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Diversity of *Meloidogyne* spp. from peri-urban areas of sub-Saharan Africa and their genetic similarity with populations from the Latin America☆

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ABSTRACT

In Africa, peri-urban vegetable production systems supply perishable vegetables to the rapidly expanding urban centers. These highly intensive systems are characterized by high levels of pests and diseases and an excessive use of synthetic pesticides to reduce their population densities. Root-knot nematodes (RKN) are especially prevalent in these systems but are often not recognized, or diagnosed correctly. The limited ability to accurately identify these pathogens likely results in the inappropriate use and misuse of control measures, such as genetic resistance, crop rotation, or synthetic chemicals. Given the perceived importance of RKN, a species characterization study was conducted in peri-urban vegetable (amaranthus, cabbage, pepper, carrot, cassava, eggplant, okra, tomato) fields and some coffee plantations, in Benin, Kenya, Nigeria, Tanzania and Uganda. *Meloidogyne* spp. were characterized from 143 field samples using esterase phenotypes (EST) and SCAR markers. Five known species were identified: three phenotypes for *M. javanica* populations (EST J3, Rm: 1.0, 1.25, 1.4; EST J2a, Rm: 1.0, 1.4; EST J2b, Rm: 1.0, 1.25), two for *M. incognita* (EST I1, Rm: 1.0; EST I2, Rm: 1.05, 1.0), one for *M. arenaria* (EST A2, Rm: 1.2, 1.3), one for *M. enterolobii* (EST E4, Rm: 0.70, 0.75, 0.90, 0.95), one for *M. izalcoensis* (EST I4 Rm: 0.86, 0.96, 1.24, 1.30) and two unusual esterase phenotypes for two unknown species, named *Meloidogyne* sp.1 and sp.2. Combinations of species were detected from numerous locations. Genetic diversity was further studied using RAPD primers, by comparing a subset of the sampled populations from Africa and some populations from Brazil and El Salvador. The analysis identified separate clusters of the more common and minor species, with low variability observed for African and American populations. The SCAR markers correctly identified all *Meloidogyne* species with the exception of *M. ethiopica*, *Meloidogyne* sp.1 and *Meloidogyne* sp.2. For *Meloidogyne* sp.1, the SCAR markers corresponded wrongly to *M. javanica* and *M. arenaria*, and for *Meloidogyne* sp.2 to *M. incognita*. This demonstrates the shortcomings of using SCAR markers alone, which can generate erroneous results for RKN species. Further morphological and molecular studies are required to clarify the identity of these two atypical species.

1. Introduction

By 2050, the population of Africa is expected to exceed two billion people and the number living in urban centers increasing by approximately 345 million by 2030 [1]. In sub-Saharan Africa (SSA), population growth will be even more dramatic [2]. To feed this escalating and urbanizing population, Africa will need to dramatically increase crop productivity and production. Nowhere is this need more pressing than in SSA, where crop productivity is effectively static and food production per capita declining [1,3]. To address this situation, cropping systems in SSA need to become more intensified [4,5], which will create greater selection pressures and aggravation of pests and diseases. To supply

Abbreviations: bp, base pair; CNPq, Brazilian National Council for Scientific and Technological Development; dNTPs, Deoxyribonucleotides triphosphate; EST, esterase; PCR, Polymerase chain reaction; RAPD, Random amplified polymorphic DNA; RKN, root-knot nematode; Rm, relative mobility; SCAR, sequence characterized amplified region; SSA, sub-Saharan Africa

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perishable fresh vegetables to urban centers, the already highly intensive peri-urban production systems become further challenged as land and water becomes scarcer [5]. These systems are characterized by high levels of pests and diseases and an excessive use and misuse of synthetic pesticides to manage this problem [6]. In order to address the pest and disease issues, researchers are faced with the need to balance the protection of the environment and also increase agricultural productivity.

Root-knot nematodes (RKN) of the genus *Meloidogyne* pose a significant threat to agricultural production in Africa due to the damage they cause on a wide range of agricultural crops, especially vegetables. The direct and indirect damage caused by these species results in decreased yield, quality, high production costs and loss of income. They are often overlooked or not recognized, and are regularly misdiagnosed due to the non-specific nature of the above-ground symptoms [7]. When recognized, diagnosis is more often not conducted to species level or is inaccurately conducted, due to limited expertise and a lack of simple techniques to reliably diagnose species [8]. However, across crops, across SSA, RKNs are viewed as possibly the greatest biotic threat to crop production [9]. This is due to a number of characteristics, including the ability of some common species to be able to parasitize and multiply on an extraordinary number and range of plant species [10]. The use of nematicides endangers food safety in peri-urban areas, including through the use of pesticides suitable only for non-food crops [11]. In order to implement management options for RKN, accurate knowledge and diagnositcs of the species present may be essential, such as for the deployment of resistance [12].

Over 100 species have been described in the genus *Meloidogyne,* of which 23 have been reported in Africa [10]; [13]. However, the characterization and identification of these species was largely conducted using morphological methods, such as the female perineal patterns. This method has since been shown to be inaccurate with several species having similar, overlapping patterns, as is the case for *M. paranaensis*, *M. incognita, M. izalcoensis* and *M. inornata*, while *M. ethiopica* presents patterns that are a mixture between *M. incognita* and *M. arenaria* [14].

Over recent decades, biochemical studies have been conducted using soluble proteins, showing that several species of RKN can be differentiated by the enzymatic phenotypes (esterases) obtained through polyacrylamide gel electrophoresis [15–19]. Since then, studies based on DNA analysis have gradually intensified, with species-specific primers (SCAR-PCR) now developed to allow the rapid identification of some species [19–23]. The combined use of isozyme esterase and SCAR markers has been advocated as the most reliable way to accurately identify *Meloidogyne* spp. from field samples [14].

The current study was conducted in order to assess the *Meloidogyne* spp. associated with some key crops in peri-urban systems and from coffee plantations in SSA, based on the esterase phenotypes and confirmed by SCAR markers, and to compare the genetic diversity of species detected in Africa and Americas (Brazil and El Salvador).

2. Materials and methods

A total of 143 populations of *Meloidogyne* spp. were collected from peri-urban sites in the countries of Benin, Nigeria, Kenya, Tanzania and Uganda from amaranthus (*Amaranthus* spp.), eggplant (*Solanum melongena*), carrot (*Daucus carota*), spinach (*Spinacia oleracea*), cassava (*Manihot esculenta*), sweet pepper (*Capsicum annuum*), okra (*Abelmoschus esculentus*), cabbage (*Brassica oleracea*), purple sage (*Salvia dorrii*) and tomato (*Solanum lycopersicum*); four populations were collected from coffee (*Coffea* spp.) plantations (Table 1). Roots were unearthed from 5 to 10 plants per field using a hand trowel and inspected for visual signs of galling. Infected roots, which presented symptoms of galling were placed in a plastic bag together with a small amount of soil from each plant, labeled and stored in a cooler box for transfer to the research station. At the station, the infected roots for each sample were rinsed free of soil, chopped finely and mixed into

Table 1
Meloidogyne spp. (RKN) in peri-urban areas of sub-Saharan African countries: locality, crop and esterase phenotypes.

N°	Locality	Crop	*Meloidogyne* spp	Esterase phenotype (Est)
Benin				
1	Cotonou	Cabbage	*M. izalcoensis*	Est I4
2	Cotonou	Carrot	*M. incognita*	Est I2
3	Cotonou	Purple sage	*M. izalcoensis*	Est I4
4	Pahou Ouidah	Amaranthus	*M. incognita*	Est I2
5	Pahou Ouidah	Okra	*M. incognita*	Est I2
Nigeria				
6	Oniboure farm -Oyo state	Tomato	*M. incognita* + *M. javanica*	Est I1 + Est J3
7	Micro plot lab. - IITA	Tomato	*M. incognita* + *M. enterolobii*	Est I2 + Est E4
8	Okobo farm – Oyo State	Tomato	*M. enterolobii*	Est E4
9	Nihort Ibadan – Oyo State	Tomato	*M. enterolobii*	Est E4
10	Akufo farm – Oyo State	Tomato	*M. enterolobii*	Est E4
11	Bagbon farm – Oyo State	Tomato	*M. enterolobii*	Est E4
12	Ilora – Oyo State	Tomato	*M. incognita*	Est I2
Kenya				
13	Mangu	Coffee	*M. javanica*	Est J3
14	Ciatundu	Coffee	*M. javanica*	Est J3
15	Kabete	Coffee	*M. izalcoensis*	Est I4
16	Lamu	Tomato	*M. incognita*	Est I2
17	Kilifi	Tomato	*M. incognita*	Est I2
18	Lamu	Tomato	*M. javanica*	Est J3/J2b
19	Lamu	Tomato	*M. incognita*	Est I2
20	Lamu	Tomato	*M. incognita*	Est I2
21	Oloitoktok	Tomato	*M. javanica*	Est J2b
22	Taveta	Tomato	*Meloidogyne* sp.	Est SP.2
23	Taveta	Tomato	*M. incognita* + *M. javanica*	Est I2 + Est J3
24	Taveta	Tomato	*M. javanica*	Est J3
Tanzania				
25	Kisse	Tomato	*M. javanica*	Est J3
26	Kisse	Tomato	*M. javanica*	Est J3
27	Donge	Sweet pepper	*M. incognita*	Est I2
28	Unguja ukuu	Okra	*M. arenaria*	Est A2
29	Unguja ukuu	Okra	*M. incognita*	Est I2
30	Unguja ukuu	Okra	*M. arenaria*	Est A2
31	Unguja ukuu	Okra	*M. incognita*	Est I2
32	Unguja ukuu	Okra	*M. incognita*	Est I2
33	Unguja ukuu	Okra	*M. arenaria*	Est A2
34	Unguja ukuu	Okra	*M. incognita*	Est I2
35	Tindini	Tomato	*M. izalcoensis* + *M. incognita*	Est I4 + Est I2
36	Kisse	Okra	*M. javanica* + *M. incognita*	Est J3 + Est I2
37	Hembeti	Tomato	*M. incognita (I2)*	Est I2
38	Tindini	Tomato	–	–
39	Hembeti	Tomato	*M. javanica* + *M. incognita*	Est J3 + Est I2
40	Kisse	Okra	*M. javanica* + *M. incognita*	Est J3 + Est I2
41	Dakawa	Tomato	*M. javanica* + *M. Incognita* + *M. arenaria*	Est J3 + Est I2 + Est A2
42	Donge chanjani	Sweet pepper	*M. incognita*	Est I2
43	Kianja	Sweet pepper	*M. arenaria* + *M. incognita*	Est A2 + Est I2
44	Unguja ukuu	Tomato	*M. incognita*	Est I2
45	Hembeti	Tomato	*M. incognita*	Est I2
46	Kwamsisi	Tomato	*M. javanica*	Est J3
47	Unguja ukuu	Tomato	*M. incognita*	Est I2

(*continued on next page*)

Table 1 (*continued*)

N°	Locality	Crop	*Meloidogyne* spp	Esterase phenotype (Est)
48	Kisse	Okra	*M. javanica*	Est J3
49	Kisse	Okra	*M. javanica*	Est J3
50	Kianga	Sweet pepper	*M. izalcoensis* + *M. incognita*	Est I4 + Est I2
51	Msongozi	Tomato	*Meloidogyne* sp.	Est SP.2
52	Fox farm	Spinach	*M. incognita*	Est I2
53	Dakawa	Tomato	*M. javanica*	Est J3
54	Hembeti	Tomato	*M. incognita*	Est I2
55	Donge	Tomato	*M. javanica*	Est J3
56	Unguja ukuu	Tomato	*M. arenaria* + *M. javanica*	Est A2 + J3
57	Unguja ukuu	Tomato	*M. arenaria* + *M. javanica*	Est A2 + J3
58	Kisse	Carrot	*M. javanica*	Est J3
59	Hembeti	Tomato	*M. incognita*	Est I2
60	Donge	Tomato	*M. javanica*	Est J3
61	Msongozi	Tomato	*M. javanica*	Est J3
62	Kisse	Tomato	*M. arenaria* + *M. incognita*	Est A2 + Est I2
63	Boko Kawe	Sweet pepper	*M. incognita*	Est I2
64	Miali	Tomato	*M. javanica*	Est J3
65	Dibamba	Tomato	*M. incognita*	Est I2
66	Hembeli	Tomato	*M. javanica*	Est J3
67	Pangani	Sweet pepper	*M. arenaria*	Est A2
68	Hembeti	Tomato	*M. javanica*	Est J3
69	Pangani	Tomato	*M. incognita*	Est I2
70	Kawe	Tomato	*M. incognita*	Est I2
71	Unguja ukuu	Tomato	*M. javanica*	Est J3
72	Fuoni mili nne	Carrot	*M.incognita* + *M. javanica*	Est I2 + Est J3
73	Kisse	Tomato	*M. javanica*	Est J3
74	Tindini	Tomato	*M. izalcoensis* + *M. javanica*	Est I4 + Est J3
75	Fuoni mili nne	Tomato	*M. incognita*	Est I2
76	Kisse	Tomato	*M. javanica*	Est J3
77	Mufindi	Coffee	*M. incognita* + *M. izalcoensis* + *M. hapla*	Est I2 + Est I4 + Est H1
Uganda				
78	Wakiso	Tomato	*M. arenaria*	Est A2
79	Wakiso	Tomato	*M. arenaria*	Est A2
80	Wakiso	Tomato	*M. arenaria*	Est A2
81	Wakiso	Tomato	*M. incognita* + *M. javanica*	Est I2 + Est J3
82	Wakiso	Tomato	*M. arenaria*	Est A2
83	Wakiso	Tomato	*M. arenaria*	EstA2
84	Wakiso	Tomato	*M. javanica* + *M. incognita*	Est J3 + Est I1
85	Wakiso	Tomato	*M. arenaria*	Est A2
86	Wakiso	Tomato	*M. arenaria*	Est A2
87	Wakiso	Tomato	*M. javanica*	Est J3
88	Wakiso	Tomato	*M. javanica*	Est J2b + Est J3
89	Wakiso	Tomato	*M. javanica*	Est J3
90	Mukono	Tomato	*M. javanica*	Est J2
91	Mukono	Eggplant	*M. javanica*	Est J3
92	Mukono	Tomato	*M. incognita* + *M. javanica*	Est I1 + Est J3
93	Mukono	Tomato	*M. javanica*	Est J3
94	Mukono	Tomato	*M. javanica*	Est J3
95	Mukono	Sweet pepper	*M. arenaria* + *M. incognita* + *M. javanica*	Est A2 + Est I1 + I2 + Est J3
96	Mukono	Cassava	*M. incognita*	Est I2
97	Mukono	Tomato	*M. incognita*	Est I2
98	Mukono	Tomato	*M. incognita*	Est I2
99	Mukono	Tomato	*M. javanica*	Est J3
100	Mukono	Tomato	*M. javanica*	Est J3
101	Mukono	Tomato	*M. javanica*	Est J3
102	Mukono	Tomato	*M. javanica*	Est J3
103	Mukono	Tomato	*M. javanica*	Est J3
104	Mukono	Tomato	*M. javanica*	Est J3

Table 1 (*continued*)

N°	Locality	Crop	*Meloidogyne* spp	Esterase phenotype (Est)
105	Luwero	Tomato	*M. javanica*	Est J3
106	Luwero	Tomato	*M. javanica*	Est J3
107	Kayunga	Tomato	*M. arenaria*	Est A2
108	Wakiso	Tomato	*M. incognita*	Est I2
109	Wakiso	Tomato	*M. arenaria*	Est A2
110	Wakiso	Tomato	*M. arenaria*	Est A2
111	Wakiso	Tomato	*M. arenaria*	Est A2
112	Mukono	Tomato	*M. javanica*	Est J3
113	Mukono	Tomato	*M. javanica*	Est J2a
114	Mukono	Tomato	*M. javanica*	Est J3
115	Luwero	Tomato	*M. javanica*	Est J3 + J2b
116	Kayunga	Tomato	*M. javanica*	Est J3
117	Kayunga	Tomato	*M. javanica*	Est J3
118	Gomba	Tomato	*M. javanica*	Est J3
119	Nakasongola	Tomato	*M. javanica*	Est J3 + Est J2b
120	Nakasongola	Tomato	*M. javanica*	Est J3
121	Wakiso	Tomato	*M. arenaria*	Est A2
122	Wakiso	Tomato	*M. arenaria*	Est A2
123	Wakiso	Tomato	*M. arenaria*	Est A2
124	Wakiso	Tomato	*M. arenaria*	Est A2
125	Mukono	Tomato + eggplant	*M. javanica*	Est J3
126	Mukono	Tomato + sweet pepper + Cassava	*M. javanica* + *M. incognita*	Est J3 + Est I1
127	Mukono	Tomato	*M. javanica*	Est J2a + Est J3
128	Mukono	Tomato	*M. javanica*	Est J3
129	Mukono	Tomato	*M. javanica* + *M. incognita*	Est J3 + Est I1
130	Mukono	Tomato	*M. javanica*	Est J3
131	Mukono	Tomato	*M. javanica* + *M. incognita*	Est J3 + Est I1
132	Mukono	Tomato	*M. javanica*	Est J3
133	Mukono	Tomato	*M. javanica*	Est J3
134	Mukono	Tomato	*M. javanica*	Est J2b
135	Luwero	Tomato	*M. incognita* + *M. incognita* + *M. arenaria*	Est I1 + Est I2 + Est A2
136	Kayunga	Tomato	*M. javanica*	Est J3
137	Kayunga	Tomato	*M. javanica*	Est J3
138	Gomba	Tomato	*M. arenaria* + *M. javanica*	Est A2 + Est J3
139	Nakasongola	Tomato	*M. javanica*	Est J3
140	Nakasongola	Tomato	*M. javanica* + *M. incognita*	Est J3 + Est I2
141	Namulonge	Tomato	*M. arenaria*	Est A2
142	Namulonge	Tomato	*M. javanica*	Est J3
143	Namulonge	Tomato	*M. incognita*	Est I2

steam sterilized soil each containing a recently transplanted tomato plant cv. Moneymaker [24]. The pots were maintained in the screenhouse for 2–3 months, until egg masses were available for collection. Between 10 and 20 egg masses were collected for each field sample, placed in 1% saline solution in Eppendorf tubes and sent to Embrapa Genetic Resources and Biotechnology (Embrapa, Cenargen), Brasília, DF, Brazil. The received egg masses were inoculated onto tomato cv. Santa Clara as above and maintained in the greenhouse for 3 months before collecting 40 females per field sample for diagnosis using biochemical analysis by esterase phenotypes according to Carneiro et al. [17,18]. When mixed species populations were encountered, the species were purified to single species [25] by inoculating individual egg masses onto tomato cv. Santa Clara, as above. At the end, the Rms were calculated, i.e., the relative migration was evaluated in relation to the first band of *M. javanica* (EST J3), used as the standard in each gel.

In a second step, 23 populations of *Meloidogyne* spp. were used for molecular studies: a subset of 14 African purified populations previously identified by isozyme esterase phenotypes and a further eight from Brazil and El Salvador that had been used as standards in previous

Table 2
Primers used in the reactions PCR-SCAR to identify *Meloidogyne* spp. from Africa, El Salvador and Brazil.

Species	Markers	Sequencies (3′-5′)	Band height (bp)	References
M. izalcoensis	iz-AB2F	GGAAACCCCTAATTAGGATACACT	670	[22]
	iz-AB2R	CGCTTGATTTGAGCAGTAGG		
M. incognita	inc-K14F	GGGATGTGTAAATGCTCCTG	399	[21]
	inc-K14R	CCCGCTACACCCTCAACTTC		
M. ethiopica	MethF	ATGCAGCCGCAGGGAACGTAGTT	350	[23].
	MethR	TGTTGTTTCATGTGCTTCGGCATC		
M. enterolobii	Mk7F	GATCAGAGGCGGGCGCATTGCGA	520	[29]
	Mk7R	CGAACTCGCTCGAACTCGAC		
M. arenaria	Far	TCGGCGATAGAGGTAAATGAC	420	[20]
	Rar	TCGGCGATAGACACTACAACT		
M. javanica	Fjav	GGTGCGCGATTGAACTGAGC	670	[20]
	Rjav	CAGGCCCTTCAGTGGAACTATAC		

studies and identified by the same method [26–28]. The populations were randomly selected as representatives of the different countries and localities, considering the esterase phenotypes similar to the selected Brazilian populations. We also emphasized species not previously detected, such as *M. izalcoensis* and cryptic species. Six species-specific SCAR primers (Table 2) were used in this work to confirm species identification. The methodology using SCAR markers was described for different species of *Meloidogyne*, according to the publications referred to Table 2.

Eggs were extracted from roots according to Carneiro et al. [30]. Total genomic DNA was extracted from 200 to 300 µl of nematode eggs using a regular phenol-chloroform extraction method as described by Randig et al. [21].

A batch of 30 random 10-mer oligonucleotide primers (Operon Technologies) was used for assessing the genetic diversity of the nematode isolates (A12, AB6, C9, F6, G2, G4, G13, J19, J20, K10, K19, K20, M20, N7, N10, P6, R4, R8, T06, U5, V7, W5, W6, W15, X16, X 20, Y5, Y16, Z4 and Z17). Amplification reactions were conducted with each primer on the DNA of the nematode isolates tested. PCR reactions were performed in a 13 µL final volume containing 1.3 µl 10X PCR reaction buffer (Phoneutria Biotechnology Services), 10 µM primer (Operon Technologies), 2.5 mM dNTPs (Invitrogen), 1 U μl^{-1} Taq DNA polymerase (Phoneutria Biotecnology and Services) and 9 ng μl^{-1} *Meloidogyne* spp. total genomic DNA amplifications were performed on a PTC-100 thermocycler: 5 min at 94 °C; 40 cycles of 30 s at 94 °C, 45 s at 36 °C, 2 min at 70 °C; and a final extension of 10 min at 70 °C [21]. PCR products were separated by electrophoresis in a 1.5% (w/v) agarose gel, stained with ethidium bromide and visualized under UV light. All RAPD analyses were repeated at least twice and only DNA fragments consistently present between replicates were recorded as present or absent directly from the gels, comparing the different populations of the same species. DNA fingerprints were converted into a 0–1 binary matrix and relationships between isolates were determined using the neighbor-joining (NJ) algorithm [31] implemented in PAUP* v. 4b10 [32], considering the data as unordered with no weighting. To test the node support of the generated tree, the analysis was performed on 1000 bootstrap replicates and only values above 50% were considered. The percentages of polymorphism were calculated among populations of the same species.

3. Results

From the five countries sampled, a total of 10 esterase profiles were found (Table 1, Fig. 1), and five species identified: *Meloidogyne arenaria* (EST A2, Rm: 1.30, 1,20), *M. enterolobii* (EST E4, Rm: 0.95, 0.90, 0.75, 0.70), *M. javanica* (EST J3, Rm: 1.4, 1.25, 1.0; EST J2a, Rm 1.25, 1.0 and EST J2b, Rm: 1.4, 1.0), *M. incognita* (EST I1, Rm: 1.0 and EST I2, Rm: 1.05, 1.0) and *M. izalcoensis* (EST I4, Rm: 1.30, 1.25, 0.95, 0.90). Two atypical species profiles were also characterized: EST SP1 (multiple weak bands) and EST SP2 (Rm: 1.02, 0.87), attributed to two unkown species named *Meloidogyne* sp.1 and sp.2, respectively (Table 1, Fig. 1). Species occurred as mixed populations in 21.4% of samples, mainly *M. javanica*, *M. incognita* and *M. arenaria. Meloidogyne javanica* was identified most frequently, with a prevalence, across countries, of 36.0%, followed by *M. incognita* (22.0%), *M. arenaria* (13.5%), *M. enterolobii* (2.8%), *M. izalcoensis* (2.1%), *Meloidogyne* sp.1 (0.71%) and *Meloidogyne* sp.2 (0.71%).

In Benin, *M. incognita* was detected in roots of carrot, okra and amaranthus, and *M. izalcoensis* in roots of purple sage and cabbage. In Kenya, *M. izalcoensis* was identified from roots of coffee and *M. javanica* and *M. incognita* from roots of tomato plants. In Nigeria *M. enterolobii* was most frequent, followed by *M. incognita* and *M. javanica* in tomato roots. In Uganda, *M. incognita*, *M. javanica* and *M. arenaria* were recorded from roots of tomato, pepper, cassava and eggplant. In Tanzania, *M. javanica, M. incognita* and *M. arenaria* occurred most frequently in roots of tomato, okra, pepper, carrot and spinach and *M. izalcoensis* was found in roots of coffee (Table 1).

The 23 populations were further characterized with molecular markers, SCAR-PCR confirmed *Meloidogyne* spp. identification by esterase phenotypes with the majority of primers validated for identification of *Meloidogyne* populations from the Americas and SSA (Table 3). The SCAR-PCR method allowed the identification of *M. izalcoensis* by amplification of specific fragments for the four populations of this species (670 bp) (Fig. 2). Other specific fragments were detected for *M. incognita* (399 bp), *M. arenaria* (420 bp), *M. enterolobii* (520 bp) and *M. javanica* (670 bp) (Fig. 3, Table 2). The SCAR markers confirmed the occurrence of three esterase intraspecific variations (Est J3, J2a, J2b) found in populations of *M. javanica* (Figs. 1 and 3). For the atypical *Meloidogyne* sp. 2 (populations 15 and 16, Table 3), a 399 bp fragment was amplified, indicating that it could be *M. incognita* (Fig. 3). The other atypical species, *Meloidogyne* sp.1, amplified two fragments: one with the same molecular weight as *M. javanica* (670 bp) and another with the same weight as *M. arenaria* (420 bp) (Fig. 3). The *M. ethiopica* population (23) was included as an outgroup, and unfortunately the SCAR marker developed for this species by Correa et al. [23] did not amplify DNA specific fragments from this species and from the atypical population *Meloidogyne* sp.1. These two populations with atypical esterase phenotypes appear to be cryptic species, therefore, in order to clarify their identity, their morphological and other molecular characters need to be further investigated.

From the 30 RAPD primers used to evaluate the genetic diversity of *Meloidogyne* spp. isolates, 409 DNA fragments were consistently amplified, of which 386 were polymorphic. The size of amplified fragments ranged from 300 to 4000 base pairs (Fig. 4). In general, all populations of the same species originating from the two continents (*M. arenaria, M. enterolobii, M. incognita, M. izalcoensis, M. javanica*) grouped together in the dendogram with high bootstrap values (Fig. 5), indicating a direct correlation between the identification with the esterase profiles, with the SCAR markers and the phylogeny analysis generated by the RAPD primers (Fig. 5). Two exceptions were

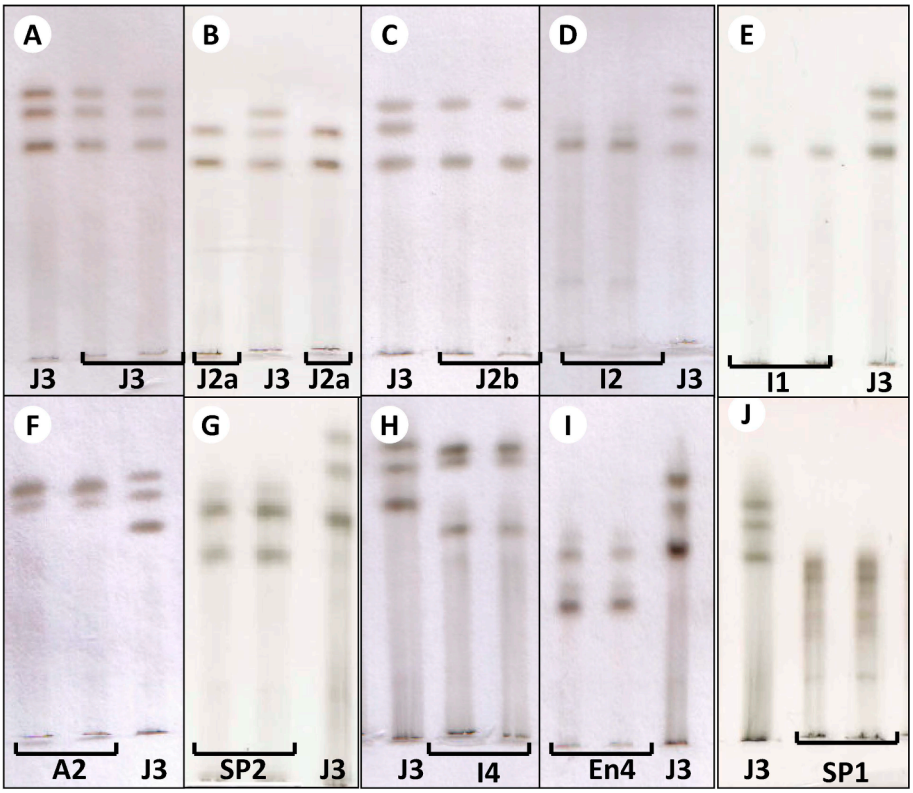

Fig. 1. Ten esterase phenotypes (Est) found in 130 populations of *Meloidogyne* spp. collected in sub-Saharan Africa. A, B, C) Est J3, J2a, J2b = *M. javanica*; D, E) Est I2, I1 = *M. incognita*; F) Est A2 = *M. arenaria*; G) Est SP2 = *Meloidogyne* sp.2; H) Est I4 = *M. izalcoensis*; I) Est En4 = *M. enterolobii*; J) Est SP1 = *Meloidogyne* sp.1. *M. javanica* (Est J3) was used as reference in each gel.

Table 3
Identification of *Meloidogyne* spp. populations from Africa, Brazil and El Salvador used in phylogenetic studies: esterase phenotypes (Est) and SCAR markers.

Nº	Country	Locality	Identification	
			EST	SCAR
1	El Salvador	Izalco	*M. izalcoensis* (I4)	*M. izalcoensis*
2	Benin	Cotonou	*M. izalcoensis* (I4)	*M. izalcoensis*
3	Tanzania	Tindini	*M. izalcoensis* (I4)	*M. izalcoensis*
4	Kenya	Kabete	*M. izalcoensis* (I4)	*M. izalcoensis*
5	Kenya	Lamu	*M. incognita* (I2)	*M. incognita*
6	Tanzania	Kawe	*M. incognita* (I2)	*M. incognita*
7	Benin	Pahou Oiadah	*M. incognita* (I2)	*M. incognita*
8	**Brazil**	**Londrina, PR**	***M. incognita* (I2)**	***M. incognita***
9	Tanzania	Msongozi	*Meloidogyne* sp. (SP 2)	*M. incognita*
10	**Brazil**	**Marechal Cândido Rondon, PR**	***Meloidogyne* sp. (SP 2)**	***M. incognita***
11	**Brazil**	**Londrina, PR**	***M. javanica* (J3)**	***M. javanica***
12	Kenya	Taveta	*M. javanica* (J3)	*M. javanica*
13	Uganda	Mukono	*M. javanica* (J2b)	*M. javanica*
14	Kenya	Oloitoktok	*M. javanica* (J2b)	*M. javanica*
15	Uganda	Luwero	*M. javanica* (J2a)	*M. javanica*
16	**Brazil**	**Brasília**	***M. javanica* (J2a)**	***M. javanica***
17	**Brazil**	**Casa Nova - PE**	***M. javanica* (J2b)**	***M. javanica***
18	Uganda	Wakiso	*M. arenaria* (A2)	*M. arenaria*
19	**Brazil**	**Recife - PE**	***M. arenaria* (A2)**	***M. arenaria***
20	**Brazil**	**Petrolina, PE**	***M. enterolobii* (E4)**	***M. enterolobii***
21	Nigeria	Oyo	*M. enterolobii* (E4)	*M. enterolobii*
22	Kenya	Taveta	*Meloidogyne* sp. (SP1)	*M. javanica and M. areanaria*
23	**Brazil**	**Farropilha, RS**	***M. ethiopica***	-

nevertheless noticed: the M4 population from *M. izalcoensis* and the M5 population from *M. incognita* (Fig. 5). The unknown species from Africa *Meloidogyne* sp. 2 (Est SP2) clustered with *Meloidogyne* sp. 2 from Brazil and *M. incognita*. *Meloidogyne* sp.1 (Est SP1) from Africa clustered with a *M. ethiopica* (Est E3) isolate from Brazil, although the esterase phenotypes of *Meloidogyne* sp.1 were distinct.

Overall, a low level of polymorphism was observed between the species from Africa and the Americas, i.e., *M. incognita* (30%), *M. javanica* (34%), *M. arenaria* (37%), *M. enterolobii* (22%), *Meloidogyne* sp. 2 (17%), *Meloidogyne* sp.1 and *M. ethiopica* (22%), with the exception of *M. izalcoensis,* which presented 50% of polymorphic bands (Table 4, Fig. 4).

4. Discussion

Knowledge surrounding the morphological, biochemical and molecular variability within a species that originates from different geographic localities is essential to determine the identity of *Meloidogyne* populations towards recognizing differential and stable characters for their diagnosis. Ultimately, the combined use of morphology, isozyme and/or molecular techniques is advocated as the most reliable way to accurately identify *Meloidogyne* spp [33]. In the current study, six known species were identified from 143 field samples collected across five countries in SSA: *M. arenaria*, *M. enterolobii*, *M. incognita*, *M. izalcoensis* and *M. javanica.* In addition, two atypical, unknown species were detected. The esterase phenotype proved the best technique to identify all species and to additionally characterize two new atypical species: *Meloidogyne* sp.1 and *Meloidogyne* sp.2. This confirms the reliability of esterase phenotyping as observed by Refs. [15,16] and Carneiro et al. [17,18]. The DNA-based SCAR-PCR technique enabled the accurate identification of five species: *M. arenaria*, *M. incognita* and *M. javanica*, *M. izalcoensis* and *M. enterolobii*, but was unable to definitively characterize *M. ethiopica* [23] and the two cryptic species, in part because the primers developed for *M. ethiopica* have been ineffective to identify this species or primers are not yet available for these unknown species [14]. Furthermore, the SCAR-PCR erronously identified *Meloidogyne* sp.1 as *M. javanica* and *M. arenaria,* and *Meloidogyne* sp.2 as *M. incognita*, all of which presented perineal patterns for *M. incognita* (Araújo Filho et al., 2016). However, *Meloidogyne* sp. 2 presented a unique esterase phenotype, and recent genome sequencing data

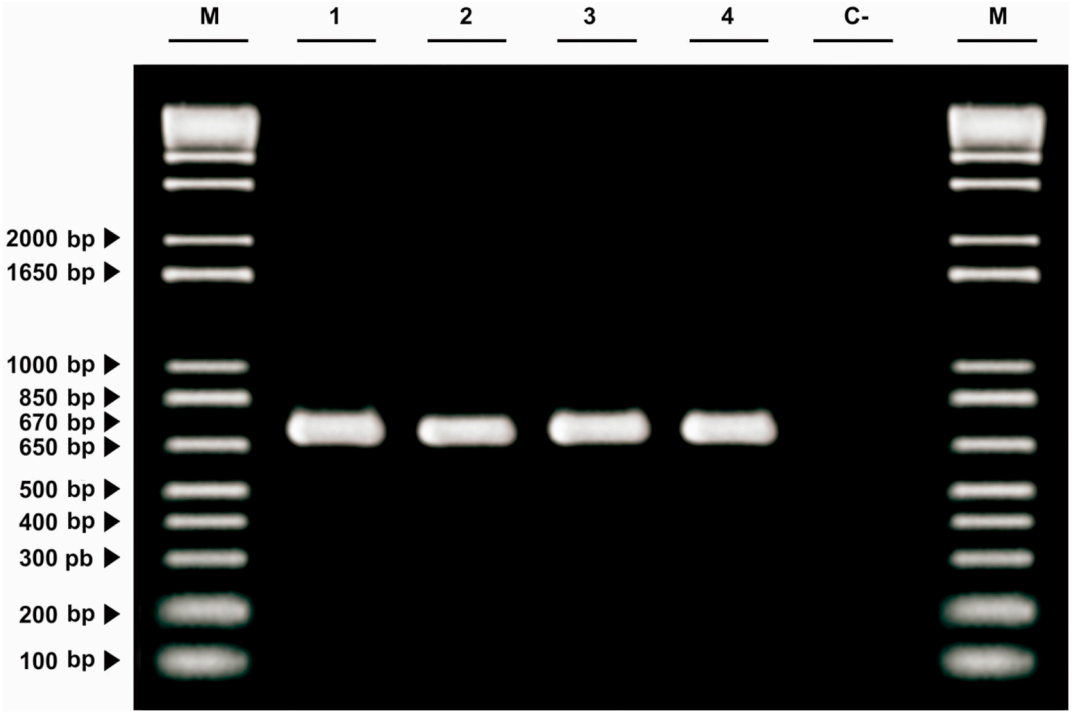

Fig. 2. PCR amplification of four *Meloidogyne izalcoensis* populations generated with specific SCAR primers iz-AB2F [22]. Populations from: 1) El Salvador, 2) Benin, 3) Tanzania and 4) Kenya.

indicated that it did not cluster with other populations of *M. incognita* (Erika V·S.A. Freire, personal information).

In the current study, *M. javanica* and *M. incognita* were identified most frequently, reflecting recent studies in which populations have been identified to species level (see Ref. [12]. However, studies involving RKN occurring in SSA have regularly reported the identification of *Meloidogyne* (and other plant-parasitic nematodes) to the genus level only, generally due to limited resources and expertise for morphological or molecular analysis. The identity, diversity and distribution of *Meloidogyne* spp. in SSA is therefore, in general lacking, but now gradually improving. Studies using isoenzymes to identify *Meloidogyne* spp. in the countries sampled in the current study in Africa are scarce or non-existent, even from relatively recent studies (e.g. Refs. [34–37]. It appears however, that *M. javanica* and *M. incognita* are indeed the most prevalent species of RKN occurring across Africa (IITA, 1981 [8,36,38,39]; [12]; [40]. A similar predominance of *M. javanica* and *M. incognita* occurs under, natural vegetation in Brazil (e.g. [26,41,42], or on crops such as potato [43]. Studies from Brazil also demonstrate low genetic diversity among the populations of these two species [27,43].

Meloidogyne enterolobii, identified in just 2.8% of total samples in the current study, was the most frequently identified species in Nigeria, and it has being increasingly recovered from Africa [8,40]. It is less common in East Africa, and while not identified during the current study in East Africa it has been identified parasitizing sweet potato [44] and nightshade [45] in Kenya. The apparent broadening distribution of this aggressive species, is cause for concern and requires attention with respect to nematode management [7,38]. Although the overall species diversity was not extensive in the current study, given that 23 *Meloidogyne* spp. have been so far recorded from Africa, the overall number of samples was relatively small. Even though two unknown species and novel records were observed, a wider diversity was anticipated, however. *Meloidogyne izalcoensis* was found in three of the five countries sampled. This species was previously reported for the first time in Africa, in Benin and Tanzania, based on initial findings from the current study [13]. The

Fig. 3. PCR amplification patterns for 22 populations of *Meloidogyne* spp. generated with specific SCAR primers: inc-K14 F/R [21], F/R jav (Ziljlstra et al., 2000), F/R ar (Ziljlstra et al., 2000) and Mk7 F/R [29]. (+inc, +jav, + are, + ent): positive controls, *M. incognita*, *M. javanica*, *M. arenaria* and *M. enterolobii* respectively. (−) DNA: negative control. M: 1 kb Plus DNA ladder (Invitrogen). Population codes are given in Table 3.

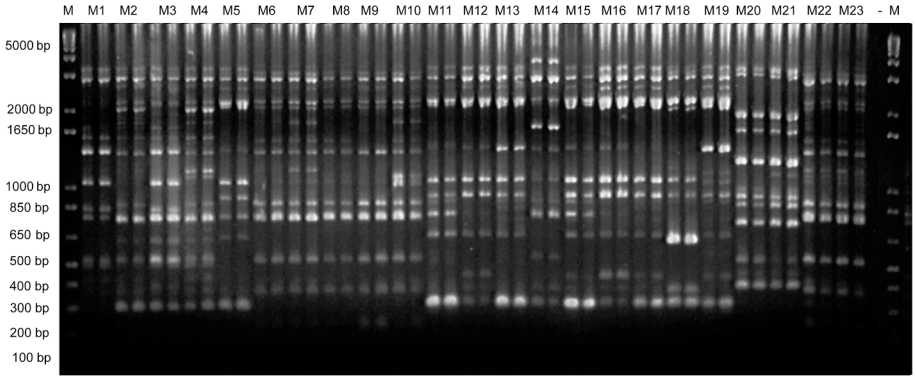

Fig. 4. Genetic diversity of *Meloidogyne* spp. analysed with primers RAPD Z17: *M. izalcoensis* (M1, M2, M3, M4), *M. incognita* (M5, M6, M7, M8), *Meloidogyne* sp.2 (M9, M10), *M. javanica* (M11, M12, M13, M14, M15, M16, M17), *M. arenaria* (M18, M19), *M. enterolobii* (M20, M21), *Meloidogyne* sp.1 (M22), *M. ethiopica* (M23). (−): DNA negative control. M: 1 kb Plus DNA Ladder (Invitrogen); bp: base pairs. For each population, two duplicate amplifications were loaded side by side on the gel.

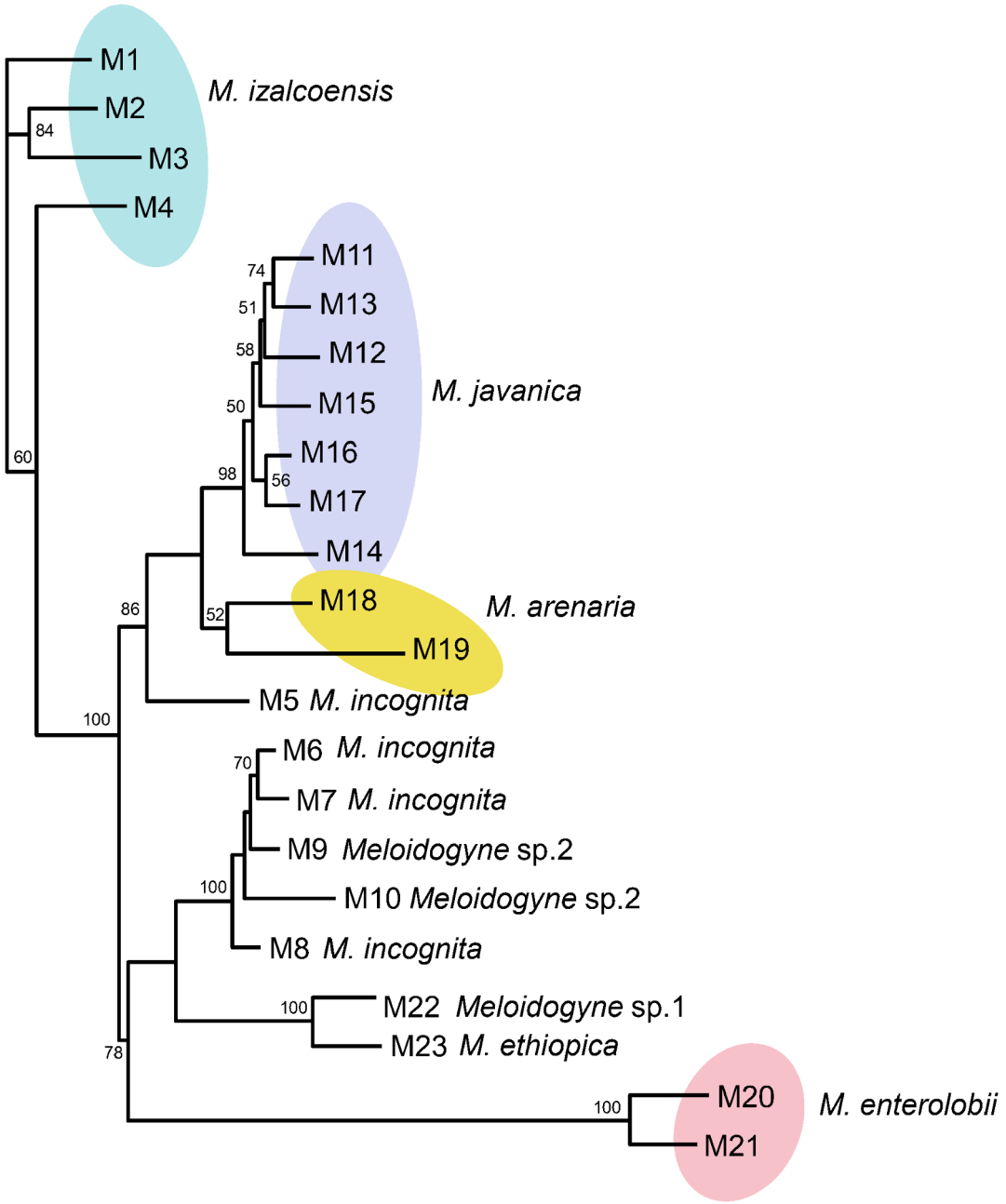

Fig. 5. Majority-rule consensus UPGMA dendrogram showing phylogenetic relationships between *Meloidogyne* spp. from Brazil, El Salvador and Africa. Bootstrap values (> 50%) based on 1000 replicates are shown. Population codes are given in Table 3.

Table 4
Percentage of polymorphisms observed in *Meloidogyne* spp. at species level *Populations were reported in Table 3.

Species and populations	RAPD fragments	
	Amplified	Polymorphic (%)
M. izalcoensis (M1, M2, M3, M4)*	173	88 (50)
M. incognita (M5, M6, M7, M8)	182	56 (30)
Meloidogyne sp.2 (M9, M10)	183	32 (17)
M. javanica (M11, M12, M13, M14, M15, M16, M17)	200	68 (34)
M. arenaria (M18, M19)	186	69 (37)
M. enterolobii (M20, M21)	172	38 (22)
Meloidogyne sp. 1 + *M. ethiopica* (M22, M23)	158	35 (22)

current study extends the country record for *M. izalcoensis* to Kenya, and to sweet pepper, in addition to cabbage, coffee and tomato [13]. The damage potential of this species on these crops in SSA is still unknown. The novelty of country and host records of *Meloidogyne* spp. in this relatively limited study demonstrates our limited knowledge on RKN occurrence in SSA, and the potential for new host, country and continental records, as well as the occurrence of new undescribed species. Given the enormity and agro-ecological diversity of the African continent, 143 field samples from various crops is, in effect, quite a limited sample size. Much broader assessment would therefore be required to enable a comprehensive understanding of *Meloidogyne* spp. occurrence and distribution in SSA. However, the magnitude of the work undertaken during the current study cannot be underestimated, involving field sampling in five countries, culturing, rearing single species cultures and shipping abroad for culturing and assessment. Consequently, numerous samples were lost along the way, reducing the ultimate number of populations examined. It is curious why some species were not recovered in the current study, such as *M. ethiopica,* in East Africa [46–48].

When examining the genetic variability of *Meloidogyne* spp., low variability was observed in *M. arenaria*, possibly due to the low number of individual populations studied. Generally, *M. arenaria* is characterized by high intraspecific variability and it is considered a swarm species [49]. The high variability detected for *M. izalcoensis*, approximately 50%, was observed for the first time during the current study, and the SCAR markers [22] used for *M. izalcoensis* were validated for the African populations.

When comparing the *Meloidogyne* spp. populations from Africa with those from the Americas, *M. javanica* populations presented the predominant esterase profile Est J3, with the populations having the esterase profiles Est J2a and J2b appearing less but in both continents [43]. A similar situation was observed for *M. incognita*, with the predominance of Est I2 compared with Est I1 [27] and a lower presence of the atypical profile Est SP2 occurring similarly in both continents. The low genetic variability observed between the African and Brazilian populations of *Meloidogyne* spp. and the occurrence of the same species and variants on both continents is intriguing.

The genetic proximity between the African and Latin American *Meloidogyne* spp. and populations can possibly be explained by the Pangaea's theory. Plant-parasitic nematodes may have existed in the Silurian or even Ordovician since the earliest known fossils are from the Devonian (416-359 million years) [50]. The genus *Meloidogyne* itself is much more recent, but there is no reliable age estimate available (Etienne Danchin, personal information). In our opinion *Meloidogyne* spp. may have evolved when the Earth's mass was organized into the Pangaea supercontinent. Consequently, with the separation of the continental land masses 175 million years ago, the *Meloidogyne* species may have spread independently within both Africa and Brazil in a similar manner as is documented for some insects, such as ants [51].

5. Conclusions

The current study confirms previous evidence that *M. javanica* and *M. incognita* are the predominant species occurring on peri-urban vegetable crops across SSA. The aggressive species *M. enterolobii* was detected in Nigeria and poses a threat to crop production across the African continent. The study also demonstrates that new host and country records will continue to be revealed as we extend our exploration of *Meloidogyne* spp. on crops in SSA using more reliable diagnostic techniques. Consequently, these studies will enable better assessment of the RKN pests that threaten crop production, and how to counter such threats.

Declaration of interest

None.

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