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Occurrence of Fungivorous Nematodes in Ngere Tea Catchment Area, Murang'a County, Kenya

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Abstract: **Tea (Camellia sinensis (L.) O. Kuntze) plant is an important source of different beverages which are claimed to be the most widely consumed fluids after water. It is grown in many countries ranging from as far north as 49° N, outer Carpathians to as far as 30° S;- Natal, South Africa and from altitudes varying from sea level in Japan to 2700 m above mean sea level (amsl) in Olenguruone, Kenya and Gisovu, Rwanda. The plant is widely adaptable to geographical areas with large variations in climate and physical features which affect rates of growth, yields and quality. A survey was conducted to determine the fungivorous nematodes associated with tea in Ngere tea catchment area in Kenya. Soil samples were collected from six electoral zones of Ngere factory (Ndakaini, Kimandi, Gatura, Karangi, Kiarutara and Mbugiti) in Gatanga division Thika district, Murang'a County, Kenya. The objectives of this research were to isolate, characterize and identify fungivorous nematodes that are associated with soil in tea growing areas of Ngere. Nematodes were extracted and recovered from soil samples using a modified baermann funnel method and identified under a light microscope based on their morphological characters. They were also classified according to their feeding habits. DNA was extracted from the isolates and PCR was performed. The amplified DNA was sequenced and the sequences compared with the sequences at NCBI database. Fungivorous nematodes recovered included Tylenchus spp., Aphelenchus spp., and Ditylenchus spp. Tylenchus spp was the most frequently occurring species in the soil (60.47%) where the population was 429 and Aphelenchus spp had frequency rating of 48.84% and a population of 530. Ditylenchus had the lowest frequency rating of 6.98%. Two genera of fungivorous nematodes were also encountered in stressed tea bushes rhizosphere. In the stressed tea bushes rhizosphere, fungivorous nematodes identified were Aphelenchus spp., and Tylenchus spp. The study showed that there is fungivorous nematodes inhabiting tea soils in Ngere tea catchment area**

Keywords: **Tea, Fungivorous, frequency, nematode population, carbon content, Aphelenchus spp., Tylenchus spp., and Ditylenchus spp.**

I. INTRODUCTION

Tea (*Camellia sinensis* (L) O. Kuntze) plant is an important source of different beverages which are claimed to be the most widely consumed fluids after water. It is grown in many countries ranging from as far north as 49° N, outer Carpathians to as far as 30° S;- Natal, South Africa and from altitudes varying from sea level in Japan to 2700 m above mean sea level (amsl) in Olenguruone, Kenya and Gisovu, Rwanda. The plant is widely adaptable to geographical areas with large variations in climate and physical features which affect rates of growth, yields and quality (Owour *et al.*, 2010). Nematodes are the most abundant multicellular animals on earth. Numerically, between 80 and 90% of all multicellular animals on earth may be nematodes (Jairajpuri and Ahmad, 1992). Nematodes can be found in different environments,

e.g. soil, sea or fresh water, as free-living, parasitic or predacious animals. Their food sources include plants, algae, bacteria, fungi and other nematode species, as well as almost all vertebrates including fishes, and invertebrates like insects and spiders (Ferris *et al.*, 2001). Fungivorous (mycophagous) nematodes are equipped with a fine mouth stylet punctures the fungal hyphae cells, allow them to feed on their contents, which kills the consumed cells. These nematodes feed on different species of soil fungi, including plant pathogenic, saprophytic and mycorrhizal fungi (Bae and Knudsen, 2001). Feeding on different groups of fungi has a differing impact on soil ecology. Highly significant is their grazing on mycorrhizal fungi, which may restrict mycorrhizal development and limit nutrient uptake by host plants, a disadvantage to plants. This can lead to a reduction in the yield of mycorrhizal host plants (Ruess *et al.*, 2000). On the other hand, when a plant pathogenic fungus is a preferred host, then disease reduction may occur. Several fungal-feeding nematodes can be cultured on media with an appropriate fungus. They can cause severe damage to fungal cultures *in vitro*. For example, it has been shown that fungivores could cause the break-up various colonies of cereal foot rot pathogens on agar cultures. They may destroy aerial hyphae and limit fungal growth (Ruess and Dighton, 1996). Fungivorous nematodes may, in some circumstances, be significant constraints on the efficacy of biocontrol fungi; therefore, further investigation of interactions between biocontrol agents and indigenous nematodes under field conditions is warranted (Bae and Knudsen, 2001). In addition, since fungal symbionts convert a portion of the roots of all trees growing under natural conditions into mycorrhizae, nematodes parasitic on fungi must be considered in any study of root or soil ecology. The most common genera of fungivorous nematodes in the soil belong to the genera *Aphelenchus*, *Aphelenchoides*, *Tylenchus*, and *Ditylenchus* (Freckman and Caswell, 1985).

II. MATERIALS AND METHODS

Rhizosphere soils were sampled from Ngere tea catchment area where forty three farms each selected in forty three tea buying centers were considered in six electoral zones of Ngere factory (Ndakaini, Kimandi, Gatura, Karangi, Kiarutara and Mbugiti) in Gatanga division Thika district, Murang'a County, Kenya. Soil sample (ca. 400 g) was a composite of 3–10 random sub-samples, taken at least 8–10 m apart, from the surface to a depth of 45 cm of the four cardinal points of the rhizosphere of the tea bush. All sub-samples were mixed together and placed in a paper bag and then a polyethylene bag to prevent water loss and kept in a cooler (ca. 15 ^{0}C) during transit to the laboratory as suggested by Kaya and Stock (1997). The samples were thoroughly mixed and 20 g of each soil samples was used for nematode extraction using modified baermann funnel technique. Nematode numbers were counted and identification to genus level was done under a compound microscope at a magnification of ×400-×1000 (Karuri *et al.*, 2010). Nematode genera were assigned to trophic groups (bacterial and fungal feeders, plant parasites, omnivores and predators) as described by Yeates *et al.* (1993). Taxonomic groups were also assigned to colonizer-persistor (cp) values according to Bongers (1990).

2.1 DNA extraction

The nematodes were picked out of the watch glass and placed in one of the drops of distilled water on a glass slide. The nematodes in the drop of water were brought into focus under the dissecting microscope at a magnification of X40. With a pipet tip on the pipetter, it was carefully brought to the end of the tip over the nematode then crashed the nematode until the nematode ruptured. The nematode-smash solution was pumped a few times before transferring it to a sterile 1.5 ml of eppendorf tube. Then the 1.5 ml eppendorf tubes were labelled and aliquot of 100 µl DNA extraction buffer was put into each tube. The nematodes smash was added in the tube and swirled thoroughly with sterile plastic pestle to homogenize the mixture. It was then incubate at 55 °C for 3 hours to overnight. Centrifuged at 13000 g for 20 sec. and added 3 µl 10 mg/ml RNase A. It was pipetted to mix and the tubes maintained at 37 °C for 15 min. One hundred micro litre of buffer saturated with phenol was added and the tubes flicked to mix the solution and the contents were placed in 55 °C water bath for 10 - 15 min with flicking every 2-3 min, followed by adding 100 µl chloroform / isoprophyl alcohol [CHCl/IAA] (24:1) to each tube. The contents in the tubes were mixed vigorously 6 times and centrifuged for 15 mins at 15,000 g. The aqueous layer was then carefully transfered to a new tube and the step repeated before adding an equal volume of phenol/chloroform isoamyl alcohol (25:24:1) mix, followed by spinning at 15000 g for 15 minutes. The aqueous layer was again carefully transfered to a new tube, where 0.7 times volume of cold isopropanol was added, followed by overnight incubation at -20 ^{o}C. The mixture was the centrifuged at 15 000 g for 10 minutes, followed by careful removal of isopropanol. 1 ml of 70% ethanol was then added followed by mixing by inversion and centrifugation at 15 000 g for 5 minutes, after which the ethanol was removed carefully, taking care not to dislodge the pellet and discarded. This

procedure was repeated twice. This was accompanied by quick spinning and pippeting to remove the residual ethanol after which the pellet was left to completely air dry on the bench at room temperature. The pellet was then resuspended in 40 µl TE. The DNA was then kept at -20^{0}c for future applications (William *et al*., 1992 and Zheng *et al.*, 2002). The DNA was semi quantified on a 1% agarose gel in 1xTAE buffer and visualized under UV by staining with ethidium bromide (Sambrook *et al*., 1989**).**

2.2 Polymerase Chain Reaction (PCR)

Amplification was performed using a model PTC-100 thermal cycler (MJ research inc., USA). The Internal Transcribed Spacer 2 (ITS2) region of ribosomal deoxyribonucleic acid (rDNA) was amplified. The primer 5.8S: 5'-ACGAGCCGAGTGATC CACCG-3' (Vrain *et al.*, 1992 and Cherry *et al.*, 1997) was used. Amplification was carried out in a 50 µl mixture containing 0.2 µl of genescript Taq, 1.0 µl (20-pmol) of 1.58 F forward primer, 1.0 µl (20-pmol) of 5.8S reverse primer, 1 µl of template DNA, 2.0 µl of dNTPs mix (2.5mM), 4.0 µl PCR 10x buffer(containing 1.5mM $MgCl_2$) (genescript) and 40.8 µl of PCR water. The control contained all the above except the DNA template. Reaction mixtures were subjected to the following temperature cycling profiles repeated for 35 cycles: Initial activation of the enzyme at 94^{o}C for 2mins 45sec., denaturation at 94^{o}C for 60 seconds, primer annealing at 52^{o}C for 60 seconds, chain extension at 72^{o}C for 2 minutes and a final extension at 72^{o}C for 5 minutes (Madani *et al.*, 2004 and De Ley *et al.*, 2002). Amplification products (7.0 µl) were separated on a 1% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining (Sambrook *et al*., 1989). The PCR products were purified using the QIAquick PCR purification Kit protocol (Qiagen).

2.3 Phylogenetic analysis

Sequencing was carried out for the ITS sequences of selected nematodes. Sequences were generated at the sequencing facility in South Korea at Macrogen Sequencing facility using the ITS-2 region of gene for ribosomal RNA that had be amplified using primer 5.8S: 5'-ACGAGCCGAGTGATC CACCG-3' (Vrain *et al.*, 1992 and Cherry *et al.*, 1997), the samples had been prepared according to the sequencer instructions. The sequences were edited by eliminating all gaps (Complete deletion option), using the CHROMAS PRO software 1.5 version. The edited sequences of the isolates were compared to the sequences in the National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST), website (htt://www.ncbi.nih.gov). Alignment was done using CLUSTAL W 1.6 software (Larkin *et al.*, 2007).The evolutionary analyses were conducted in Mega 5 (Tamura *et al*., 2011). To show the evolutionary relationships of these taxa, the evolutionary history was inferred using the Neighbor-Joining method (Tamura *et al.*, 2004 and Tamura *et al*., 2007). The optimal tree was drawn and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) shown next to the branches. Felsenstein, (1985), bootstrap analysis was performed to attach confidence estimates for the tree topologies. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes and Cantor method (Jukes and Cantor, 1969).

III. RESULTS

Three genera of fungal feeding nematodes were encountered in rhizosphere soil samples collected from Ngere tea catchment area. Fungivorous nematodes identified were Aphelenchus spp., Tylenchus spp., and Ditylenchus spp., Tylenchus spp was the most frequently occurring species in the soil (60.47%) where the population was 429/20 g of soil which was followed by Pratylenchus spp with 55.81% frequency rating and a population of 404/20 g of soil while Aphelenchus spp had frequency rating of 48.84% and a population of 530/20 g of soil (**Table 1**). Ditylenchus had the lowest frequency rating of 6.98% and 4.65% (**Table 1**). Two genera of fungivorous nematodes were also encountered in stressed tea bushes rhizosphere. In the stressed tea bushes rhizosphere, fungivorous nematodes identified were Aphelenchus spp., and Tylenchus spp. Tylenchus spp., was the most frequently occurring fungivorous nematode in the soil (50%) with a population of 21/20 g soil and Aphelenchus spp., with 16.67% frequency rating and a population of 10/20 g. (**Table 2**). The amplified DNA yielded 750 bps bands as show in **figure 1.** The **Table 4** below show BLAST analysis of the partial sequences showed that the isolates: N3-rDNA2, N11-rDNA2 and N14-rDNA2 had sequence similarity of between 95-99 % while N9 - rDNA3 had sequence similarity of 89 % and these could represent novel species. Among these were Aphelenchus spp., Tylenchus spp., and Ditylenchus spp.

IV. DISCUSSION

Tylenchus are small to medium sized (0.4-1.3mm), ventrally curved upon relaxation. Cuticle moderately thick (1-2µm), distinctly annulated. Lateral fields each with four incisures. Cephalic region continuous, annulated; framework with light or no sclerotization. Stylet 8-21µm long, with conus comprising between one third and half of the stylet length and round, posteriorly sloping basal knobs. Median oesophageal bulb oval, muscular, anterior to middle of oesophagus and with refractive valve plates; basal bulb pyriform. Cardia distinct. Excretory pore usually opposite basal bulb. Vulva a transverse slit, usually at 60-70% of body length, lips not modified. Tail ventrally arcuate, often hooked, regularly tapering to a point or minutely rounded terminus (Kimenju et al., 2004; Luc et al., 2005). Tylenchus spp had the highest frequency of occurrence among the healthy tea bushes according to **Table 1**, it was highest in the stressed tea bushes occurring three times **(Table 2**) this could be attributed to the fact that Tylenchus spp., belongs to cp value of 2 (**Table 3**). They have a high reproduction rate, a short life cycle, high colonization ability and are tolerant to disturbances (Bonger, 1990). It also belongs to the trophic group of a fungal feeder according to Bonger, (1990), see Table 4. Isolate N14-rDNA2 displayed the typical morphological characteristics of the genus Tylenchus. Sequences analysis by BLAST search system on the NCBI website showed that the isolate N14-rDNA2 was most closely related to Tylenchus arcuatus isolate wb8 EU306348.1with 99% sequence similarity (**Table 4**).

Aphelenchus can be described as 0.4–1.2 mm long slender, with the body almost straight when relaxed. Labial region weakly sclerotized; stylet weak, with or without basal swellings. Oesophageal bulb well developed, spherical to rounded–rectangular in shape and more or less filling the body diameter. Dorsal oesophageal gland duct opening within bulb, just anterior to the valve plates. Oesophageal gland lobe overlapping intestine dorsally. Tail medium conoid. Ectoparasitic on leaves, stems and other parts of higher plants. Most species can also be readily cultured on various fungal hyphae (Kimenju et al., 2004; Luc et al., 2005). It also belongs to the trophic group of a fungal feeder according to Bonger, (1990), see **Table 3**. Recent studies have illustrated that Aphelenchus avenae can be used as a biological control of phytopathogenic fungi. This is because several investigators have found that A avenae is a fungivorous nematode which feeds on hyphae of more than 90 species of soil fungi (Ishibashi et al., 2005). Aphelenchus spp had the third highest frequency of occurrence according to **Table 1** this could be attributed to the fact that Aphelenchus spp., belongs to cp value of 2 (**Table 3**). They have a high reproduction rate, a short life cycle, high colonization ability and are tolerant to disturbances (Bonger, 1990). Isolates N3-rDNA2 displayed the typical morphological characteristics of the genus Aphelenchus. Sequences analysis by BLAST search system on the NCBI website showed that the isolates N3-rDNA2 and N11-rDNA2 were most closely related to Aphelenchus avenae isolate: 2010_026, AB630232 with 99% sequence similarity (**Table 4**).

Ditylenchus are slender nematodes dying straight or slightly curved ventrally on heat relaxation. Skeleton of labial region weakly sclerotized. Stylet of moderate strength and with small basal knobs. Oesophagus with a muscular median bulb; isthmus gradually expanding to form the basal bulb, which may extend as a lobe over the intestine. Ectoparasites of plant stems and leaves but also found within the tissues. Infected stems and leaves are often stunted and deformed. They are fungal feeders (Kimenju et al., 2004; Luc et al., 2005). Among more than 60 species presently recognized in the genus Ditylenchus (Siddiqi, 2000), only a few are parasites of higher plants, whilst the majority of species are mycophagous (Vovlas et al., 2011). It also belongs to the trophic group of a fungal feeder according to Bonger, (1990), see **Table 3**. Isolate N9-rDNA2 displayed the typical morphological characteristics of the genus Ditylenchus. Sequences analysis by BLAST search system on the NCBI website showed that the isolate N9-rDNA2 was most closely related to Ditylenchus SZ-2011, JN594665 with 89% sequence similarity (**Table 4**).

V. CONCLUSION

Tea fields in Ngere tea catchment area of Kenya inhibits fungivorous nematode with the predominant ones being in the genera Tylenchus spp., Aphelenchus spp., and Ditylenchus spp. More work is required to determine the economic importance of the nematodes associated with tea in Kenya.

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APPENDIX

TABLES AND FIGURES

Table 1: Nematodes extracted from rhizosphere soil sample of healthy tea bushes in Ngere

Nematode Genera	C-P Values	Population/20 g of soil	Frequency of Occurrence	% Frequency Rating*	% Nematode population**
Aphelenchus	2	530	21	48.8	25.9
Cervidellus	2	140	12	27.9	6.8
Ditylenchus	2	39	3	7.0	1.9
Dorylaimus	4	126	5	11.6	6.2
Hoplolaimus	2	77	6	14.0	3.8
Helicotylenchus	3	127	6	14.0	6.2
Pratylenchus	3	404	24	55.8	19.8
Rotylenchus	3	33	2	4.7	1.6
Tylenchus	2	429	26	60.5	21.1
Xiphinema	5	139	10	23.3	6.8

* n/N x 100 (n = number of times individual nematodes occurred and N = Sample size (43).
** In/TN x 100 (In = Individual nematode in all the samples and TN = Total Population of all the nematodes extracted in all the samples).

Table 2: Nematodes extracted from stressed tea bushes rhizosphere

Nematode Genera	Population/20g of soil		Frequency of Occurrence	% Frequency Rating*	% Nematode population**
Pratylenchus	25		3	50	28.1
Tylenchus	21		3	50	23.6
Helicotylenchus	10		1	16.7	11.2
Aphelenchus	10		1	16.7	11.2
Xiphinema	3		1	16.7	3.4
Hoplolaimus	20		1	16.7	22.5

* n/N x 100 (n = number of times individual nematodes occurred and N = Sample size (6).
** In/TN x 100 (In = Individual nematode in all the samples and TN = Total Population of all the nematodes extracted in all the samples).

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Table 3: Extracted classified according to their Trophic Group

FAMILY	GENERA	C-P VALUES	TROPHIC GROUP
Anguinidae	Ditylenchus	2	[d]FF
Pratylenchidae	Pratylenchus	3	[a]PF
Tylenchidae	Tylenchus	2	[a]PF
Longidoridae	Xiphinema	5	[a]PF
Aphelenchidae	Aphelenchus	2	[d]FF
Cephalobidae	Cervidellus	2	[b]BF
Hoplolaimidae	Hoplolaimus	2	[a]PF
Dorylaimidae	Dorylaimus	4	[c]OM
Hoplolaimidae	Rotylenchus	3	[a]PF
Hoplolaimidae	Helicotylenchus	3	[a]PF

C-P: Colonizer-persister scale 1-5 where cp 1 are colonizers characterized by short generation time and cp 5 are persisters characterized by long generation time (Bongers, 1990). [a]Plant feeders [b]Bacteriovores [c]Omnivores

Table 4: Showing the BLAST isolates, nearest neighbours in the data bank and their % relatedness

Isolate	Length	Nearest relative	Accession number	% similarity
N3-rDNA2	404bp	Aphelenchus avenae 2010_017	AB630201.1	100%
		Aphelenchus avenae st1_47	AB631013.1	99%
		Aphelenchus avenae st1_43	AB631010.1	99%
		Aphelenchus avenae st1_35	AB631003.1	99%
		Aphelenchus avenae st1_34	AB631002.1	99 %
N9-rDNA2	765bp	Ditylenchus SZ-2011 isolate DityPHBQA-1	JN594665.1	89%
		Ditylenchus SZ-2011 isolate DityPSDLW-1	JN635037.1	89%
		Ditylenchus SZ-2011 isolate DityPHBQA-2	JN605348.1	89%
		Ditylenchus destructor strain Ch2	EU188727.1	89%
		Anguina tritici clone 2	JF826516.1	87%
N14-rDNA2	270bp	Tylenchus sp. FL-SType-6	EU040130.1	100%
		Tylenchus arcuatus isolate wb8	EU306348.1	99%
		Tylenchus sp. JH-2003	AY284589.1	99%
		Tylenchus arcuatus isolate wb9	EU306349.1	98%
		Filenchus filiformis isolate FileFil	AY284592.1	98%

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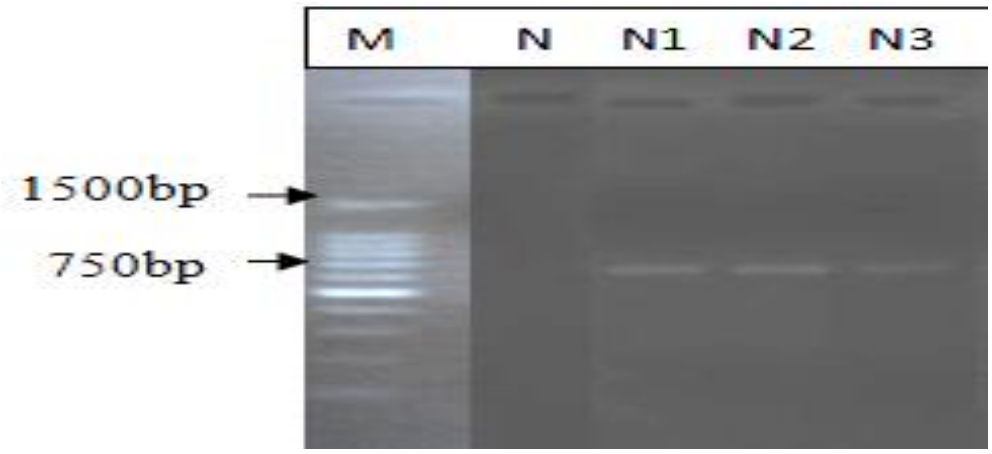

Figure 1: Showing PCR products for fifteen isolates run in 0.8% (W/V) agarose gel. M- Marker and N- Negative control

Legend: Lanes N1*(**N3-rDNA2**), N2*(**N9-rDNA2**), N3*(**N14-rDNA2**), (**N**) negative control and (**M**) M-1500bp Molecular marker size. *The figures outside the brackets are the isolate numbers