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Original Research Article

Molecular Characterization of Sweet Potato Plant Parasitic Nematodes in Different Agroecological Zones of Kirinyaga County, Kenya

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Abstract: Plant parasitic nematodes (PPNs) are widely distributed in all agroecological zones (AEZs) within Kenya. Plant parasitic nematodes are important pests of many cultivated crops and they exert a detrimental influence on a wide range of vascular plants, leading to significant crop losses by reducing both quantity and quality of the yield. However, due to inadequate taxonomic descriptions and a low number of diagnostic features, the morphological diagnosis of many species remains a challenge. The objective of this study was to characterize PPNs associated with sweet potatoes in different agroecological zones of Kirinyaga County, Kenya, using 18S rRNA gene sequencing. Seventy-seven soil samples from sweet potato tubers rhizosphere were collected from different agroecological zones and nematodes were extracted using Baermann's technique. Thirteen nematode isolates were obtained and only seven could be identified morphologically to their genus level based on their distinct phenotypes. Seven isolates that could not be identified through the microscope were advanced to molecular sequencing. The nematode DNA were extracted and the PCR amplification and sequencing of 18S rRNA gene carried out. The study identified six PPN species, including *Mylonchulus hawaiiensis, Aporcelaimellus nigeriensis, Rotylenchulus reniformis, Rotylenchulus borealis, Aporcella femina, Heterodera dunensis,* and a predatory nematode (*Dorylaimus aff. stagnalis*) as part of the soil biota. This study showed significant distribution of plant parasitic nematodes across the agroecological zones. Thus, the occurrence of a nematode species complex in sweet potato farms requires the development of specific and appropriate sustainable control strategies.

Keywords: Plant parasitic nematodes, Molecular characterization, Implied by sweet potatoes, *Mylonchulus hawaiiensis, Aporcelaimellus nigeriensis, Rotylenchulus reniformis, Rotylenchulus borealis, Aporcella femina, Heterodera dunensis.*

INTRODUCTION

Sweet potatoes (*Ipomoea batatas* L. (Lam.) form a major staple food in the diet providing food security in many rural households (Omotobora *et al*., 2014; Onchari *et al*., 2023). Despite sweet potato playing an important role in ensuring food security and household income sources for local communities, its production has decreased by 37% from 2015 to 2021 (FAOSTAT, 2022). The reduced production of sweet potato in Kenya is attributed to various biotic, abiotic and social factors (Karuri *et al.*, 2017). Biotic factors include parasitic nematodes (Ezin *et al*., 2018). Worldwide, food production has reduced by 20% due to plant parasitic nematodes (PPN) especially the root knot nematodes (Devi *et al.*, 2018). These plant parasitic nematodes are known to lower yield and quality of sweet potato tubers by about 12% annually (Briar *et al*., 2016).

Plant parasitic nematodes exist in all agroecological zones, but their distribution and abundance differ according to climatic factors, soil characteristics, the availability of plant hosts and farming systems (Anusha *et al.*, 2021). Approximately 4,100 PPN species have been identified, each with a unique

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adaptation for infecting plants in various habitats (Decraemer & Hunt, 2006). *Pratylenchus* spp, root knot nematodes, cyst nematodes, *Heterodera* and *Globodera* species, *Radopholus similis*, burrowing nematodes, and root-lesion nematodes are some of the major detrimental PPN taxa (Jones *et al.*, 2013).

Parasitic nematodes attack plant roots as parasites that cause galling, skin lesions, and impaired absorption of nutrients and water resulting to leaf wilting, stunted growth and death of the plant. The rate to which crop yield experiences significant yield losses depends on PPN infestation. For instance, root-knot nematodes solely are thought to affect a wide range of crop species and result in output losses of 5–10% (Singh *et al*., 2013). Identifying the prevalent nematode species and the variables influencing their diversity in various climatic and cropping zones is essential to develop comprehensive pest control methods appropriate for the specific agroecosystems (Anusha *et al.,* 2021). Comprehending the PPN species and how agronomic and environmental factors affect their spread is essential for their sustainable management (Anusha *et al.,* 2021).

The growth, survival, reproduction, and propagation of PPNs are all highly influenced by environmental conditions such as humidity, temperatures, moisture and soil texture (Anusha *et al*., 2021). Tropical and subtropical areas offer excellent humid and warm environments for population expansion. For most species, the ideal temperature for reproduction is between 25°C and 30°C (Luc *et al*., 2005). With adjustments to endure colder temperatures as low as 10°C and severe drought through anhydrobiosis, plant parasitic nematodes may also flourish in temperate and desert zones (Luc *et al*., 2005). This adaptation of PPN may lead to evolution of new strains.

The classification of nematodes is mainly based on morphological features (Dorris *et al*., 1999; Van den Elsen *et al*., 2009; Khadka *et al*, 2019), which may be inadequate in this large and diverse genus (Janssen *et al*., 2017). Most morphological characteristics studied are homoplasious, consequently the resulting classification may be misleading (Dorris *et al*., 1999; Van den Elsen *et al*., 2009; Viney & Diaz, 2012; Khadka *et al*, 2019). Moreover, due to continued formation of new strains, molecular techniques are more reliable in identifying parasitic nematode species (Blok *et al.*, 2002; Hu *et al.*, 2011; Niu *et al.,* 2012).

Phylogenetic studies are now employing recombinant DNA (rDNA) (Ye *et al.,* 2019), microRNAs (Medina *et al.,* 2017), nuclear ribosomes and mitochondrial gene sequence (Janssen *et al.,* 2017; Bogale *et al*., 2020) to accurately identify and classify nematodes. These numerous molecular characterisation approaches are expected to complement morphology-based techniques. The use of nematode ribosomal DNA sequencing provides an additional identification tool, a better understanding of nematode evolution and relationships among nematode species (Bogale *et al*., 2020). This study employed 18S rRNA gene sequencing to identify and classify PPNs sampled from various agroecological zones in Kirinyaga County, Kenya.

METHODS AND MATERIALS

Nematode Sampling and Extraction

Nematodes were sampled from sweet potato soil rhizosphere in three agroecological zones (UM3, UM4 & LM3) of Kirinyaga County – Kenya as documented by Onchari *et al*., (2023). In the study, there were thirteen isolates with diversity and distribution across the three agroecological zones. Seven isolates (*Rotylenchulus, Meloidogyne, Pratylenchus, Helicotylenchulus, Scutellonema, Heterodera* and *Aporcelaimellus*) were identified morphologically based on their distinct features. However, *Rotylenchulus* species, due to its overlapping features within the genus level was advanced to molecular analysis among others that could not be identified through the microscope (Onchari *et al*., 2023).

The nematode DNA were extracted following the procedure described by Huang *et al.* (2017). Nematodes were placed in 0.5 ml sterile Eppendorf tubes containing 10 µl buffer solution made of 2 µl of 10x PCR buffer, 2 µl Proteinase K (600 µg/ml) and 6 µl distilled water. Contents in the eppendorf tube were incubated at -20ºC for 30 minutes followed by 65ºC for 1 hour, then 95ºC for 10 minutes. The extracted DNA was stored at -80°C. Amplification of the 18S rRNA was done using Nem_18S forward and Nem_18S reverse primers (Table 1).

Table 1: The length in base pairs (bp), melting temperature and self-complementarity of the Forward and Reverse Primers for Amplification of 18S rRNA gene

Primes	Sequence (5'-3')	Length	Melting temperature	Self-complementarity
Forward	GCGATCAGATACCGCCCTAGTTC	23 bp	62.75	5.00
Reverse	AGGGCAGGGACGTATTCAGGACG	23 bp	66.05	4.00

All PCR reactions were performed in 25 µl volumes containing 100 mg DNA, 200 µM dNTPs, 10 pmol of each primer, 1·5 mM $MgCl_2$ and 1·5 U *Taq* DNA polymerase (Fermentas). PCR amplification conditions were: an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation (30 s at 94°C), primer annealing (20 seconds at 60°C) and primer extension (1 min at 72°C). Termination cycle of the PCR reaction was at 72°C for 7 minutes. The negative control comprised double distilled water in place of genomic

DNA and was included in all PCR amplifications to test for contaminants in the reagents. Amplification of the primers were carried out in a thermal cycler (Model/ manufacturer). Aliquots (3·0 µl) of PCR products were analysed by electrophoresis in 1·2% (w/v) agarose gels, containing 1 X TBE buffer, stained with ethidium bromide (0·5 µg mL^{-1}), then photographed under ultraviolet (UV) light. The lengths of the DNA fragments were estimated by comparison with MassRuler Low Range 1 kb DNA ladder (Marek *et al.*, 2010).

The PCR end-product was purified using Quickclean PCR purification kit (from Gene Script). Purified PCR products were then sent for sequencing at International Livestock Research Institute (ILRI), Kenya. Using the obtained sequence data, Basic Local Alignment Search Tool (BLAST) was run in NCBI database to identify organisms with similar sequences. A phylogenetic tree was constructed based on the Neighbour-Joining method (Saitou and Nei, 1987) on MEGA X version 11.0 (Tamura *et al.*, 2021). The evolutionary distances of the species on the phylogenetic tree were calculated following Tamura 3-parameter method (Tamura, 1992), with the output being units of the number of base substitution per site.

RESULTS

Molecular Characterization of Plant Parasitic Nematodes Associated with Sweet Potato

The analysis of DNA PCR products using 1kb ladder on agar rose gel (*18S rRNA*) formed bands between 750 to 1000 base pairs (Figure 1).

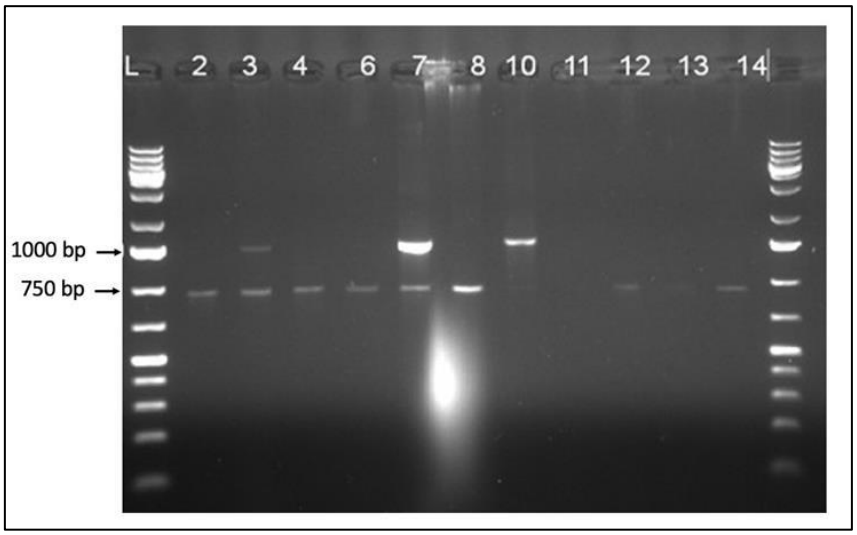

Figure 1: Gel analysis of PCR products amplified using the *18S rRNA* primers on genomic DNA of nematode isolates extracted from sweet potato farms

Where L= ladder (1.5kb), 2= N02, 4= N04, 6= N06, 11= N11, 12= N12, 13= N13, 14= N14

On the basis of similarity as determined through BLAST analysis, the isolates were identified as follows; *Mylonchulus hawaiiensis* (N14) - a predatory nematode, *Aporcelaimellus nigeriensis* (N13), *Rotylenchulus reniformis* (N12), *Rotylenchulus borealis* (N11), *Aporcella femina* (N06), *Dorylaimus aff. stagnalis* (N04) and *Heterodera dunensis* (N02) (Table 2).

Table 2: Molecular identification of nematodes associate with sweet potato in Kirinyaga County

Isolate	Close match	GenBank Accession No.	% Similarity	County
N02	*Heterodera dunensis*	MT509424.1	100	Spain
N04	*Dorylaimus aff. stagnalis*	MF409839.1	99.02	Germany
N13	*Aporcelaimellus nigeriensis*	MN605663.1	97.31	Nigeria
N14	*Mylonchulus hawaiiensis*	AB361442.1	94.79	Japan
N11	*Rotylenchulus borealis*	KJ636293.1	94.29	Netherlands
N06	*Aporcella femina*	MW237832.1	94.11	Nigeria
N12	*Rotylenchulus reniformis*	JX406343.1	90.27	Alabama

The phylogenetic tree constructed from alignment of 18S rRNA sequences of the isolated PPN grouped the isolates into two clades (Figure 2). This grouped *Dorylaimus aff. Stagnalis, Aporcelaimellus nigeriensis, Aporcella femina* and *Mylonchulus hawaiiensis* into one clade, while *Rotylenchulus borealis (N11), Rotylenchulus reniformis (N12) and Heterodera dunensis* (N02) were grouped into the osecond clade (Figure 2). The evolutionary distance matrix of the species (Table 3).

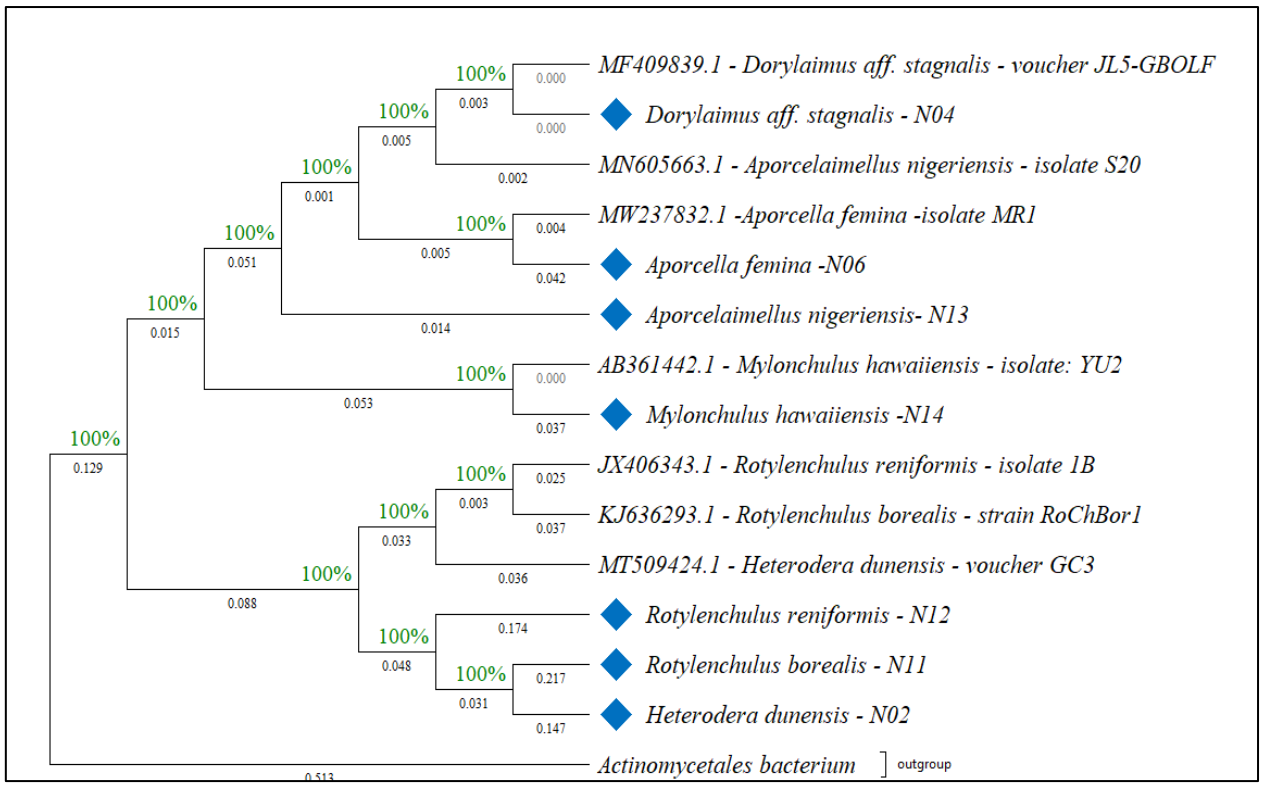

Figure 2: Phylogenetic tree of extracted nematode species from sweet potato farms and their counter parts in the NCBI gene bank

Table 3: Evolutionary distance matrix of sweet potato nematodes in Kirinyaga County and their counter parts in the NCBI gene Bank based on the p-distance method

Nematode species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
[1]AB361442.1 - *Mylonchulus hawaiiensis*	0													
[2]*Mylonchulus hawaiiensis* - N14	0.035													
[3]MN605663.1 - *Aporcelaimellus nigeriensis*	0.112	0.154												
[4]*Aporcelaimellus nigeriensis*- N13	0.113	0.154	0.020											
[5]JX406343.1 - *Rotylenchulus reniformis*	0.173	0.218	0.218	0.218										
[6]*Rotylenchulus reniformis* - N12	0.372	0.413	0.380	0.380	0.280									
[7]KJ636293.1 - *Rotylenchulus borealis*	0.179	0.224	0.238	0.246	0.062	0.325								
[8]*Rotylenchulus borealis* - N11	0.423	0.450	0.502	0.505	0.356	0.401	0.333							
[9]MW237832.1 -*Aporcella femina*	0.118	0.160	0.010	0.030	0.232	0.381	0.252	0.522						
[10]*Aporcella femina* -N06	0.153	0.185	0.057	0.057	0.266	0.375	0.310	0.482	0.046					
[11]MF409839.1 - *Dorylaimus aff. Stagnalis*	0.106	0.147	0.005	0.025	0.218	0.389	0.231	0.491	0.015	0.062				
[12]*Dorylaimus aff. stagnalis* - N04	0.106	0.147	0.005	0.025	0.218	0.389	0.231	0.491	0.015	0.062	0.000			
[13]MT509424.1 - *Heterodera dunensis*	0.192	0.238	0.239	0.254	0.067	0.297	0.073	0.359	0.240	0.290	0.232	0.232		
[14]*Heterodera dunensis* - N02	0.379	0.389	0.387	0.397	0.279	0.373	0.286	0.364	0.388	0.363	0.396	0.396	0.286	
[15]*Actinomycetales bacterium* {outgroup}	0.735	0.766	0.720	0.719	0.858	0.949	0.811	0.932	0.718	0.750	0.706	0.706	0.777	1.016

DISCUSSION

Molecular Characterization of Plant Parasitic Nematodes isolated from Sweet Potato Farms in Kirinyaga County

Many plant parasitic nematodes are easily distinguishable by their morphological characteristics (Shokoohi & Moyo, 2022; Onchari *et al*., 2023). However, some species have characteristics that overlap hence morphological identification becomes uncertain (Ahmad & Jairajpuri, 2010; Shokoohi & Moyo, 2022). Molecular characterization of nematodes has been successful in identifying PPN species (Blok *et al*., 2002; Hu *et al*., 2011; Niu *et al.,* 2012). More so, where there is development of new strains due to resistance and adaptation to new environments (Schleker *et al*., 2022). Molecular characterization is therefore an important and reliable way of identifying PPNs to species level. In this study, molecular markers, that is 18S rRNA, aided in identification of PPN such as *Mylonchulus hawaiiensis*. Similarly, molecular identification using18S rRNA, 28S rDNA and D2-D3 was used to identify *Aporcelaimellus nigeriensis* (Rashidifard *et al*., 2020). Moreso, characterization of nematodes to species level will lead to development of effective and specific PPN management strategies (Mokrini *et al.,* 2019).

Some species such as *Rotylenchulus* species are described as semi endo-parasites especially in woody and herbaceous plants (Van *et al*., 2016). They exhibit their parasitism in a wide range of crops and landscapes within farms in both tropical and subtropical areas. Due to almost similar morphological relation between *R. borealis* and *R. macrosoma*, phylogenetic approach confirms the difference in their nuclear ribosomal analysis using molecular markers (Palomares-Rius *et al*., 2020; Onchari *et al*., 2023). A study on *R. reniformis* and *R. parvus* reveal that they are both ranked among the pathogenic species of reniform nematodes. These species exhibit a very close morphological relationship and are commonly found in warmer regions characterized by high annual mean temperatures as described by Palomares-Rius *et al*., (2020). *Rotylenchus* species affect production of crops by causing stunted growth as well as hypersensitivity reactions to plants (Khanal *et al*., 2018).

According to Liu *et al.* (2016) *Heterodera* species are known to cause cell death in plants by injecting nematode effector proteins through the stylet into the plants resulting in the death of a plant and or loss of production. *Heterodera dunensis* can be easily distinguished from other species in a phylogenetic tree by molecular data (ITS, D2-D3, COI and 18S) as described by Singh *et al*., (2020). Similar to the current study, *Heterodera dunensis* nematodes were reported in Gran Canaria, Spain (Singh *et al*., 2022) and in other parts of the world such as United States and Canada (Tylka & Marett, 2021). These species commonly affect potatoes by forming cysts and furthermore, they are reported to be present in cabbage (Mwamula *et al*., 2018) and Arabica coffee (Singh *et al*., 2023).

Nematode survey in 2016 discovered presence of previously unidentified species in watermelon farm in Nigeria (Rashidifard *et al*., 2021) as well as in this study. Molecular characterization confirmed the monophyly and phylogeny relation of the new species to *Aporcella* genus (Rashidifard *et al*., 2021). The genus *Aporcella* is diversely distributed and its taxonomy has been subjected to a number of changes including its description of the new species (Naghavi *et al*., 2019), its definition (Álvarez-Ortega *et al*., 2013) and a recent update of its taxonomy (Vazifeh *et al*., 2020). Further, the closer evolutionary relationship of *Aporcella* with non-aporcelaimid taxa (tylencholaims, discolaims) than with other aporcelaims has also been reported by various researchers (Álvarez-Ortega & Peña-Santiago 2016; Imran *et al*., 2019; Naghavi *et al*., 2019, Vazifeh *et al*., 2020; Rashidifard *et al*., 2021).

Predatory nematodes have been reported to reduce PPN populations in all soil biomes by releasing nutrients in forms readily available to plants (Khan & Kim, 2007). Molecular characterization revealed the presence of *Dorylaimus aff stagnalis*. It is possible that these reductions are linked to changing climate and cropping practices. The low diversity of PPN observed by Onchari *et al*., (2023) in sweet potato farms was similar to that reported by Nolan & Callahan (2006).

Further, according to Fournet *et al.*, (2016) and Onchari *et al*., (2023), characterization of PPN forms a baseline for continuous survey to determine the change in host parasite evolutionary trajectories and their potential for speciation under different agroecological zones. Moreover, associations observed from previous studies provide basis for appropriate PPN control and management strategies that can be adopted for future use (Mokrini *et al.,* 2019).

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