N1a (7%), and N3 (4%), and seven patterns, A2 (4%), E3 (2%), I (5%), I1 (19%), I2 (36%), J3 (29%), and M2 (5%) were observed for the Mdh and the Est activities, respectively (Fig. 1). Pattern combinations and association to RKN species are illustrated in Fig. 1 and Table 2 and correspond to A2-N3 (100%) for *M. arenaria*, M2-N1a (75%) and E3-N1a (25%) for *M. enterolobii*, I2-N1 (60%), I1-N1 (32%), and I-N1 (9%) for *M. incognita*, and J3N1 (100%) for *M. javanica* (Fig. 1). Along with the Mdh staining, patterns of the glucose-6-phosphate dehydrogenase (G6PDH) were observed in some cases (Fig. 1).

Fifty-nine sequences of *Meloidogyne* species from the Nad5 were newly generated from 29 populations corresponding with 28 samples (Table 2). The sequences alignment was 516 bp in length. The results showed that virtually all obtained sequences were identical to one of the known reference sequences (Janssen et al., 2016) (Table 3). Except for one new haplotype for *M. incognita*, named *M. incognita* haplotype H4, which differed only in one nucleotide from haplotype 1 (H1), i.e., the Guanine (G) had been substituted with the Adenine (A) (Janssen et al., 2016). For *M. enterolobii*, which is divergent from other tropical RKN species, the sequences obtained from the nematodes in yam were identical to the reference sequence.

For *M. incognita*, except for the new haplotype H4 (14%), all sequences corresponded to *M. incognita* haplotype 1 (84%). For *M. javanica*, all sequences except one were the same as for the reference haplotype. All the *M. enterolobii* and *M. arenaria* sequences corresponded, respectively, to the only haplotype of *M. enterolobii* (100%) and to *M. arenaria* haplotype 2 (100%) (Table 3).

Meloidogyne incognita was the most widely distributed species, recorded in all three agroecological zones surveyed (Fig. 7). In the Southern Guinea, Savanna, all four RKN species were recorded on yam. *Meloidogyne arenaria* was not recorded from the Humid Forest or the Derived Savanna.

Discussion

Accurate identification of tropical RKN species has previously proved a challenge. In the current study however, the RKN species affecting yam were unequivocally identified using the mtDNA barcode-based technique, correlated with the enzyme phenotype analysis. Consequently, the Nad5 gene fragment of the mtDNA appears to be a highly useful barcode for the diagnosis of tropical RKN, at least based on the four species occurring in the current study on yam. Each species could be assigned to one haplotype, except for a new haplotype in *M. incognita*, despite variation in the enzyme patterns. In most of the cases, migration of one or two minor bands from the major band caused these variations (Carneiro et al., 2000). Esterase patterns were more species-specific than the Mdh as they easily differentiated *M. incognita* from *M. javanica*, whereas

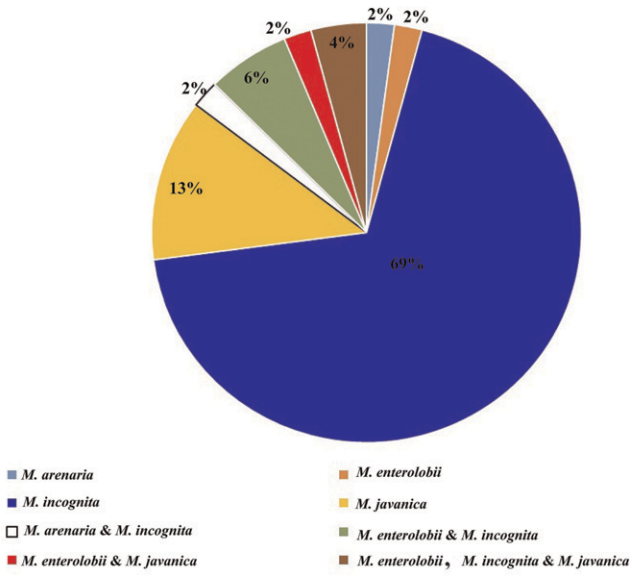

FIG. 6. Frequency of *Meloidogyne* species identified on galled yam tubers (n = 48) from Ghana and Nigeria.

for these species the same pattern was found for Mdh. Intraspecific enzyme patterns did not correlate with different DNA-based haplotypes; for instance, the E3_N1a and M2_N1a of *M. enterolobii* resulted in one haplotype. The same observation was made for the patterns I_N1, I1_N1, I2_N1 of *M. incognita*, which all corresponded to the *M. incognita* haplotype 1 (*M. incognita* H1). In addition to the Est and Mdh activity, we also observed patterns of a third enzyme, the G6PDH, occurring occasionally with the Mdh staining as a result of the catalytic activity of the G6PGH on the Mdh. These patterns, whenever present, were very helpful in the identification of the four RKN species when one or both reference isozymes (Est and Mdh) were not clearly displayed. Optimal conditions for its stabilization therefore need to be investigated.

It is well known that competition between species may result in the dominance of one species after several generations of culturing (Manzanilla-Lopez and Starr, 2009). The dominance of one species over others can be favored by numerous factors, such as the environmental conditions, the inoculum level and the host suitability. Therefore, to enhance the chance of having initial species for further use within mixed populations, if any in a given sample, pure populations were established using single egg masses after one generation. Further studies using the pure-species populations will help clarify the interactions between species on yam.

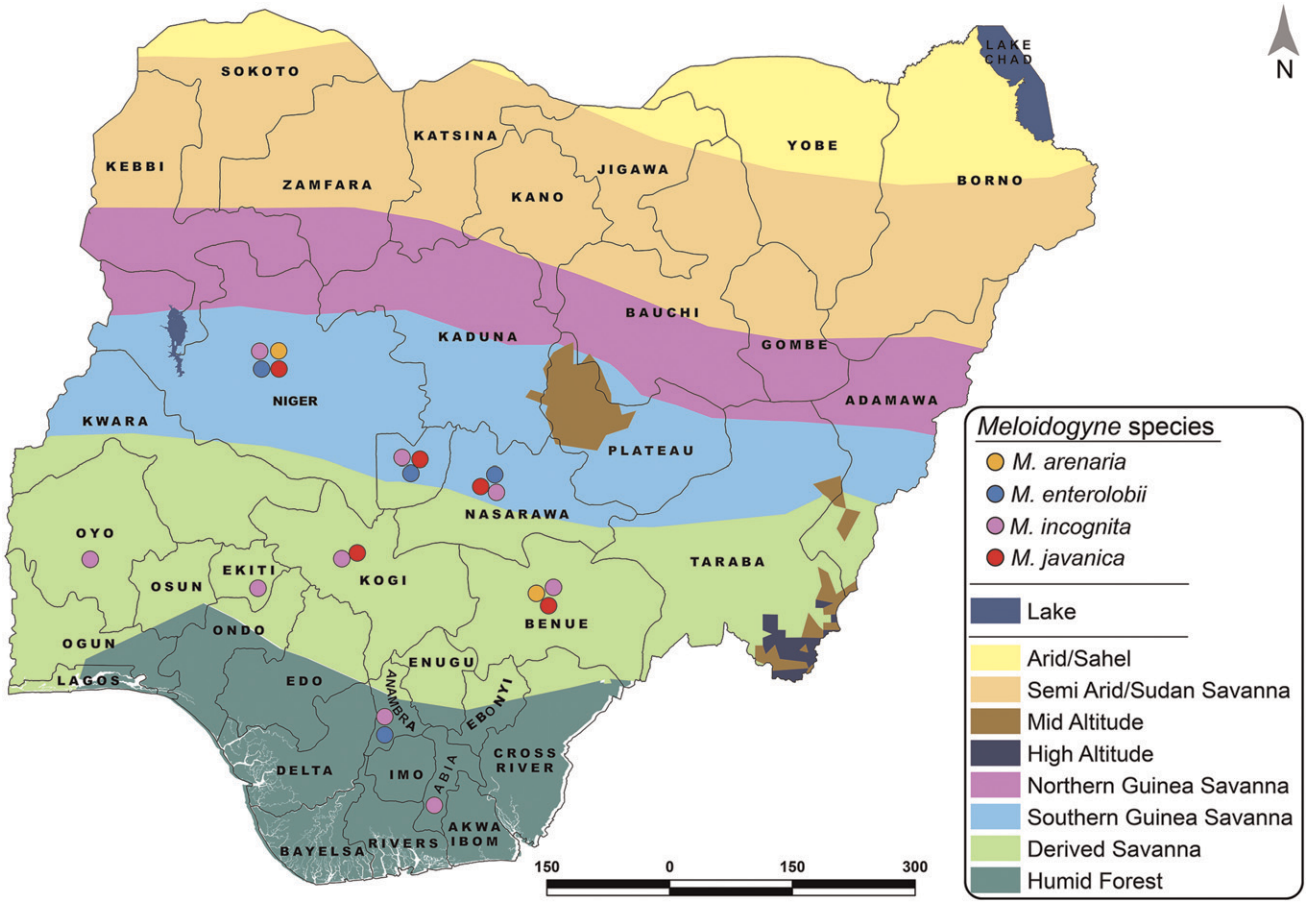

FIG. 7. Distribution of *Meloidogyne* species isolated from yam in different agroecological zones in Nigeria.

TABLE 3. Polymorphic nucleotide positions of Nad5 in *Meloidogyne* species from *Dioscorea alata* and *D. rotundata* with haplotypes in reference to Janssen et al. (2016).

Samples	*Meloidogyne* spp. and haplotype[a]	Enzyme pattern[b]	Mutation positions																	
			26	63	71	72	86	97	113	200	221	242	254	273	355	395	396	427	461
KY522746, KY522747, KY522748	***M. enterolobii***	**E3_N1a, M2_N1a**	**A**	**T**	**A**	**C**	**A**	**G**	**T**	**G**	**T**	**A**	**T**	**G**	**A**	**A**	**A**	**T**	**A**
			5[c] 1				*3 5*					*20*	*2 1*	*2 2*	*9 7*	*8 1*			
All *M. javanica* from yam	***M. javanica***	**J3_N1**	**T**	**T**	**A**	**T**	**G**	**A**	**T**	**A**	**A**	**G**	**T**	**G**	**G**	**G**	**T**	**T**	**A**
KY522790	***M. javanica***	**J3_N1**	**T**	**G**	**A**	**T**	**G**	**A**	**G**	**A**	**A**	**G**	**T**	**G**	**G**	**G**	**T**	**T**	**A**
KU372392 (T347)	*M. javanica*	**J3_N1**	**T**	**T**	**A**	**T**	**G**	T	**T**	**A**	**A**	**G**	**T**	**G**	**G**	**G**	**T**	**T**	**A**
KY522763, KY522764, KY522771, KY522772	***M. incognita* H4**	I2_N1	T	T	A	T	A	A	T	A	A	A	T	G	A	A	T	T	A
All other *M. incognita* from yam	***M. incognita*** H1	I_N1, I1_N1, I2_N1	T	T	A	T	G	A	T	A	A	A	T	G	A	A	T	T	A
KU372373 (T384), KU372380 (M8), KU372383 (M20), KU372384 (M21), KU372386 (T532))	*M. incognita* H2	I1-N1	T	T	A	T	G	A	T	A	A	A	T	G	A	G	T	T	A
KU372366 (T540), KU372388 (T161)	*M. incognita* H3	I1-N1	T	T	A	T	G	A	T	A	A	**G**	T	G	A	R	T	T	A
KU372349 (M41), KU372350 (T393), KU372356 (T411)	*M. arenaria* H1	A2-N1	T	T	A	T	G	A	T	A	A	A	C	A	G	G	T	T	A
All *M. arenaria* from yam, KU372351 (T453), KU372354 (T332)	***M. arenaria*** H2	**A2_N3, A2_N1**	**T**	**T**	**A**	**T**	**G**	**A**	**T**	**A**	**A**	**A**	**C**	**G**	**G**	**G**	**C**	**A**	**A**
KU372357 (T311), KP202350 KU372352 (T461)	*M. arenaria* H3	A3N1, A2N3	T	T	A	T	G	A	T	A	A	A	C	G	G	G	T	T	A
KU372421 (T585)	*Meloidogyne*. sp 1 H1[d]	A2-S1-M1_N1	T	T	T	T	G	A	T	A	A	A	C	G	G	G	T	T	A
KU372420 (T473)	*Meloidogyne* sp 1 H2[d]	A2-S1-M1_N1	T	T	A	T	G	A	T	A	A	A	C	G	G	G	T	T	A
KU372423 (T316), KU372422 (T576)	*Meloidogyne*. sp 2[d]	Ala-S1_N1	T	T	A	T	G	A	T	A	A	A	C	G	G	A	T	T	A
KU372417 (T326), KU372419 (T693)	*M. luci* H1	L3_N1	T	T	A	T	G	A	T	A	A	A	C	G	G	G	T	T	A
KU372418 (T459),	*M. luci* H2	L3_N1	T	T	A	A	G	A	T	A	A	A	C	G	G	G	T	T	**G**
KU372390 (T638), KU372389 (T695)	*M. inornata*	I3_N1	T	T	A	T	G	A	T	A	A	A	C	G	G	G	T	T	A
KU372360 (T612)	*M. ethiopica*	E3_N1	T	T	A	T	G	A	T	A	A	A	C	G	G	G	T	T	A

[a] Haplotypes in reference to Janssen et al. (2016), except for *M. incognita* H4, a new haplotype.
[b] Enzyme patterns: Esterase, Malate dehydrogenase.
[c] Additional mutation in *M. enterolobii*. Results from this study are mentioned in bold.
[d] Unknown *Meloidogyne* species in Janssen et al. (2016).

Given that the mitochondrial barcoding and enzyme patterns provide confirmatory results, the preferred method to determine tropical *Meloidogyne* species depends on the available laboratory equipment and the availability of young egg-laying females. The mitochondrial barcode method has some obvious advantages in comparison with enzyme-based identification, such as (i) being considerably faster, (ii) regardless of lifestage is sufficient, (iii) resulting sequences can be analyzed in a comparative population genetic framework; and (iv) results are highly reproducible between laboratories (Janssen et al., 2016). Nevertheless, for unknown lineages or species, the combination of all available methods, including

morphological data, will allow a more comprehensive description.

Detection of two and even three *Meloidogyne* species from the same yam sample, using both techniques, indicates that multiple RKN infection of yam tubers occurs, as has been determined for other crops (Moens et al., 2009). This illustrates again that species identification must be performed on several individuals obtained from the same plant or field sample, to establish accurate diagnosis, toward determining suitable management practices, such as crop rotation and plant resistance.

Globally, *M. incognita* and *M. javanica* have been recorded from yam and are being viewed as major pests damaging tubers (Jenkins and Bird, 1962; Unny and Jerath, 1965; Adesiyan and Odihirin, 1978; Bridge, 1998; Bridge et al., 2005; De Moura, 2006). Both species were identified in the current study, with *M. incognita* being the most prevalent and widespread in Nigeria. Adesiyan and Odihirin (1978), showed a clear demarcation in the distribution of RKN species in Nigeria, *M. javanica* in the western part of the southern region and *M. incognita* in the eastern part of the southern region. However, the present study revealed that *M. incognita* is widespread across the country and that the geographical demarcation does not exist anymore, possibly due to the dissemination of infected seed materials.

Meloidogyne arenaria was previously recorded from yam in the Caribbean, Central and Latin America (Schieber and Lassmann 1961; Jenkins and Bird, 1962; De Moura, 2006; De Moura et al., 2010), and Asia (Park et al., 1998; Gao et al., 2000). In Nigeria, Caveness (1967) recorded *M. arenaria* in the western side of the Derived Savanna, but only in the rhizosphere soil and not on the yam itself. Here, *M. arenaria* is reported for the first time from yam tubers in Nigeria in the Derived Savanna and in the Southern Guinea Savanna. To the best of our knowledge, this is the first record of this species on yam tubers in Africa.

Until recently, *M. enterolobii* was not recorded from yam. It was established as a causal agent of galling damage on white yam (*Dioscorea rotundata*) in the same study framework (Kolombia et al., 2016). Three months after reinoculation, heavy galling damage was observed on yam tubers with a nematode reproduction factor of 29. *Meloidogyne enterolobii* is a particularly damaging and aggressive species, able to reproduce on crops with *Mi* resistance genes effective against other tropical species, such as *M. incognita* and *M. javanica* (Castagnone-Sereno, 2012). In addition, it has a quarantine status in the European and Mediterranean (EPPO) region (Anonymous, 2016), calling for special attention to yam tubers traded with countries in these regions. *Meloidogyne hapla*, a species reported from yam in South Korea and Japan (Kawamura and Hirano, 1961; Park et al., 1998), was not detected in the current study, likely as it is more commonly associated with temperate climates or at higher altitudes in the tropics (Hunt and Handoo, 2009) and therefore less probably found to occur in Nigeria.

Despite the well-known importance of RKN on yam in general (Bridge et al., 2005), relatively little is known about species-specific effects or the interactions of the four identified species. Inoculation of white yam with *M. incognita* at a rate of 1,250 nematodes per plant, resulted in a reduction of 40% of the marketable value (Atu et al., 1983). The interspecific diversity of RKN species parasitizing yam in Nigeria requires broad-range screening of wild yam germplasm species to identify sources of resistance with a broad spectrum of resistance. More investigations are required to establish the virulence and the damage threshold level of each *Meloidogyne* species and their combined effect on yam.

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