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Research Article

Molecular detection of root-knot nematodes (Nematoda: Meloidogynidae) infecting okra in Ghana.

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Abstract

Root-knot nematode infect okra making the crop struggle to yield marketable fruits. Proper root-knot nematodes identification is critical in deciding management options. Morphometric identification procedures do not provide consistent and reproducible results. Molecular identification techniques are efficient and precise tools used to identify root-knot nematodes. Five universal primers were used to detect root-knot nematodes infecting okra in two agro-ecologies of Ghana. The DNA fragments yielded 760, 910, 950, 835, and 879-bp major bands for the primers; TW81-F/AB28-R, ITS-F/ITS-R, 18S-F/26S-R, F194-F/F195-R, and D2A-F/D3B-R, respectively. Four main clusters (a, b, c and d) and two outliers (e and f) were obtained when the agglomerative hierarchical clustering dendrogram was used to illustrate neighbor groups among the root-knot nematode populations. This study has shown a useful molecular technique in the efficient and timely detection of root-knot nematodes infecting okra to aid in strategic management decisions against the pest.

1. Introduction

Okra (*Abelmoschus esculentus* L. Moench) is a nutritious [1] and economically important fruit-vegetable crop [2] popularly found worldwide. It is cultivated under both tropical and subtropical conditions. It also has medicinal properties [3]. Okra has a high value in African markets ranging from $3.45/ kg to $7.07/ kg when sold fresh [4]. These attributes make okra suitable for cultivation by both small-scale and commercial vegetable producers. In Ghana, okra is being promoted as a non-traditional export crop to generate more foreign exchange revenue.

Okra is, however, susceptible to plant parasitic nematode attack, particularly root-knot nematodes, *Meloidogyne* species. Proper root-knot nematode identification is critical for the successful management of infections [5, 6]. This will depend on rapid and accurate detection so that appropriate remedial measures could be taken. Morphometrics identification techniques do not provide consistent and reproducible results due to genetic, climatic, conserved morphology, and the similar nature of nematodes generally. DNA molecular techniques have become efficient and precise tools of increasing importance to identify plant parasitic nematodes. This is because, they are faster, easier, less subjective, and applicable to all life stages of a nematode population [7, 8]. The objective of this study was to detect root-knot nematodes infecting okra using five universal primers.

2. Materials and methods

2.1 Materials

Galled okra roots were sampled from okra farms. Root-knot nematode juveniles' extraction was done at the CSIR-Crops Research Institute Nematology Lab., Ghana.

Nematode DNA extraction kit was procured from ClearDETECTIONS® (Wageningen, The Netherlands). Five primers based on rDNA sequences targeting the *18S* and *28S* genes were synthesized and supplied by METABION® International (AG, Germany). PCR and electrophoresis were performed at the KNUST Agric. Biotechnology Lab., Ghana.

2.2 Root-knot nematodes maintenance and extraction

Okra roots with root-knot nematode infection symptoms were randomly sampled from the Forest-Savanna Transition and Moist Semi-deciduous Forest agroecologies between May and October 2020, in Ghana. The sampling areas spanned six okra-growing districts (Table 1).

Table 1. Sources of root-knot nematode isolates in two agroecologies of Ghana

District	Agro-ecology	Number of isolates
Kintampo North	Forest-Savanna Transition	23
Atebubu Amantin	Forest-Savanna Transition	14
Wenchi	Forest-Savanna Transition	12
Ejura Sekyedumase	Moist Semi-deciduous Forest	25
Atwima Nwabiagya	Moist Semi-deciduous Forest	13
Offinso North	Moist Semi-deciduous Forest	9
	Total samples	96

On each okra farm, five root-knot nematode-infected (galled) okra plants were collected. Nematode eggs were extracted from the infected okra root samples, following [9]. The nematode eggs were applied on three-week-old okra (*Abelmoschus esculentus* L, cv. 'Lady fingers') seedlings in steam-sterilized topsoil building sand (3:1) mixture in plastic pots (43 cm x 25 cm x 14 cm). The pots were widely spaced in a plant house at the CSIR-Crops Research Institute, Kumasi (AE-06809724; 6º 43 'N,1º 36 'W) to avoid cross-contamination. The inoculated plants were periodically watered whenever necessary. Care was taken to avoid water spillage from the pots during watering. Average air temperature and relative humidity inside the plant house were maintained at 24 °C±2 and 87±2 %, respectively over the study period. Root-knot nematode infective juveniles (IJs) were extracted from the okra rhizosphere soils in the pots following the modified Baermann funnel extraction method at eight weeks after inoculation. Caution was taken to ensure that nematode-water suspensions were devoid of plant and soil debris. Each nematode-water suspension was concentrated to 20 ml for downstream activities.

2.3 Root-knot nematodes DNA extraction and PCR

Nematode DNA extraction kit was procured from ClearDETECTIONS® (Wageningen, The Netherlands). Fifty microliters of each nematode–water suspension was added to 150 µl of the working extraction buffer. The tubes were closed gently and mixed by vortex and were incubated for two hours at 65 °C in a water bath. Samples were taken from the water bath, vortexed thoroughly, and spun quickly down. The DNA extract was transferred to the DNA extraction tube containing a white pellet. Each sample was mixed carefully by handshaking to re-suspend the white pellet in the tube and centrifuge for 1 min at 350 g at room temperature. The DNA purification tube was taken and kept on top of the accompanying waste collection tube. The samples were incubated for 5 min at room temperature and centrifuged for 1 min at 350 g at room temperature. The waste collection tube was discarded and the DNA purification tube was placed on the DNA collection tube. The samples were further incubated for 3 min at room temperature and centrifuged for 1 min at 350 g at room temperature. The flow-through contained the purified DNA ready for downstream activities. The freshly extracted nematode DNA was stored at -20 °C for subsequent use. Three DNA extracts were randomly selected from each district to determine their nucleic acid concentrations and purity, using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Incorporated, USA). Readings were taken in triplicates and means were determined. Five primers based on rDNA sequences targeting the *18S* and *28S* genes were synthesized and supplied by METABION® International (AG, Germany) (Table 2)

PCR was carried out in 20 µl reaction mix containing 6.6 µl PCR water, 10.0 µl PCR buffer, 2x with dNTPs, 0.04 µl $MgCl_2$ (25 mM), 0.6 µl (10 mM) each for the forward and reverse markers and 0.16 µl Taq DNA polymerase. A 2.0 µl DNA was added to 18.0 µl of each reaction mix PCR tube. The PCR tubes were covered in a thermocycler (Mycycler-BIO-RAD, USA)

Table 2. Primer sequences

Primer code	Primer sequence (5'-3')	Source
ITS-F/-R	TGT AGG TGA ACC TGC TGC TGG ATC CCT ATT TAG TTT CTT TTC CTC CGC	[13]
F194-F/195-R	CGT AAC AAG GTA GCT GTA G TCC TCC GCT AAA TGA TAT G	[7]
18S-F/26S-R	TTG ATT ACG TCC CTG CCC TTT TTT CAC TCG CCG TTA CTA AGG	[13]
TW81-F/AB28-R	GTT TCC GTA GGT GAA CCT GC ATA TGC TTA AGT TCA GCG GGT	[13]
D2A-F/D3B-R	ACA AGT ACC GTG AGG GAA AGT TG TCG GAA GGA ACC AGC TAC TA	[7]

using the following cycles; an initial denaturation cycle of 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 2 min, final extension at 72 °C for 10 mins before being held at 4°C. The amplified fragments were separated by electrophoresis through 2% agarose gel in a 1x TAE (Tris-acetate-EDTA) buffer at 120 volts for 45 minutes. The gel resolution was stained with 0.01% ethidium bromide and visualized under UV light and photographs were taken. The banding patterns were visualized and compared between two individuals. The PCR products (genotypes) were scored for the presence (1) and absence (0) of bands. For every marker, alleles for the data set were scored based on the size of the base pairs of the 100 bp ladder DNA marker. This process was followed for every marker until all alleles were scored with the smallest and largest sized alleles representing the start of the first scoring and the end of the last scoring, respectively. The data was subjected to NTSYSpc (2.20) (Jaccards dis/similarity coefficient) to generate structure dendrogram using the neighbor joining approach.

3. Results and discussion

Purity and nucleic acid concentrations of the selected samples ranged between 1.80 nm and 1.94 nm; and 162.20 ng/µl and 315.20 ng/µl, respectively (Table 3).

The primers TW81-F/AB28-R, ITS-F/ITS-R, 18S-F/26S-R, F194-F/F195-R, and D2A-F/D3B-R yielded amplification products 760, 910, 950, 835, and 879-bp major bands, respectively (Fig. 1-6).

The performance of the primers in amplifying the root-knot nematode isolates have been summarized in Fig. 6. Four major neighbor groups and two outliers were obtained when an agglomerative hierarchical clustering dendrogram was used to illustrate the

Table 3: Nucleic acid concentrations in selected root-knot nematode DNA extracts

Sample	Purity (260: 280) nm	Concentration (ng/µl)
1D	1.80	162.20
2D	1.80	191.50
5D	1.81	206.90
11D	1.94	186.50
13D	1.85	215.60
38D	1.84	162.30
23D	1.84	177.40
26D	1.86	315.20
16D	1.89	169.70

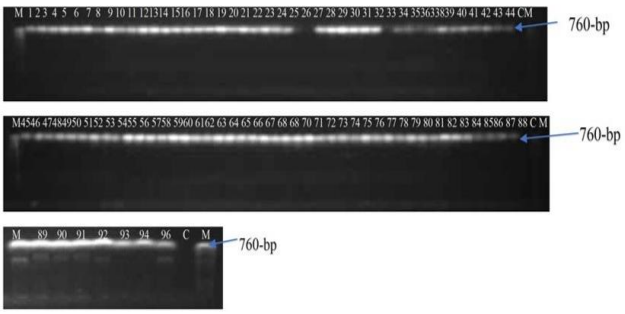

Figure 1. PCR amplification of 96 root-knot nematode isolates using primer TW81-F/AB28-R. M (100-bp molecular ladder), C (No DNA template control), lanes 1-96 (root-knot nematode DNA PCR products)

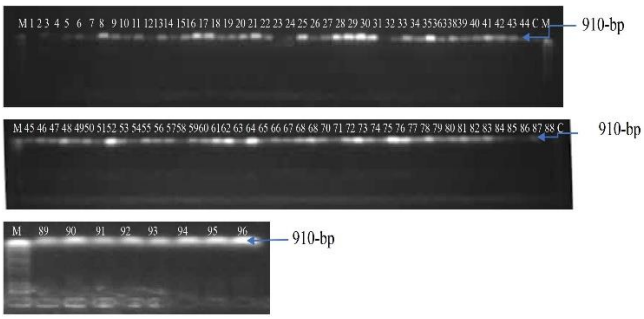

Figure 2. PCR amplification of 96 root-knot nematode isolates using primer ITS-F/-R, M (100-bp molecular ladder), C (No DNA template control), lanes 1-96 (root-knot nematode DNA PCR products)

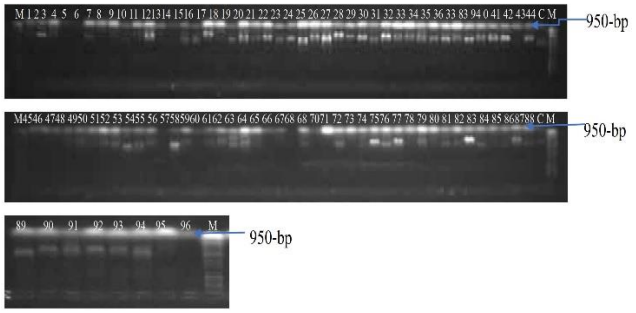

Figure 3. PCR amplification of 96 Root-knot nematodes isolates using primer 18S-F/26S-R, M (100-bp molecular ladder), C (No DNA template control), lanes 1-96 (root-knot nematodes DNA PCR products)

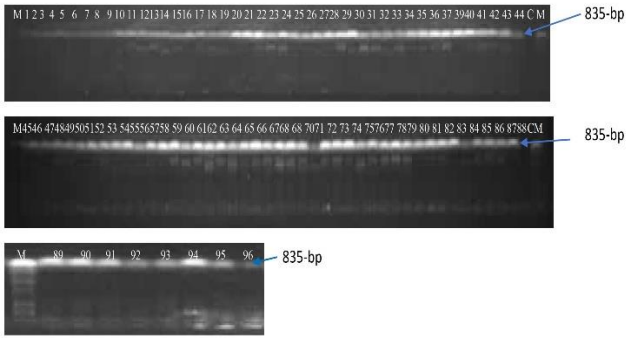

Figure 4. PCR amplification of 96 root-knot nematode isolates using primer F194-F/F195-R, M (100-bp molecular ladder), C (No DNA template control), lanes 1-96 (root-knot nematodes DNA PCR products)

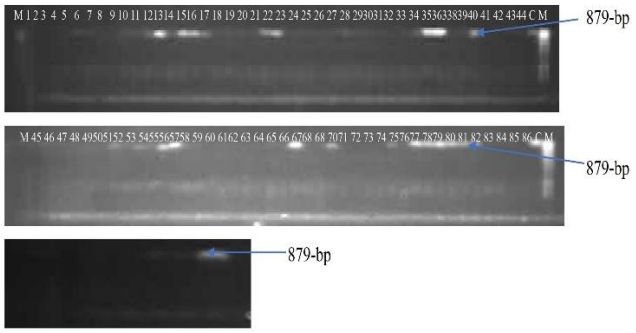

Figure 5. PCR amplification of 96 root-knot nematodes isolates using primer D2A-F/D3B-R, M (100-bp molecular ladder), C (No DNA template control), lanes 1-96 (root-knot nematodes DNA PCR products)

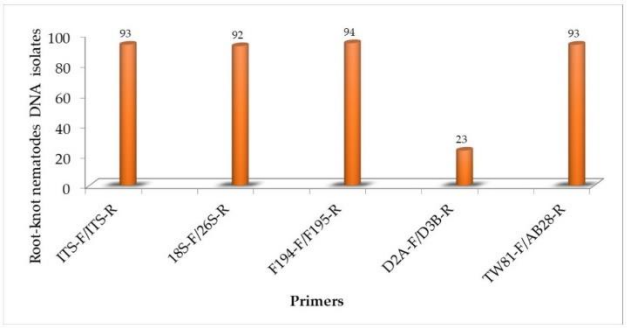

Figure 6. Performance of five primers in amplifying 96 root-knot nematode isolates

relationship among the root-knot nematode populations. The four groups (a, b, c, and d) contained 22 (22.9%), 26 (27.1%), 25 (26.0%), and 21 (21.8%) members (Fig. 7), respectively. The two outlier samples (29 and 49) were picked from the Atebubu-Amantin district of the forest-savanna transition agroecology.

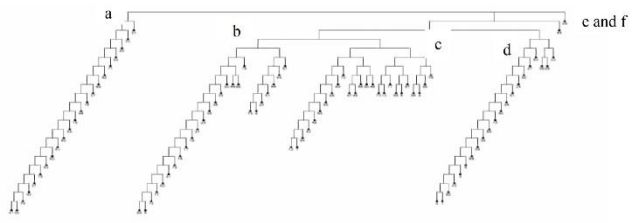

Figure 7. Root-knot nematodes infecting okra population structure from two agroecologies of Ghana using the neighbor-joining group method

The primers yielded approximately 760, 910, 950, 835, and 879-bp major fragments. Contrarily, [10] used the same primers and produced 750-bp major fragments for *Meloidogyne incognita*, *M. javanica,* and *M. arenaria*. [11] also used primers (D2A-F/D3B-R and F194-F/F195-R) to amplify *M. incognita*, *M. javanica*, *M. arenaria* and produced 750 and 720-bp single amplicon sizes, respectively. This reflected no variation in size among the populations. Four major neighbor groups and two outliers were determined from the root-knot nematode populations in our study. This is in concert with [12] who found that 99% of root-knot nematode populations identified in the International *Meloidogyne* Project (IMP) from cultivated crops around the world were represented by four major species (*M. incognita*, *M. javanica*, *M. arenaria,* and *M. hapla*). In a similar study, [13] found same four major root-knot nematode species; *M. incognita*, *M. javanica*, *M. arenaria,* and *M. hapla* in tropical and sub-tropical soils on cassava in single and multiple species infections. The use of species-specific primers is recommended to identify tropical root-knot nematode species. This is because tropical root-knot nematode species genes are too conserved to identify them reliably even by DNA sequencing and blast search.

4. Conclusions

The advent of DNA molecular diagnostic techniques is facilitating faster and more efficient root-knot nematodes detection. Our study has reported on PCR optimization for accurate and routine identification of

tropical root-knot nematodes infecting okra in Ghana. Further molecular studies should incorporate the use of sequencing to identify root-knot nematode species to complement management efforts.

Authors' contributions

Designed, supervised the study and wrote the manuscript, Y.D.; conducted analysis of the PCR products, B.A.D.; performed DNA extraction, PCR and gel electrophoresis, B.A.; performed nematode sampling and extractions, B.A.

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Availability of data and materials

All data will be made available on request according to the journal policy.

Conflicts of interest

Authors have declared that no competing interests exist.

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