Molecular Characterization and Genetic Variation of Root-knot Nematode (Meloidogyne spp.,) in Selected Legume Production Areas of Eastern Kenya

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Abstract

Root-knot nematodes are sedentary endoparasites of plant roots and the primary nematode pathogens of most cultivated crops worldwide, including legumes. Root- Knot Nematodes of the genus *Meloidogyne* is the most economically important nematode pests affecting cowpea and pigeon pea in Eastern Kenya. This study sought to identify the *Meloidoigyne* species of root-knot nematodes on selected legumes in Mbeere district and characterize the genetic diversity of the species using small subunit (SSU) rDNA. PCR amplifications of the extracted purified DNA were carried out using primers specific for the intergenic spacer region between the 5S and 18S ribosomal DNA and the expected size of about 720bp was obtained. Purified PCR products were then sequenced and thirteen 5S-18S rDNA sequences obtained. The sequences were aligned using CLUSTALW2, Sequence statistics, pairwise differences, and estimates of divergence were determined with MEGA5. Nucleotide diversities were estimated in DnaSPv5. Phylogenetic tree was drawn using Phylowin and edited in MEGA5. From the findings of the study it has been established that root knot nematodes affecting the cowpea and pigeon pea in Mbeere district are *M. javanica, M. incognita* and *M. arenaria*. Judging from the extent of differences in base composition biases between sequences, it was concluded that the sequences under study have not evolved with the same pattern of substitution.

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Sequences from the species under study were closely related to sequences retrieved from sequences databases especially those sequences which were less divergent due to less substitutions, deletions and insertions. It can be concluded that SSUrDNA are useful in identification, inferring genetic diversity and phylogenetic relationships between the isolated root knot nematodes. There is need for a rapid and reliable method to identify populations of root-knot nematodes in order to design effective control programs.

Keywords: Phylogenetic relationships between the Meloidogyne species; intergenic region between 18S and 5S genes; SSU rDNA analysis; Mbeere**.**

1. Introduction

Cowpea plays a critical role in the lives of millions of people in Africa and other parts of the developing world, where it is a major source of dietary protein that nutritionally complements staple low-protein cereal and tuber crops, and is a valuable and dependable commodity that produces income for farmers and traders [1, 2]. Dry grain for human consumption is the most important product of the cowpea plant, but fresh or dried leaves (in many parts of Asia and Africa) [3, 4], fresh peas (the southeastern USA and Senegal), and fresh green pods (humid regions of Asia and in the Caribbean) may be the most important in some local situations.

Pigeon pea (*Cajanus cajan* (*L*) Millsp.) is an important grain legume crop of rain-fed agriculture in the semi-arid tropics. Cowpea and pigeon pea are grown in many regions where root-knot nematodes (RKNs) are a major problem in production fields. Root-knot nematodes (*Meloidogyne* spp.) are economically important plant pathogens, displaying marked sexual dimorphism. Males are vermiform and active. Females are pyriform or saccate and sedentary, laying eggs in a gelatinous matrix ("egg sac"). Usually only the roots are attacked, and these are induced to form characteristic galls ("knots") on many host plants. They are the primary nematode pathogens of most crop species worldwide, including many cultivated legumes [5]. The major root-knot nematode (RKN) species, *Meloidogyne incognita, M. javanica, M. arenania,* and *M. hapla,* are geographically widespread and capable of infecting and damaging a wide range of plant hosts, making them economically important agricultural pests [6].

Control of root-knot nematodes, *Meloidogyne* spp., by crop rotation or through the use of resistant cultivars can be accomplished only if the species and host races to be controlled are known. Accurate identifications are necessary because some species or races attack certain crop plants, whereas others do not, and resistance developed in one crop cultivar is not necessarily effective against all species or races of root knot nematodes. Because of the importance of identification in the design of effective control programs, there is need for a rapid and reliable method to identify populations of root-knot nematodes.

Though root-knot nematodes (RKNs) are of worldwide economic importance, they are the most difficult to identify to species level due to their high level variability within the species and existence of biotypes whose identities cannot be verified morphologically. Identification of RKN has heavily relied on morphological features and morphometric attributes of the females, males, and second stage juveniles [6,7], also through karyotype aspects and host preferences [8]. Use of these for species identification has limitations of unreliability

and imprecision: The morphological variability of the perineal patterns may be absent or difficult to observe [9]; in morphometric studies, most characters show overlapping ranges while others have limited usefulness. In karyological studies; the small chromosomes used are difficult to observe and count. Use of host range is useful yet time consuming as it requires a minimum of 30 days to produce RKN inoculums [8]. These are further compounded by the occurrence of biotypes. Due to the above shortcomings, nucleic acid based techniques have been developed to overcome these limitations. In this study; identification and characterisation of RKN was done using nematode small-sub unit ribosomal DNA (rDNA). The phylogenetic relationship between the *Meloidogyne* spp. isolated from the selected sites in Mbeere district was also established.

2. Materials and methods

2.1. Study sites and sampling

Sampling was done in three selected sites in Mbeere district (Gachoka, Mwea, and Siakago zones). Each of the sites was divided into 2 localities from which 10 cowpea and 10 pigeon pea plants infected by RKNs were randomly selected and uprooted. They were packaged in paper bags and transported to Kenyatta University where infected plants were used to raise pure cultures [10].

2.2. DNA extraction and purification

Single females were handpicked from the infected cowpea and pigeon pea plants from each site, and inoculated on tomato (*Lycoperscion esculentum*) seedlings variety Moneymaker. The seedlings were maintained under greenhouse conditions at 20-28°C for 35 days. Plants were then harvested and females used for DNA extraction according to a protocol used for cyst nematodes [11] and adjusted to optimize laboratory conditions following [12]. DNA pellet was stored at -20 °C and used for further analysis. DNA concentration was calculated using the Gene Quant spectrophotometer (Biochrom,Cambridge-UK).

2.3. SSUrDNA PCR amplification

PCR amplifications of the extracted DNA were carried out for each isolate in a reaction volume of 10 µl. The primers used were code 194 and code 195[13]; specificity of these primers is 5S- 18S ribosome region (figure 1).
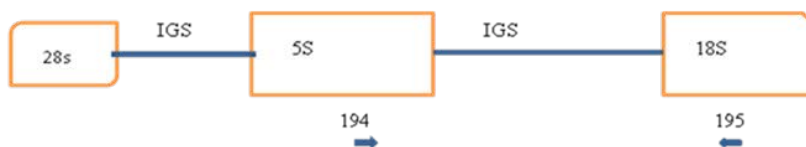

Figure 1: Meloidogyne rDNA cistrons showing the position of the PCR primers. Schematic diagram (not to scale) showing the position of the PCR primers (194/195) used to amplify the intergenic (IGS) region between 18S and 5S genes. The annealing locations of the oligonucleotide primers and their orientations are indicated by arrows.

PCR was carried out in a thermal cycler under the following conditions: Initial preheating at 94ºC for 5 minutes, 40 cycles of (94 ºC for 1 minute; 52ºC for 2 seconds; 72ºC for 90 seconds) and final extension step at 72ºC for 5 minutes. The amplified PCR products were electrophoretically fractionated in 1X TAE buffer in 1% agarose gel (Seakem®, Cambrex Bio science; Rockland, USA) and visualized by staining with 0.003% ethidium bromide (0.02 µg/ml). Hyperladder I DNA ladder (bioline) was used as molecular size markers. Gels were viewed on a UV transilluminator.

The bands were excised from the gel and purified with the Qiaquick PCR Cleanup kit (Qiagen Operon, Alameda, CA). The amplicons were concentrated by ethanol precipitation for direct sequencing. The amount of DNA obtained was quantified using a spectrophotometer [14].

2.4. Phylogenetic analysis

Nucleotide sequences were aligned using CLUSTAL W [15]. Sequence statistics, pairwise differences, and estimates of divergence were determined with MEGA5 [16]. Nucleotide diversities were estimated with another computer program; DnaSPv5 [17]. Nucleotide diversities and divergences were based on observed numbers of differences only. Gaps were treated as missing data with pairwise exclusion. Molecular phylogenetic relationships were constructed using discrete character-based (maximum parsimony and maximum likelihood respectively) algorithms implemented in the Phylowin with all characters unordered and unweighted and edited in MEGA5. During the analyses involving alignment from the secondary structure, phylogeny trees were rooted alternately with the most divergent of the species under study in order to compare tree topologies.

3. Results

3.1. SSU rDNA analysis

Using primers 194 and 195, there was no obvious size polymorphisms evident in the PCR products produced from the *Meloidogyne* species under study. The size of the single bands is about 700bps (figure 2a). It was therefore not possible to identify the species using PCR hence the need for purifying the PCR products for sequencing (Figure 2b).

3.2. Genetic diversity between the Meloidogyne species

3.2.1. rDNA sequences

Two microlitres (2µl) of the purified PCR product using forward primer (code 194) was sequenced with the applied Biosystems Big Dye Terminator cycle sequencing in a DNA automated sequencer (SegoliLab, International Livestock Research Institute - ILRI, Nairobi Kenya). The partial 5s rDNA genome sequences were assembled and edited using BioEdit sequence alignment editor [18]; gaps and ambiguities were eliminated from the final sequences.

A total of 13 complete sequences were generated (named J1 to J13). The thirteen 5S rDNA sequences obtained
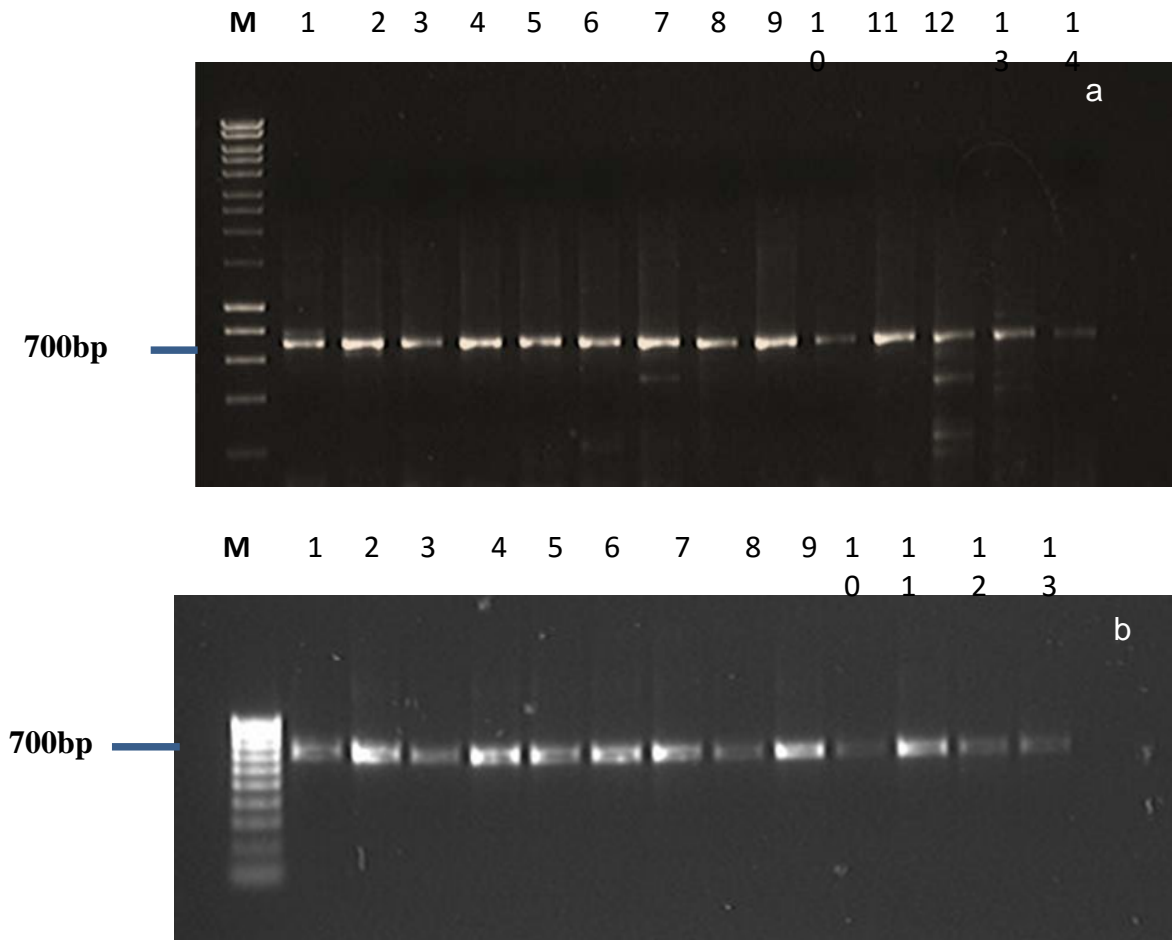
in this study varied from 663 to 802 bp.

Figure 2(a): 1% agarose gel of PCR products amplified using primers specific for the 18S and 5S SSUrDNA of Meloidogyne species M: Hyperladder 1 (bioline) (b): 1% agarose gel of purified PCR products of representative Meloidogyne species ready for sequencing: M: Hyperladder IV (bioline).

3.2.2. Sequence alignments

The sequences of 5s rDNA were aligned using the default parameters of Clustalw2.1 [15]. A total of 13 sequences were aligned. Conserved regions have stars (*) below the nucleotide bases and the rest show the variable regions (Figure 3A).

3.2.3. Blasted and aligned sequences

The sequences of 5S rDNA were used as queries to search similar sequences in sequences databases at NCBI via the blast algorithms. The retrieved sequences were aligned against the sequences obtained from this study (J1 to J13). Alignments were done using the default parameters of CLUSTALW2.1 [15]. Samples J1 to J13 are *Meloidogyne* species from cowpea and pigeon pea from Mbeere while the rest are sequences selected and downloaded from GenBank with accession numbers (GQ395506.1, FJ555690.1, GQ395518.1, and

GQ395510.1). They were selected because they had a species identity of between 98 to 99%. The sequences under study aligned to the GenBank downloaded sequences showing high level of conservation as shown by the stars (∗) under the base sequences. The results are shown below

A)

J10

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAAGATT 151

J12

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 173

J5

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAAAAACTCGTGGAGAGAAATAATAGGATT 173

J1

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 176

J7

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 171

J8

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 172

J4

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 169

J6

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 169

J2

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 168

J3

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 173

J9

GTTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 171

J13

GTTTAAAGGAGAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATT 174

J11

GGTTAAAAGAAAAATCCAATTGGGCCAAACCAAAAACCCCGGGAAAAAAATTAAAAGAAT 172

          * ***** ** ** ** ******** ** * **** * *** * **** * * ** *

J10   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAAAAT
211

J12   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAAAAT
233

J5   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAAAAT
233

J1   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAGAAT
236

J7   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAGAAT
231

J8   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAGAAT
232

J4   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAGAAT
229

J6   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAGAAT
229

J2   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAGAAT
228

J3   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAGAAT
233

J9   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAATAAGCTTTGTTTTTTGAAGAAT
231

J13   AAAAAATTTTTTGAAAAATTTAAAGTTTATTATTATAAAAACTTTGTTTTTTGAAGAAT
234

J11   AAAAAATTTTTTTAAAAATTTAAAGTTTATTAATAAAATAAACTTTGTTTTTTGAAAAAA
232

73

************ ****************** ** ** ** ************** **

J1
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CGATGTTCGCTGTTCGCGGGAATGG 117

J7
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CGATGTTCGCTGTTCGCGGGAATGG 112

gb|GQ395506.1
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CGATGTTCGCTGTTCGCGGGAATGG 105

J8
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CGATGTTCGCTGTTCGCGGGAATGG 113

gb|FJ555690.1
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGCTTTTCGATGTTCGCTGTTCGCGGGAATGG 100

J12
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGCTTTTCGATGTTCGCTGTTCGCGGGAATGG 114

gb|GQ395518.1
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGCTTTTCGATGTTCGCTGTTCGCGGGAATGG 106

gb|GQ395510.1|
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGCTTTTCGATGTTCGCTGTTCGCGGGAATGG 106

J5
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGCTTTTCGATGTTCGCTGTTCGCGGGAATGG 114

J2
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CGATGTTCGCTGTTCGCGGGAATGG 109

J4
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CGATGTTCGCTGTTCGCGGGAATGG 110

J6
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CGATGTTCGCTGTTCGCGGGAATGG 110

J3
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CGATGTTCGCTGTTCGCGGGAATGG 114

J9
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-
CCATGTTCGCTGTTCGCGGGAATGG 112

J10
A---ATTTCGCTGAGGCAA-GTGGGCGTGGATTT-CCATGTTCGCTGGTCGCGGGAATGG 92

J13
ACAAATTTCGCTGAGGCAAAGTGGGCGTGGATTT-CCATGTTCGCTGGTCTCGGGAATGG 115

J11
ACAAATTTCGCTGAGGCAAAGTGGCCGTGGATTT-CCAAGGTCCCCGGTCCCGGGAAAGG 113

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J1
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 177

J7
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 172

gb|GQ395506.1
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 165

J8
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 173

gb|FJ555690.1
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 160

J12
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 174

gb|GQ395518.1
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 166

gb|GQ395510.1|
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 166

J5
TTTAAAGGAAAACTCAAATTGGGCTAATCTAAAAACTCGTGGAGAGAAATAATAGGATTA 174

J2
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 169

J4
TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 170

J6

TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 170

J3

TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 174

J9

TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 172

J10

TTTAAAGGAAAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAAGATTA 152

J13

TTTAAAGGAGAACTCAAATTGGGCTAATCTAGAAACTCGTGGAGAGAAATAATAGGATTA 175

J11

GTTAAAAGAAAAATCCAATTGGGCCAAACCAAAAACCCCGGGAAAAAAATTAAAAGAATA 173

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Figure 3: Nucleotide sequence alignments showing conserved and variable regions. **A)** J1 to J13 are from selected legumes in Mbeere district. **B)** Sequences under study and GenBank generated sequences. Conserved regions have stars (∗) below the nucleotide bases and the rest show the variable regions.

3.3. *Pairwise distance matrix*

The pairwise distances among the *Meloidogyne* spp. 5S rDNA sequences are shown in table 1 below. Judging from the extent of differences in base composition biases between sequences, it was concluded that the sequences under study have not evolved with the same pattern of substitution. Sequence J11 has a higher base substitution per site from between the rest of the sequences, which is 0.26 while sequence J13 has a low base substitution between all the other sequences except between J11. The analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 466 positions in the final dataset. The overall average evolutionary divergence (mean distance) over all sequence pairs was **0.042.** Evolutionary analyses were conducted in MEGA5 using the Maximum Composite Likelihood model [16].

3.4. *Nucleotide substitution*

The probability of transitional substitutions (between purine to purine or pyrimidine to pyrimidine) is high compared to the transversional substitutions (purine to pyrimidine or vice versa). The pattern of nucleotide substitution was done using alignments of *Meloidogyne* species under study (J1-J13) compared to the sequences from the GenBank in MEGA5; hence a total of 20 aligned sequences (Table 2). Each entry is the probability of

substitution (*r*) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei model [16] with rates of different transitional substitutions in bold and those of transversional substitutions are shown in italics in the table below. C/T transitional substitutions having the highest incidence. Relative values of instantaneous *r* should be considered when evaluating them. The sum of *r* values is made equal to 100. The nucleotide frequencies were A = 32.83%, T = 38.46%, C = 12.75% and G = 15.95%. Codon positions included were 1st+2nd+3rd+Noncoding. The transition/transversion rate ratios are k_1 = 3.169 (purines) and k_2 = 73.15 (pyrimidines). The overall transition/transversion bias is R = 13.739, where R = [A*G*k_1+ T*C*k_2]/[(A+G)*(T+C)].

Table 1: Estimates of Evolutionary Divergence between 13 aligned Sequences

	1	2	3	4	5	6	7	8	9	10	11	12	13
J1													
J2	0.00												
J3	0.00	0.00											
J4	0.00	0.00	0.00										
J5	0.01	0.01	0.01	0.01									
J6	0.00	0.00	0.00	0.00	0.01								
J7	0.00	0.00	0.00	0.00	0.01	0.00							
J8	0.00	0.00	0.00	0.00	0.01	0.00	0.00						
J9	0.01	0.01	0.0O	0.01	0.01	0.01	0.01	0.00					
J10	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01				
J11	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26			
J12	0.00	0.00	0.00	0.01	0.01	0.01	0.00	0.00	0.01	0.02	0.26		
J13	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.02	0.03	0.25	0.03	

Table 2: Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution for 20 aligned sequences

	A	T/U	C	G
A	-	*0.98*	*0.27*	**1.19**
T	*0.82*	-	**19.96**	*0.38*
C	*0.82*	**71.38**	-	*0.38*
G	**2.59**	*0.98*	*0.27*	-

3.5. Nucleotide diversity for the 13 aligned sequences

The thirteen 5S-18S rDNA Sequences obtained in this study varied from 663 to 802 bp. They had a total of 663 polymorphic sites excluding sites with gaps and missing data, 14 singleton variable sites and 649 parsimony

informative sites as shown in the table 3 below. The sequences had a nucleotide diversity of 0.62919 as analyzed in DNA Sequence Polymorphism (DnaSP version 5 [17] and MEGA5.

Table 3: Results from Tajima's Neutrality Test for 13 aligned sequences

m	S	p_s	Θ	Π	D
13	663	1.000000	0.322247	0.629191	4.399088

The abbreviations used are as follows: m = number of sites, S = Number of segregating sites (polymorphic sites), p_s = S/m, Θ = p_s/a_1, and π = nucleotide diversity. D is the Tajima test statistic. Statistical significance: ***, P < 0.001 [19].

3.6. Phylogenetic relationships between the Meloidogyne species

Small subunit Ribosomal DNA has been a popular target, because highly conserved sequences are interspersed with less conserved regions, enabling phylogenetic studies at various taxonomic levels.

Sequences J8 and J4 are sister taxa to *M. incognita* (GQ395506) while J6 and *M. arenaria* (GQ395523) are sister taxa. Sequence J2, J1 and J7 are sister taxa to *M. javanica* (GQ395510 and 395513 respectively) but in different subgroups. J5 and J12 seem to be sister taxa within the same subgroup. *Meloidogyne* spp. (J11) is depicted as a sister taxon to the remaining species, being more divergent it forms the basal taxon to the rest of the species. The relationships are not supported by high bootstrap analyses.

The samples under study did not give identical sequences for the blasted sequences for the same species this could be due to substitutions mostly transitional as shown in table 2 above. The species did not take the same rate of substitutions. This is shown in table 1, figure 3A and 3B which showed that J11 was more divergent followed by J13 which were both from Gachoka infecting cowpea plant. Some species could not be identified since they did not cluster with the GenBank downloaded sequences hence could only be referred to as *Meloidogyne* spp.

4. Discussion

PCR amplifications of the extracted purified DNA were carried out using primers 194 and 195 [13]. The 194/195 ribosomal primers successfully amplified fragments of the expected size from extracted DNA. The size of the PCR product obtained following amplification of the intergenic spacer region between the 5S and 18S ribosomal genes is about 720 bp, this agrees with work done by [20] whose findings grouped *M. incognita*, *M. javanica*, *M. arenaria* as having 720 bp. The purified PCR products were sequenced with the forward primer (code 194) and thirteen 5S rDNA sequences obtained in this study varied from 663 to 802 bp. Sequence J13 was the longest sequence with 802 bp followed by 750 bp.
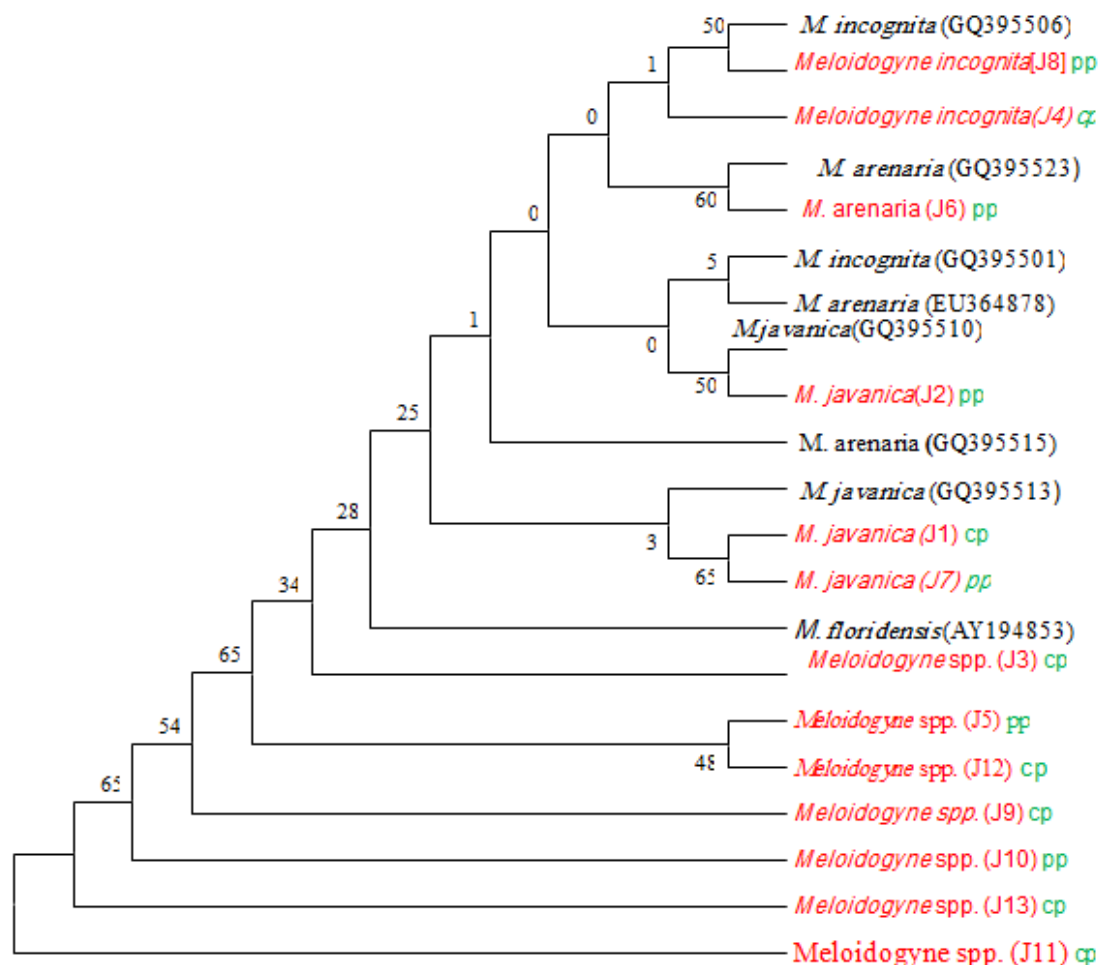
Figure 4: Maximum likelihood tree based on 5s-18s rDNA gene sequences. The accession numbers of the GenBank-downloaded sequences are shown in parentheses. Numbers next to branches are bootstrap values from MP analyses, isolates under study are shown in red colour while selected legumes cowpea (cp) and pigeon pea (pp) are shown in green.

The others had lengths varying from 669 to 690 bp. Sequences have not evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences. Sequence J11 seems to have undergone high rate of substitutions compared to the rest of the sequences under study followed by J13 with the least being sequence J4 and J8. The study favoured a higher rate of transitional substitution compared to transversional substitution with C/T substitution having the highest degree of incidence. Some of the substitutions of J11 are at 17, 59, 89,99,93,97,107,111,138,141,155 base pairs. This explains why it is more divergent than the rest of the species and it does not cluster with the other species in the phylogram.

Within the rDNA repeats from a wide variety of organisms, the IGS region is the least conserved. However, in this study little sequence variation was found in the region between the 18S and 5S genes for the group comprising *M. incognita, M. arenaria,* or M. *javanica,* despite the populations originating from two different legumes from Mbeere district. This rather low variability among the isolates could be related to the mitotic

parthenogenetic mode of reproduction of *Meloidogyne* species that theoretically give rise to clonal progenies [8]. Nevertheless, the existence of variation in the form of mixtures of clones originated by mutation cannot be discarded.

The samples under study did not give 100% identical sequences for the blasted sequences for the same species this could be due to substitutions mostly transitional, they seemed more divergent may be because of deletions , insertions and substitutions as observed in the alignments done by clustalW2.

Several isolates of *Meloidogyne* clustered separately in the phylogram and J11 was depicted as the basal taxon to the rest. Some differences between the sequences may be due to a natural variation within the population [21]. However, artefacts during amplification may cause some variations and these also explain some dissimilarity between the sequences under study and GenBank downloaded sequences.

Some authors have reported differences within rDNA sequences among isolates belonging to the same species, implying that some *Meloidogyne* spp. are more molecularly heterogeneous than previously thought [22,23].

From the findings of the study it has been established that root knot nematodes affecting the selected legumes are *M. javanica, M. incognita* and *M. arenaria*. The species under study were closely related to the blasted sequences especially sequences which were less divergent due to less substitutions , deletions and insertions. Sequence J4 and J8 aligned well with blasted sequence accession number GQ395506 whose identity is *M. incognita*. Sequence J11 was the basal taxon being more divergent than the other species.

Extent of sequence divergence is used as a parameter to estimate relatedness of taxa [24]. SSU sequence information has been used to estimate the phylogenetic history of phylum Nematoda [25].

5. Conclusion

The SSUrDNA has been a useful tool for identifying, showing variability and inferring phylogenetic relationships between the species collected from Mbeere district. SSUrDNA also does not rely on the expressed products of the genome unlike the isozyme phenotypes.

The *Meloidogyne* species affecting cowpeas and pigeon peas in the selected sites in Mbeere district are *M. arenaria, M. incognita* and *M. javanica.* The farms where the infected samples were collected practiced mixed cropping hence the *Meloidogyne* species affecting the cowpea and pigeon pea in Mbeere did not seem to favor either of the legumes**.**

The obtained data showed that despite reproduction among the studied species being mitotic parthenogenesis, intrapopulation variations have occurred within the isolated *Meloidogyne* spp.

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Recommendations

More identification can be done on other crops in Mbeere in order to establish the species diversity of *Meloidogyne* species.

For a study of differences between very similar isolates, as observed in the study, some less conserved DNA sequence will be more useful. Although rDNA region is specific at the species level in the genus *Meloidogyne* for example, and may be used for species identification, it is unlikely to be useful for the identification of very similar isolates as it is highly conserved.

References

[1] A.S. Langyintuo, J. Lowenberg-DeBoer, M. Faye, D. Lambert, G. Ibro, B. Moussa,et al. "Cowpea supply and demand in West Africa." Field Crops Resource,vol. 82, pp. 215–231, 2003.

[2] B. B. Singh. "Recent genetic studies in cowpea," In: Challenges and Opportunities for Enhancing Sustainable Cowpea Production, C.A. Fatokun, S.A. Tarawali, B.B. Singh, P.M. Kormawa, M. Tamo, Ed. Ibadan, Nigeria:IITA,2002,pp. 3–13.

[3] K. Ahenkora, H. K. Adu-Dapaah, and A. Agyemang. "Selected nutritional components and sensory

attributes of cowpea (Vigna unguiculata [L.] Walp.) leaves." Plant Foods Human Nutrition,vol. 52, pp. 221–229, 1998.

[4] S. S. Nielsen, T. A. Ohler, and C. A. Mitchell. "Cowpea leaves for human consumption: production, utilization, and nutrient composition." In: Advances in Cowpea Research, B.B. Singh, D.R. Mohan Raj, K.E. Dashiell, L.E.N. Jackai, Ed. Sayce, Devon, UK: Copublication of International Institute of Tropical Agriculture (IITA) and Japan International Research Center for Agricultural Sciences, 1997, 326–332.

[5] K. Barker. "Introduction and synopsis of advancements in nematology" In: Plant Nematode Interactions, K.R. Barker, G.A. Pederson , G.L. Windham, Ed. Madison, WI: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, 1998, 1–20.

[6] J. D. Eisenback and H. H. Triantaphyllou. "Root-knot nematodes: Meloidogyne species and races" Manual of Agricultural Nematology, pp. 191-274, 1991.

[7] S. B. Jepson. Identification of Root-knot Nematodes (Meloidogyne species). Wallingford,UK: CABI Publishing, 1987, pp. 265.

[8] A. C. Triantaphyllou. "Cytogenetics, cytotaxonomy and phylogeny of root-knot nematodes." In : An

advanced treatise on Meloidogyne, Biology and control vol. 1. J. N. Sasser, C. C. Carter, Ed. Raleigh: North Carolina State University Graphics, 1985, pp. 113 126 .

[9] T. J. Baum, P.M. Gresshoff, S. A. Lewis and R. A. Dean. "Characterization and phylogenetic analysis of four root- knot nematode species using DNA amplification fingerprinting and automated polyacrylamide gel electrophoresis." Molecular Plant-Microbe Interactions,vol. 7, pp. 39-47,1994.

[10] J. M. Kavuluko, C. Gichuki, J.W. Waceke and S.M. Runo. "Characterisation of root-knot nematodes (Meloidogyne spp.) from selected legumes in Mbeere District Kenya using isoenzyme phenotypes." In "Transforming Agriculture for improved livelihoods through Agricultural Product Value Chains: The Proceedings of the 12th KARI Biennial Scientific Conference" Nairobi Kenya, 8- 12th Nov 2010, pp. 92- 97

[11] S. Bekal, J.P. Gauthier and R. Rivoal. "Genetic diversity among a complex of cereal cyst nematodes inferred from RFLP analysis of the ribosomal internal transcribed spacer region." Genome, vol. 40, pp. 479-486, 1997.

[12] O. Randig, F. Leroy, M. Bongiovanni and P. Castagnone-Sereno. "RAPD characterisation for single females of the root-knot nematodes, Meloidogyne spp." European Journal of Plant Pathology ,vol. 107 pp. 639–643. 2001.

[13] V. C. Blok, M.S. Phillips and M. Fargette. "Comparison of sequences from the ribosomal DNA intergenic region of Meloidogyne mayaguensis and other major tropical root-knot nematodes." Nematology, vol. 2, pp. 16-21,1997.

[14] Sambrook and Russel. Molecular cloning. A laboratory manual. New York: Cold spring harbour

publishers. 2001

[15] J. D. Thompson, T. J. Gibson, F. Plewniak , F. Jeanmougin and D. G. Higgins. "The Clustal-x Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools." Nucleic Acids

Research,vol. 25, pp. 4876–4882, 1997.

[16] K. Tamura, J. Dudley, M. Nei and S. Kumar. "MEGA4: Molecular Evolutionary Genetics Analysis

(MEGA) software version 4.0." Molecular Biology and Evolution,vol. 24 pp. 1596- 1599, 2007.

[17] P. Librado and J. Rozas. "DnaSP v5: A software for comprehensive analysis of DNA polymorphism data."Bioinformatics,vol 25 pp. 1451-1452, 2009.

[18] T.A. Hall. "BioEdit: a user-friendly biological sequence alignment editor and analysis program for

Windows 95/98/NT." Nucleic Acids Symposium Ser,vol. 41 pp. 95-98, 1999.

[19] F. Tajima. " Statistical methods to test for nucleotide mutation hypothesis by DNA polymorphism."

Genetics,vol. 123, pp. 585-595, 1989.

 [20] M. A. M. Adam, M. S. Phillips and V. C. Blok . "Identification of Meloidogyne spp. from north east Libya and comparison of their inter- and intro-specific genetic variation using RAPDs." Nematology , vol. 7, pp. 599–609,2000.

[21] S. A. Subbotin, P.D. Halford, A. Warry and R.N. Perry. "Variations in ribosomal DNA sequences and phylogeny of Globodera parasitising solanaceous plants." Nematology , vol. 2, pp. 591–604, 2000.

[22] M. S. Tigano, R. M. D. G. Carneiro, A. Jeyaprakash, D. W. Dickson and B. J. Adams. "Phylogeny of Meloidogyne spp. based on 18S rDNA and the intergenic region of mitochondrial DNA sequences." Nematology , vol. 7,pp. 851–862, 2000.

[23] P. De Ley and W. Bert. "Video capture and editing as a tool for the storage, distribution and illustration of morphological characters of nematodes." Journal of Nematology , vol.34, pp. 296– 302,2002.

[24] J. G. Files and D. Hirsh . "Ribosomal DNA of Caenorhabditis elegans." Journal of Molecular Biology, vol. 149, pp. 223, 1981.

[25] M. L. Blaxter, P. Deley, J. R. Garrey,   L. X. Liu, A. V. Scheldeman, J.R. Vanflenten et al. "Molecular evolutionary framework for the phylum nematode." Nature , vol. 392, pp. 71-75**,** 1998.