

Preliminary Molecular Characterization of Root-knot Nematode Species Associated with Tomato Grown in Three Ecozones of Nigeria

*¹C.O. Eche and ²J.J. Atungwu

¹Department of Crop and Environmental Protection, University of Agriculture, P.M.B. 2373, Makurdi, Benue State, Nigeria, **Corresponding** Author's e-mail: elqris@gmail.com

²Department of Crop Protection, Federal University of Agriculture, P.M.B. 2240 Abeokuta, Ogun State, Nigeria

ABSTRACT

Background: Root-knot nematodes (RKN) have been reported as one of the major constraints of tomato production worldwide. Identification of RKN to species level based on morpho-anatomy is cumbersome, misleading in the absence of taxonomic expertise and consequently results in poor management. However, use of molecular-based approaches promises to increase precision of identification and reduce taxonomic bias. **Materials and Methods:** Root-knot nematode-infected roots of tomato were collected from nine local government areas (LGAs) spread across three distinct agro-ecological zones in Nigeria between 2015 and 2016 from a total of forty-six (46) farmers' fields. RKN genomic deoxyribonucleic acids (gDNA) of specimen were extracted based on ecozones, followed by amplification of the intergenic spacer region of ribosomal DNA. Sequences obtained from amplicons were subjected to phylogenetic analysis to determine their evolutionary relationship. **Results:** Molecular data from the Intergenic Spacer region of ribosomal DNA expansion segment revealed formation of topology with five distinct clades, well-supported by bootstrap confidence values ranging from 90% to 100% and representing *M. incognita* (Mi), *M. enterolobii* (Me), *M. arenaria* (Ma), *M. javanica* (Mj) and *M. hapla* (Mh) groupings. The analysis showed that seven (77.78%) out of 9 nematode populations clustered in the Mi clade while isolates from Gboko and Ajingi clustered in the Mj and Ma clades, respectively. No

RKN species clustered in the Me and Mh clades. Calculated Tajima's Statistics for Neutrality Test was 2.125 and indicates low levels of low and high frequency genetic polymorphism within and between clades. **Conclusion:** This investigation provides information on genetic variation within RKN species across the ecozones and can form the basis for determining efficient management strategies through indigenous breeding programmes for RKN resistance in tomato planted in these areas.

Keywords: Tomato, Intergenic Spacer Region, *Meloidogyne* species, Phylogeny, Nigeria, Molecular Taxonomy, Agro-ecological zones, Speciation

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INTRODUCTION

In Africa, Nigeria is ranked second largest producer of tomato after Egypt and 13th in the world, producing about 1.2 million tons at an average of 25 to 30 tons per hectare (NARICT¹). Although the crop is grown in all agro-ecological zones in Nigeria, in the Southwest comprising Rain Forest and Derived Savannah, it is majorly cultivated under smallholdings mostly under rain-fed conditions and in the North, comprising Northern Guinean Savanna, under irrigation systems (Ayandiji *et al.*²). Commercial production relies mostly on exotic introductions and its production is largely cultivated under the Northern Guinea Savanna and the Sudan ecologies compared to other agro-ecologies. This is due to favourable climatic conditions, particularly high insolation, low relative humidity and rainfall

in the Northern agro-ecological zones (Etebu *et al.*³). In Nigeria, tomato accounts for about 18 % of the average daily consumption of vegetables (Babalola *et al.*⁴). Hence this makes it a very important food crop to an average Nigerian (Adepoju⁵). It can be eaten fresh or processed into products — including canned tomatoes, tomato sauce, tomato paste, tomato soup, tomato juice and ketchup – with tremendous health benefits.

However the pestiferous activities of root-knot nematodes (RKN) have been reported as one of the causes of low return on investment in tomato cultivation within the country. Their highly adaptable, obligate, and polyphagous natures endow them with the where withal to cause vast damages on tomato root architecture and structural proteins within the roots in course of feeding thereby interfering with root functions. They are distributed worldwide and parasitize nearly every species of higher plants including tomato.

While identification based on morphological diagnosis is possible given the availability of an in-depth taxonomic skill and high throughput equipment, it is often saddled by certain limitations. First, there is a high chance of species misidentification due to both phenotypic plasticity as well as genetic variability (Hebert *et al.*⁶). Second, certain life stages or sex may be needed before a complete identification is possible in some cases; hence in the absence of such a criterion, identification is either impossible or subjective (Jarman and Elliott⁷). Third, the identification of cryptic species i.e. two or more distinct species that were classified as the same due to morphological similarity (Bickford *et al.*⁸), is most times overlooked. This is because of the difficulty of identifying such species based on homogenous phenotypic morphological characters (Xiao *et al.*⁹). As a result of all these inherent limitations associated with morpho-based identification technique, a new approach which overcomes these limitations has been the key focus for molecular taxonomists in recent times.

Different molecular techniques such as Real-Time Polymerase Chain Reaction (PCR), PCR-Restriction Fragment Length Polymorphism, Multiplex PCR, Random Amplified Polymorphic DNA and Amplified Fragment Length Polymorphism, have been tested towards assessment of nematode diversity and rapid identifications (Floyd *et al.*¹⁰; De Ley *et al.*¹¹; Bhadury *et al.*^{12,13}). In delineating species of *Meloidogyne* genus several fragments of DNA have been exploited such as Intergenic Spacer (IGS) region of the ribosomal DNA (Wishart *et al.*¹⁴; Adam *et al.*¹⁵); Internal and External Transcribed Spacer regions, mitochondrial DNA among others. The primary goal is to accurately identify RKN to species level, eliminating “taxonomist’s judgemental bias” or errors which are frequent, hence leading to incongruent conclusions on species type. Since resistance genes have been reported to be species- or cultivar-specific and only effective against one or a few nematode species or races, successful management of root-knot nematodes will therefore require accurate identification of the nematodes present in the field. Hence this study was undertaken to provide detailed molecular phylogenetic basis for species delineation of RKN populations across three agro-belts in Nigeria for the first time.

MATERIAL AND METHODS

Nematode Sample Collection and Extraction

Nematode-infected root gall of tomato were collected from nine (9) local government areas (LGAs) spread across three distinct agro-ecological zones in Nigeria between 2015 and 2016 as shown in Table 1 and Fig 1. Tomato root samples for identification of root-knot nematode females were collected from a total of forty-six (46) farmers’ fields. In each field, five (5) to ten (10) tomato roots were obtained. Infected roots and soil were put into polythene bags,

labelled appropriately according to their geographic origin and brought to Advanced Plant Pathology Laboratory, Department of Crop and Environmental Protection, University of Agriculture, Makurdi for identification and extraction of root-knot nematode females. *Meloidogyne* females were separated from infected tomato roots using a nematode needle under a stereomicroscope (Leica, S8APO) at 40x magnification. *Meloidogyne* females separated from gall-infected roots from farmers' fields within same LGA were where grouped to form a root-knot nematode (RKN) population. Each RKN population was then transferred into well-labelled Eppendorf tube containing 20 μ L worm lysis buffer (50mMKCl, 10mTris pH 8.3, 2.5 mM MgCl₂, 0.45% NP40, 0.45% Tween 20). Specimens were kept at 4°C in a refrigerator.

Table 1: Local government areas from where nematode populations were obtained

AEZ	State	LGA	Nematode Population Code	Geographical Coordinate of Location (Longitude and Latitude)	Number of Composite Samples Collected
DS	Oyo	Akinyele	M-Ak	3° 54'59"E and 7°35'50"N	5
DS	Oyo	Ido	M-Id	6°51'0"E and 4°44'0"N	3
DS	Ogun	Abeokuta*	M-Ab	3°21'0"E and 7°9'0"N	4
SGS	Benue	Otupko	M-Ot	8°40'0"E and 6°49'0"N	3
SGS	Benue	Makurdi	M-Mk	8°32'0"E and 7°44'0"N	4
SGS	Benue	Gboko	M-Gb	10°6'0"E and 7°26'0"N	4
NGS	Kano	Bunkure*	M-B	8°33'0"E and 11°42'0"N	6
NGS	Kano	Ajingi*	M-Aj	9°2'21"E and 11°58'12" N	10
NGS	Kano	Kura*	M-K	8°13'0"E and 23°34'0"N	7

*Samples collected under irrigated fields; AEZ: Agro-ecological zone; DS = Derived Savannah; SGS: Southern Guinea Savannah; NGS: Northern Guinea Savannah

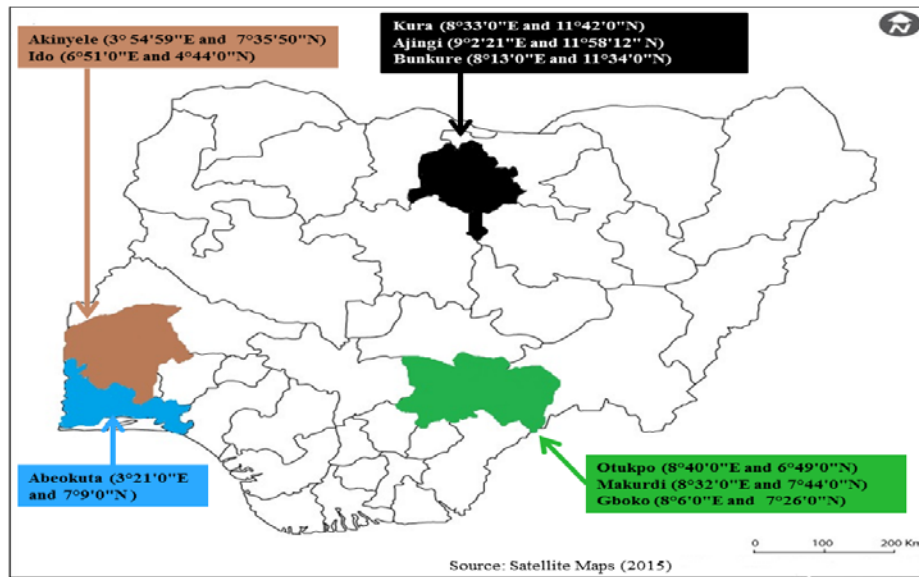


Figure 1: Sampling points along three agro-ecological zones in Nigeria in 2014, 2015 and 2016. Black, Green, Brown and Blue areas represent Kano, Benue, Ogun and Oyo States respectively.

DNA extraction

To extract genomic deoxyribonucleic acid (gDNA) of each specimen, proteinase K (1 μ l of 10g/ml) was added into each Eppendorf tube containing a single female nematode in worm lysis buffer (50Mm KCl, 10Mm Tris pH 8.2, 2.5Mm MgCl₂, 0.45% NP₄O, 0.45% Tween 20 (Sigma) and 0.01% gelatine. This was followed by incubation at 65°C for one hour and the proteinase K was denatured at 95°C for 10minutes. Finally, the extracted DNA was centrifuged for one minute at 20°C and stored at 4°C.

Amplification of intergenic spacer (IGS) region of rDNA

Amplification of the IGS region of the ribosomal DNA (rDNA) was done by polymerase chain reaction (PCR) using a pair of forward (194: 5'TTAACTTGCCAGATCGGACG3') and reverse (195: 5'TCTAATGAGCCGTACGC3') primers according to Blok *et al.*¹⁶. PCR-Mix was prepared in a total volume of 25 μ L containing 15.13 μ L distilled water, 2.5 μ L of 10x PCR

buffer, 2.5µL dye, 2µL MgCl₂, 0.5µL dNTP, 0.25µL of each primer (25 nM), 0.125µL TopTaq polymerase (Qiagen) and 2µL DNA. Three test samples were randomly chosen each time to test the amplification success of the primer set and optimize the PCR Machine. The optimal amplification conditions were: initial denaturation of 3 minutes at 94°C, 45 cycles of (94°C for 30s; 50°C for 30s; 72°C for 90s) and a final extension of 10 minutes at 72°C. PCR products (amplicons) were loaded on 1% agarose gel containing 0.003% ethidium bromide and visualized using BioDoct-It™ Imaging System (UVP). Gel contained one lane with 4µL of DNA Mass Ladder (Invitrogen) and one lane which contained 4µL of negative control (PCR product without DNA) while other lanes contained 4µL PCR products of the specimens under scrutiny. Amplifications were considered successful when a band of the expected size was observed on agarose gel and when aspecific bands were absent. A total of eleven (11) lanes were activated on the gel.

Sequencing

5µL of PCR products which gave reliable bands and void of aspecific bands were added into 96-well plates containing 5µL of primers: 194 and 195 were used as forward and reverse primers respectively to sequence the 18S gene. Sequencing plates were then sent to Macrogen, The Netherlands for sequencing.

Phylogenetic analyses of sequences

To investigate the biological origin of the sequences as being nematode sequences, all sequences were first subjected to a Basic Local Alignment Search Tool search (Altschulet *al.*¹⁷) using BLASTn for 5S-18S gene fragment. Reliability and base calling of sequences obtained were checked using BioEdit Sequence Alignment Editor. The obtained chromatogram for each sequence was used as a yardstick in the selection of usable and non-usable sequences.

Chromatograms with clearly defined nucleotide peaks and high fluorescent signals were considered usable while those with multiple peaks and low signals were considered non-usable. Forward and reverse sequences of IGS rDNA region were edited, assembled and merged into consensus sequences using ChromasLite Version 2.1.1. Software. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model to reconstruct Neighbour-joining trees using MEGA v. 6.0. (Tamura *et al.*¹⁸) with bootstrap analysis at 1000 replications of both DNA extracted from this study and additional sequences from Genbank (Table 2). Tajima Neutrality Test statistic was used to determine polymorphism between sequence data.

Table 2: List of sequences of each species of the genus *Meloidogyne* obtained from NCBI was used in the study

S/N	Species	Accession No.
1	<i>Meloidogyne incognita</i>	GQ395504
2	<i>Meloidogyne incognita</i>	GQ395506
3	<i>Meloidogyne incognita</i>	GQ395505
4	<i>Meloidogyne incognita</i>	GQ395505
5	<i>Meloidogyne enterolobii</i>	GQ395530
6	<i>Meloidogyne enterolobii</i>	GQ395529
7	<i>Meloidogyne arenaria</i>	GQ395522
8	<i>Meloidogyne arenaria</i>	GQ395523
9	<i>Meloidogyne javanica</i>	GQ395512
10	<i>Meloidogyne javanica</i>	GQ395511
11	<i>Meloidogyne hapla</i>	AJ421708
12	<i>Meloidogyne hapla</i>	AJ421704
13	<i>Meloidogyne thailandica</i>	HF568829
14	^x <i>Bursaphelenchus xylophilus</i>	AB500152

x represents sequence used as outgroup in the study

RESULTS

The primer set – 194/195 used for the amplification of the IGS region produced clearly marked bands for all nematode populations used in the study (Plate 1). However amplification of the fragment between 5S and 18S across all nine root-knot nematode populations was unable to delineate populations into species since amplicons sizes obtained for all nematode populations were found to tally with the 710bp mark of the mass ladder on electrophoretic gel. Absence of band in the control (c) lane as shown in Plate 8 indicated that polymerase chain reaction (PCR) was void of foreign DNA contamination. Aspecific bands were absent under optimized thermal cycler conditions and this was reflected on the image capturing of the electrophoretic gel.

A total of 23 sequences were used in this study, nine consensus sequences of which were from DNA samples obtained from the nine nematode populations and 14 from Genbank. Consensus sequences were subsequently compared with the Genbank database to check their nematode origin. All hits reported hereafter had a coverage of 99% or 100%. Sequences were most similar to nematodes. No stop codons or frame shift mutations were observed in the alignment.

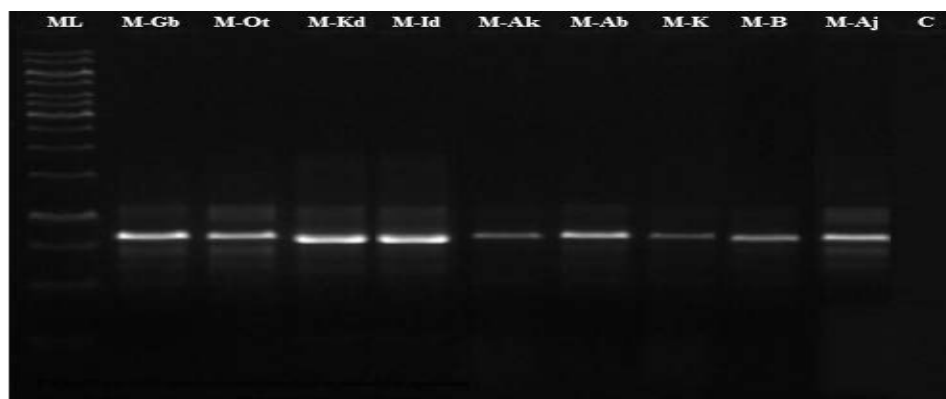


Plate 1:Electropheric gel imaging showing Amplicons of IGS region (between 5S-18S) of rDNA using 194/195 primer-set on eleven root-knot nematode populations from Gboko (M-Gb), Otukpo (M-Ot), Makurdi (M-Mk), Ido (M-Id), Akinyele (M-Ak), Abeokuta (M-Ab), Kura (M-K), Bunkure (M-B), Ajingi (M-Aj). C=control; ML = Mass Ladder.

Phylogenetic Analysis of Sequence Data based on IGS rDNA

To investigate if differences exist in the nucleotide compositions between nematode populations and ascertain their species identity, phylogenetic analysis was performed which resulted in five nucleotide clouds represented by five distinct clades including *M. incognita* group, *M. enterolobii* group, *M. arenaria* group, *M. javanica* group and *M. hapla* group. The analysis showed that 7 out of the nine nematode populations clustered in the incognita clade. They include isolates from Ido, Otupko, Kura, Abeokuta, Akinyele, Bunkure and clade was significantly supported by a bootstrap value of 96 (Figure 2). Only population from Ajingi fell in the arenaria group and supported by a bootstrap value of 99. Isolate from Gboko clustered in the javanica clade supported by a bootstrap value of 100. From the study no isolate clustered within the hapla and enterolobii clades. *Bursaphelenchus xylophilus* which was chosen as the outgroup formed the basal node. However, genetic polymorphism within each clade showed low levels of low and high frequency polymorphism as calculated by Tajima's Neutrality Test (Table 3).

Table 3: Neutrality Test on the rDNA sequences of *Meloidogyn* populations isolated from three distinct ecozones of Nigeria

<i>m</i>	<i>S</i>	<i>ps</i>	Θ	π	D
23	290	0.979730	0.265451	0.404417	2.125059

m = number of sequences, *n* = total number of sites, *S* = Number of segregating sites, *ps* = *S/n*, Θ = *ps/a*, π = nucleotide diversity, and D is the Tajima test statistic.

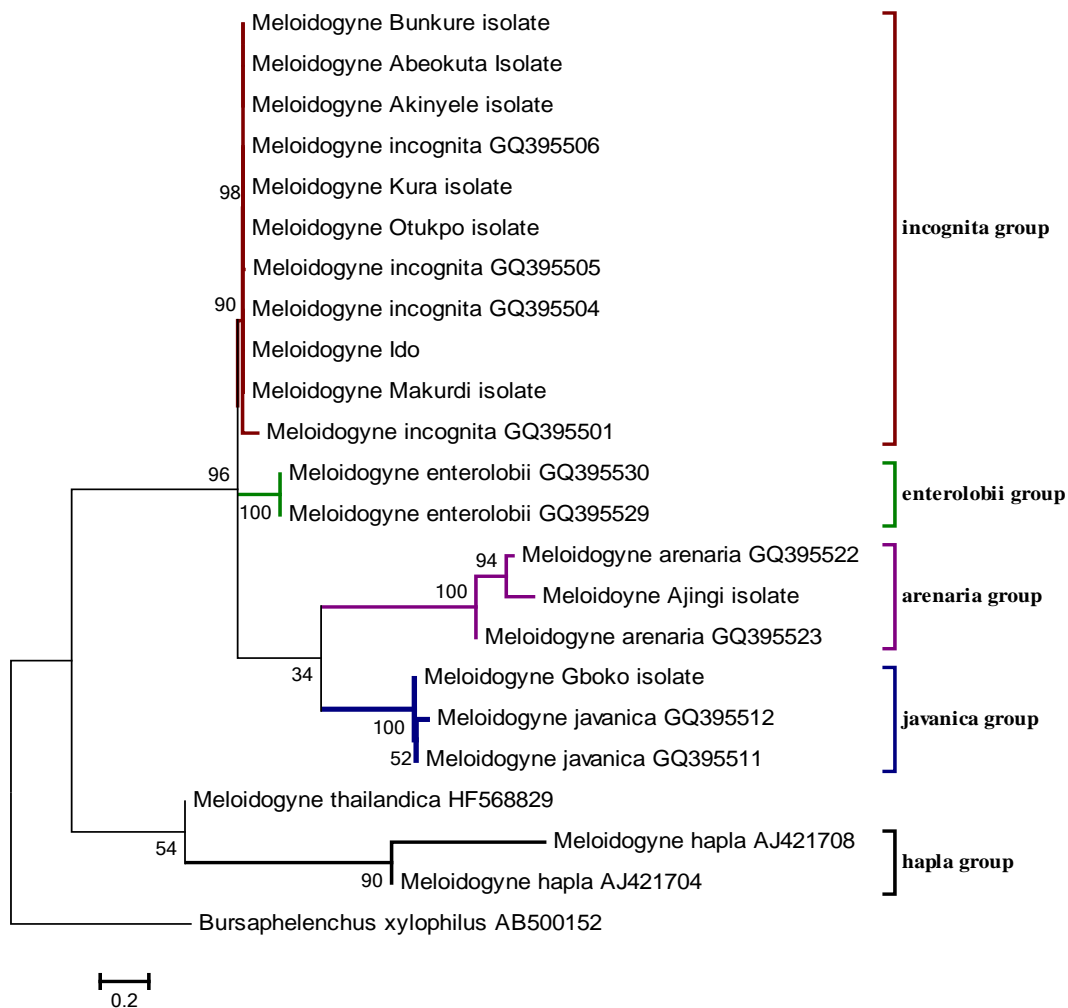


Figure 2. Molecular Phylogenetic analysis by Maximum Likelihood method.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-3139.9712) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 24 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 296 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

DISCUSSION

Molecular identification based on the intergenic spacer region of the ribosomal DNA (IGS-rDNA) region has been used in previous studies as a diagnostic target to differentiate various *Meloidogynes* species (Blok *et al.*¹⁶; Wishart *et al.*¹⁴). The IGS-rDNA was chosen in this study as the target site because it has relatively conserved and highly variable regions which have been used in identification and in reconstruction of phylogenetic relationships for RKN and other nematode species (Landa *et al.*¹⁹). Amplification of this target genes from findings obtained from this study indicated that identification of RKN based only on IGS-rDNA amplicons fragment size did not delineate RKN to species level since electrophoretic bands of all nematode populations were aligned at the 710bp mark of the mass ladder. This finding is consistent with Akyazi and Felek²⁰ who reported that amplification amplicons of all 17 nematode populations of RKN used in their study gave the same fragment size and could not separate species at this level.

Furthermore, Adam *et al.*¹⁵ reported that with the 194/195 primers the amplification products from tropical species (*M. incognita*, *M. javanica* and *M. arenaria*) and *M. hapla* are of very similar sizes (720 and 700 bp, respectively) and inclusion of positive controls could be helpful to resolve the different products on an agarose gel. Nevertheless previous study by Wishart *et al.*¹⁴ showed that polymerase chain reaction amplification of the intergenic spacer region between the 5S and 18S genes from *Meloidogyne chitwoodi*, *M. fallax*, and *M. hapla* enabled these three important temperate species to be differentiated. Length polymorphism was found between *M. chitwoodi* and *M. fallax* as a result of differing numbers of short repeats located between the 5S and 18S genes when 194/195 primer set was used.

The results of phylogenetic analyses of RKN populations isolated from Kano (Bunkure, Kura and Ajingi), Benue (Gboko, Otukpo and Makurdi), Oyo (Akinyele and Ido) and Ogun (Abeokuta) States indicated five (5) groups – *incognita*, *enterolobii*, *arenaria*, *javanica* and

hapla groups spread across the three ecozones involved in the study. In general, all population sequence data were clustered independent of geographic origin into clear separations of *M. hapla*, *M. enterolobii* (Syn = *M. mayaguensis*), *M. incognita*, *M. javanica* and *M. arenaria*. All populations collected from Derived Guinea zone clustered within the *M. incognita* clade alongside isolates from Bunkure, Kura, Otupko and Makurdi. This finding validates previous reports based on RKN morphometrics by Chindo and Bello²¹ that *M. incognita* is the most prevalent root-knot nematode in northern Nigeria. It is however not consistent with findings of Bridge²², Bos²³ and Caveness²⁴ that *M. javanica* is the most prevalent RKN in northern Nigeria. In addition, Onkendi *et al.*²⁵ reported that *M. incognita* was the most reported RKN in Nigeria. According to the sampling results of the international Meloidogyne project, *M. incognita* was reported to be the most distributed RKN species and investigations at different times and in different countries also showed that *M. incognita* is the most encountered species (Khan and Ahmad²⁶; Guzman-Plazola *et al.*²⁷; Anwar and McKenry²⁸; Kayani *et al.*²⁹). Chindo and Bello²¹ further stated that this discrepancy in *M. incognita* occurrence in northern Nigeria could be linked to changes in crop culture due to the introduction of new crop varieties which may be more efficient hosts of *M. incognita* than *M. javanica*. In addition, the time differences and soil edaphic factors arising from soil fertilization may account for this difference as well.

The study reports identification of *M. javanica* and *M. arenaria* for the first time based on molecular data in Gboko and Ajingi respectively, although confirmatory studies will enhance available information on RKN distribution in these areas as little or no research findings are available in these regions. One of the root-knot nematodes currently of global concern is *M. enterolobii* because it can reproduce on cultivars with the Mi resistance gene (Fargette³⁰; Berthou *et al.*³¹; Carneiro *et al.*³²; Cetintas *et al.*³³). The Mi resistance gene confers resistance to the three major tropical-subtropical nematode species, such as *M. incognita*, *M. javanica*

and *M. arenaria* (Zoon *et al.*, 2004). It is highly virulent and produces more root galls compared to other root-knot nematodes (Onkendi *et al.*²⁵). In Africa, it has been reported in Burkina Faso, Ivory Coast, Malawi, Senegal, South Africa and Togo. It is for this reason that *M. enterolobii* sequence data were compared with those used in this study, to determine if any population forms a clade with enterolobii group. Finding based on phylogenetic analyses showed that none of the nematode populations clustered with this devastating species of Meloidogyne. However, the spectrum of survey cum isolates collection may have to be broader in future studies to improve the sample size. *M. hapla* was not also found in any of the ecozones from which isolates of RKN were collected. Again ecological variations could have influenced the non-recovery of this nematode within the sampling locations under tropical conditions. This could be further explained in terms of varying local soil and weather conditions, weed types, cropping and land history, and farming practices.

Results showed that the three RKN species viz, *M. incognita*, *M. javanica* and *M. arenaria* were closely related. Results further revealed that *M. javanica* and *M. arenaria* grouped more closely and corroborates findings of Ishrat³⁴, who had similar result using Random Amplified Polymorphic DNA (RAPD-PCR) using different primer set. Adam *et al.*¹⁵ reported that *M. javanica* and *M. arenaria* grouped together with 62.5 % average similarity. The fact that all the three species grouped more closely than the rest could also be explained by the fact that the three species viz., *M. incognita*, *M. javanica* and *M. arenaria* have mitotic parthenogenetic mode of reproduction and are polyploid that should theoretically lead to clonal. Results clearly separated between mitotic and meiotic species. Similar results were found by Blok *et al.*¹⁶ and Adam *et al.*¹⁵. These results also agreed with other studies which investigated the relationships between Meloidogyne species using different approaches including electrophoretic mobility (Ef) values of several enzymes (Esbenshade and

Triantaphyllou³⁵), RAPD and RFLPs (Blok *et al.*¹⁶), AFLPs and tandem repeats in mtDNA (Dautova *et al.*³⁶).

CONCLUSION

This investigation provides information on genetic variation within RKN species across the ecozones and can form the basis for determining efficient management strategies through indigenous breeding programmes for RKN resistance in tomato planted in these areas.

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