

Nematode 18S rRNA gene is a reliable tool for environmental biosafety assessment of transgenic banana in confined field trials

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Abstract Information on relatedness in nematodes is commonly obtained by DNA sequencing of the ribosomal internal transcribed spacer region. However, the level of diversity at this locus is often insufficient for reliable species differentiation. Recent findings suggest that the sequences of a fragment of the small subunit nuclear ribosomal DNA (18S rRNA or SSU), identify genera of soil nematodes and can also distinguish between species in some cases. A database of soil nematode genera in a Ugandan soil was developed using 18S rRNA sequences of individual nematodes from a GM banana confined field trial site at the National Agricultural Research Laboratories, Kawanda in Uganda. The trial was planted to evaluate transgenic bananas for resistance to black Sigatoka disease. Search for relatedness of the sequences gained

with entries in a public genomic database identified a range of 20 different genera and sometimes distinguished species. Molecular markers were designed from the sequence information to underpin nematode faunal analysis. This approach provides bio-indicators for disturbance of the soil environment and the condition of the soil food web. It is being developed to support environmental biosafety analysis by detecting any perturbation by transgenic banana or other GM crops on the soil environment.

Keywords Nematodes · Biosafety assessment · Environmental biomarkers · Barcoding

Introduction

Banana production in Uganda is challenged by a number of biotic constraints and banana is very difficult to improve by conventional breeding because of the high gamete sterility and lack of native resistance genes. Genetic transformation of banana offers a viable alternative tool for complementing conventional breeding for the improvement of banana for pest and disease resistance. Genetic transformation of EAHBs is now possible in several cultivars and technologies have been developed that may offer resistance to pests and diseases, improved nutrition via bio-fortification, delayed ripening or prolonged shelf life, yield enhancement and overall production enhancement (Shotkoski et al. 2010). However, to

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execute field evaluation of genetically modified banana plants, a reliable tool is needed for assessment of safety to the environment.

According to the regulations governing the release of GMO plant to the farmers and market in Uganda, there is need to determine the effect of transgenic plants, on the environment since Uganda is a signatory of the Cartagena protocol on biosafety to the Convention of biological diversity and is hence required to comply with its guidelines for risk assessment when introducing GM plants into the environment. The Cartagena protocol was developed for subscribing countries to ensure an adequate level of protection in the field during transfer, handling and use of living modified organisms resulting from modern biotechnology so that they do not exert adverse effects on the conservation and sustainable use of biological diversity. Uganda has developed a working regulatory framework for research in GMO which is implemented by the Uganda National Council of Science and Technology (UNCST) as the Competent Authority. On top of building physical and personnel capacity for biotechnology intervention in agricultural research (Clarke 2003), already three confined field trials (CFTs) of transgenic banana transformed for resistance against black Sigatoka, banana bacterial wilt, and enhanced pro-vitamin A and iron have been established in Uganda (Nordling 2010). In order to devise approaches that would evaluate the biosafety of products before any release to farmers could be contemplated, investigations were initiated to develop protocols for detection of any unintended effects of GMO bananas on soil quality.

Soil nematode communities participate in many functions at different levels of the food web. These nematodes are stable in response to moisture, temperature, and land management in predictable ways that reflect changes in soil microenvironments (Ingham 2000). They are therefore useful indicators of soil health, previously defined as the capacity for a particular soil to function optimally in the ecosystem, to sustain biological productivity, diversity and maintain environmental quality (Doran and Parkin 1996). Other research scientists have also identified soil nematodes as good indicators of soil and environmental health (Ferris et al. 1999; Ritz and Trudgill 1999) but only Pattison et al. (2004) have studied these communities in banana cropping systems. Nematodes interact very closely with the biological, chemical and physical properties of soil.

They exhibit a wide range of specialization, trophic levels, diversity, and abundance and are easy to extract from the soil compared to other microorganisms so that assessing their change in diversity can be related easily to soil health. These attributes make soil nematodes suitable candidates for evaluation of soil status in the CFT of genetically modified banana.

In the past, morphologically based taxonomy which requires expert knowledge for accurate identification of genera was drawback to utilizing nematode diversity studies to predict soil health. Nevertheless, categories of different trophic levels as applied to faunal analysis included the integration of nematode feeding groups (Yeates et al. 1993) and colonizer persister scaling (cp) that ranges from cp1 to cp5 into a matrix classification of functional guilds (Bongers and Bongers 1998). Guild is an assemblage of species with similar biological attributes. Also response to environmental conditions helps to reduce the need for advanced taxonomic skills (Ferris et al. 2001). Advances in DNA based molecular techniques have reduced the need for taxonomic expertise and are also superior to morphological based taxonomy for identification of nematode species (Carta et al. 2001). DNA barcoding technology approach involves sequencing taxonomically important gene, the small subunit nuclear ribosomal RNA (SSU or 18S rRNA) and provides an efficient method for identifying genera of soil nematodes.

The objective of this study was therefore, to develop a molecular tool that can be used to assess changes in the nematode community structure in CFT sites in Uganda. Here we report the development of genus specific primers designed from sequences of samples of the 18S rRNA gene isolated from a nematode sample collection from the trial site before CFT establishment. We discuss application of the potential use of the molecular tool in assessing soil environment effects of transgenic banana plants through evaluating the changes in soil nematode community structure.

Materials and methods

Study site

The CFT site used was located at the National Agricultural Research Laboratories, Kawanda (NARL) one of the institutes of the National Agricultural

Research Organization (NARO). It is located 0°25'N, 32°32'E, 13 km north of Kampala, along Kampala-Bombo road. NARL is 1,195 m above sea level, receives 1,250 mm of rainfall annually, with a daily average temperature of 15 °C minimum and 29 °C maximum and mean relative humidity of 76 %.

Nematode samples for molecular identification

Transgenic banana CFT occupied a total area of 60 m by 39 m (2,340 m²). Soil samples were collected before CFT planting, using a garden trowel following two transects in an X sampling design as described by Abawi and Gugino 2007. An inch of top soil was discarded from each of the four randomly selected points along transect before collecting the soil samples. This was repeated to provide two samples per transect. At least 1 kg soil sample was carefully placed in the bucket and mixed thoroughly using a garden trowel. Plant debris was removed and approximately 1 kg of constituted composite soil sample mixture was placed in a polythene bag. The bag was labeled, sealed and transported from the field for storage in a refrigerator at about 4 °C until nematode extraction. A total of 16 composite soil samples were collected to represent the whole field.

Single nematode isolation, digestion and PCR

Nematodes were isolated from soil samples following the standard filter extraction procedure (Southey 1986). About 100 g of moist soil was spread thin over double layer of fine facial tissue (Kleenex), placed on a wire mesh. The mesh was placed in 0.5 cm deep tap water within a shallow plate of about the same size as the mesh. After incubation at 28 °C for 48 h, nematodes present within the water per plate were collected. A Compound microscope at a magnification of 40× was utilized to determine nematode morphological characters that were coded.

Individual nematodes (adults or larvae) were picked and placed into 20 µl of 0.25 M NaOH in 0.2 ml tubes, then kept at room temperature for 3–16 h (Stanton et al. 1997). The lysate was then heated for 3 min at 99 °C. 4 µl of 1 M HCl and 10 µl of 0.5 M Tris–HCl buffered at pH 8.0 were added to neutralize the base. 5 µl of 2 % Triton X-100 was also added, and the lysate was heated further for 3 min at 99 °C. Lysates were stored at –20 °C.

For PCR, 4 µl of each lysate was added to a 50 µl PCR reaction comprising of 10× buffer at 1×; 1.6 mM MgCl₂; 0.2 mM each nucleotide; 1.3U of Taq polymerase (Fermentous); and 0.16 pmol/µl each primer. The primers used were SSU18A (AAAGATT AAGCCATGCATG) and SSU26R (CATTCTTGGC AAATGCTTTTCG), SSU9R (AGCTGGAATTACCG CGGCTG) (Blaxter et al. 1998), giving a ~1,000 bp PCR product. The reaction conditions were: 94 °C for 4 min; 35 cycles of (94 °C for 1 min; 52 °C for 1 min; 72 °C for 1 min); 72 °C for 10 min. Products (10 µl) were visualized on 1 % agarose gels and stained with ethidium bromide. PCR-available DNA cleaned using wizard clean up kit (Promega) was sequenced using Applied Biosystems (ABI) 3130xl capillary sequencer.

Sequence analysis

The sequence results were compared to nematode sequences in the online genomic databases through the Basic Local Alignment Search Tool (BLAST) available at the website of The National Center for Biotechnology Information (NCBI; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and the nematodes were identified to genera or species. The BLAST tool finds similarity between sequences and compares them to known sequences in online sequence databases. It also calculates statistical significance of the matches found and therefore can infer evolutionary relationships between sequences and organisms.

Primer design and testing

The 18 s rRNA sequence data from NCBI that provided the best match of 95–100 % with the sequences obtained from the different individual nematodes were aligned using MegAlign software (DNA Star Inc. Madison WI, USA) which also provided a phylogenetic tree to guide primer design. The nematode genera in the same group were selected and primers that differentiated them designed. The primers were tested for unwanted self and paired primer dimers, and then BLAST searches of the primers was done to rule out annealing to other non-intended gene targets. The primers that registered minimal or no dimers were advanced to testing using the quantitative PCR (qPCR) (Stratagene Mx3005P™ running MxPro software, version 4.10).

qPCR primer testing

For qPCR, single nematode DNA and pooled nematode DNA was used to test the primer specificity, the initial data used in the analysis to obtain dissociation and amplification plots was carried out by MxPro

software, version 4.10 (Stratagene, California, USA). The dissociation curve was accepted if the DNA template from both a single nematode of the desired genus and that from a pool of nematodes including the genus provided a single peak of amplified DNA at the same temperature. A fully optimized primer pair with

Table 1 Nematode genera identified at a GM banana CFT in Uganda

Genus name	GenBank accession no.	% Sequence similarity	qPCR designed primer name	Primer sequence	Product size length (bp)	CP value
<i>Acrobeloides</i>	AY284673.1	100	Acrob Acrod Chil F1	TGATCACGCGTAACGGAGAATA	362	2
			AcrodR1	TACGACGGGAAAACCAGCAAAAA		
<i>Aphelenchus</i>	AY284641.1	100	AphF1	GTGACGGGTAACGGAGGATAA	366	2
			AphR1	GTAAAGGCCACAACGGGAAAA		
<i>Paraphelenchus</i>	AY284642.1	96	AphF1	GTGACGGGTAACGGAGGATAA	366	2
			AphR1	GTAAAGGCCACAACGGGAAAA		
<i>Ceratoplectus</i>	FJ474097.1	92	PleCerF2	ATCAGCGTGCTAAAACCATT	128	2
			PleCerR2	ACGCGAGCTTATGACCCAC		
<i>Plectus</i>	FJ474097.1	97	PleCerF2	ATCAGCGTGCTAAAACCATT	128	2
			PleCerR2	ACGCGAGCTTATGACCCAC		
<i>Helicotylenchus</i>	FJ969124	97	HelF1	TTTGTGCGCACTATACTTCTTA	337	3
			HelR2	GCAACCAGACCAACAATCCC		
			HelR1	GAAAGCAACCAGACCAACA		
<i>Rotylenchulus</i>	EU306342.1	95	RotylsF2	TTAGAGGGATTTTCGGTGTTTTC	329	3
			RotylsR1	GCCCCGTATGGAGCCGTTA		
<i>Radopholus similis</i>	AJ966502.1	99	Rado F1	GCCATTGTGCACGCCGACTT	373	3
			RadoR2	GCGATTAAGCCGGTTCTCAC		
<i>Rotylenchus</i>	EU306356.1	95	RotyF2	TTAGAGGGACAAGGGGTAT	329	3
			RotyR1	CTTTCCAGACTAGGTTTCAATG		
<i>Tylencholaimus</i>	AJ966510.1	96	Tyle03F1	TAGACGGTATATCGAAAAGTA	149	4
			Tyle03R2	TAGGTATTGCGCACTCTGAT		
			Tyle03R3	AGGTATTGCGCACTCTGAT		
Paractinolaimus	AY993978.1	98	DoryLabrF1	TAGCCGCATGAAATAGAGCA	100	4
			DoryR2	AACTAGGTATCACGCCCACTG		
Anatonchus	AJ966474.1	95	AnF2	TGGTAAGAATTGGTAAACACGA	188	4
			AnR2	CGAGTCCAGTCCGAAGAATT		
Labronema	AY552972.1	96	DoryLabrF1	TAGCCGCATGAAATAGAGCA	100	4
			DoryR2	AACTAGGTATCACGCCCACTG		
<i>Dorylaimoides</i>	EF024986.1	97	DoryLabrF1	TAGCCGCATGAAATAGAGCA	100	4
			DoryR2	AACTAGGTATCACGCCCACTG		
			DoryR3	CTAGGTATCACGCCCACTG		
<i>Alaimus</i>	AJ966514.1	93	AlaiF2	AATCAGCGTGCTTGTCTTTC	128	4
			AlaiR2	CGAAGGCCGGAAGTCAAT		

Single nematode amplification, sequencing of the 18S small subunit of ribosomal RNA gene and BLAST-National Center for Biotechnology Information (NCBI), searched to identify the genus by percentage sequence identify. Corresponding quantitative PCR primers designed and tested to distinguish the nematode genera

no dimers and specific to the target gene gives a single melting product. The dissociation curve determines if any other gene rather than the one of interest was amplified in the qPCR reaction. The presence of a single homogeneous melting product peak for all the samples confirms a specific amplification. 3–5 μ l of digested nematode lysate was added to a 25 μ l qPCR reaction comprising of 2 $\times$ SYBR Green buffer at 1 $\times$ concentration (Stratagene, CA, USA); and 0.3 μ mol/ μ l each primer. The primers used were the primers designed for the different genera (Table 1). The reaction conditions were: 95 $^{\circ}$ C for 10 min; 40 cycles of (95 $^{\circ}$ C for 30 s; 56–75 $^{\circ}$ C for 1 min; 72 $^{\circ}$ C for 1 min). The primers that tested positive with qPCR were used to amplify with ordinary PCR (TProfessional Thermocycler—*Biometra*, Goettingen, Germany) and the products (10 μ l) were visualized on 1 % agarose gels stained with ethidium bromide. Ordinary PCR was done to confirm single PCR product of the qPCR.

Results

Single nematode PCR

A total of 170 nematodes were isolated from the site to be used for future CFTs. Using the universal primers (SSU18A and SSU26R) spanning the conserved region of the nematode 18S ribosomal gene, product sizes of $\sim$ 1,000 bp were obtained as expected. In

addition the primers SSU18A and SSU9R provided $\sim$ 600 bp amplicons as expected.

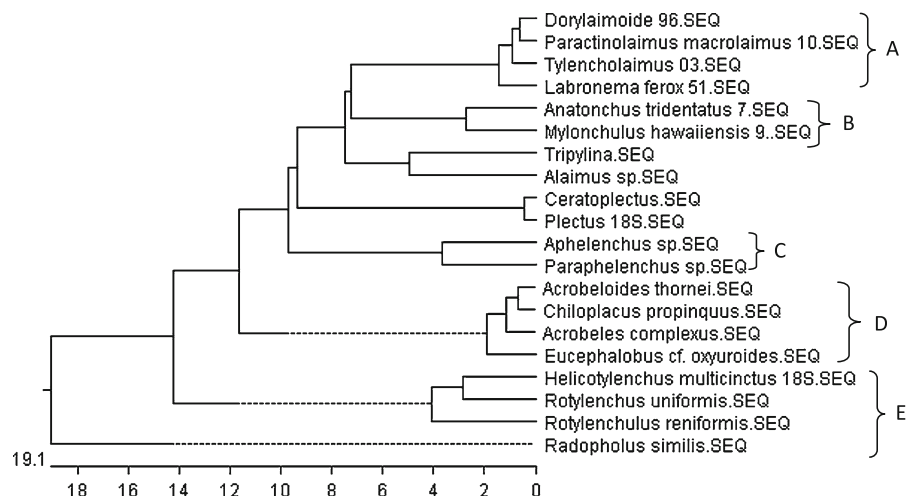
DNA sequencing and BLAST search

A total of 60 sequences were obtained from the nematodes and 20 different nematode genera were identified after BLAST search from NCBI data base (Table 1). The sequence similarity ranged between 90 and 100 %.

Primer design and testing

The NCBI sequences for the 20 nematode genera were organized into a cladogram of five groups, A–E using MegAlign software (Fig. 1). Twelve primer pairs were confirmed as neither generating primer dimers nor self priming and they covered 16 nematode genera out of 20 genera of which four are plant parasitic nematode genera and 12 are other members of the soil nematode community. All the primers provided product sizes ranging from 100 to 400 bp. The primer length ranged between 17 and 30 bp and melting temperature ranges of 60–75 $^{\circ}$ C (Table 1). Some nematode genera shared a very high sequence similarity, the same feeding habit and colonizer-persister (CP) scale value. One such group was, *Dorylaimoides*, *Labronema*, *Paractinolaimus*. with a CP value of 4. The second pair was *Plectus* and *Ceratoplectus* which also share the same CP value of 2. All primer pairs that registered minimal or no such dimers were advanced to testing using qPCR (Fig. 2).

Fig. 1 Phylogenetic tree of the 20 nematode genera obtained after BLAST search to guide primer designing and alignment with MegAlign (DNASar). The tree shows the different groups of the nematode genera according to the similarity of their sequences of the 18S rRNA gene. A–E are the different groups with the members of each having a high similarity in their sequences



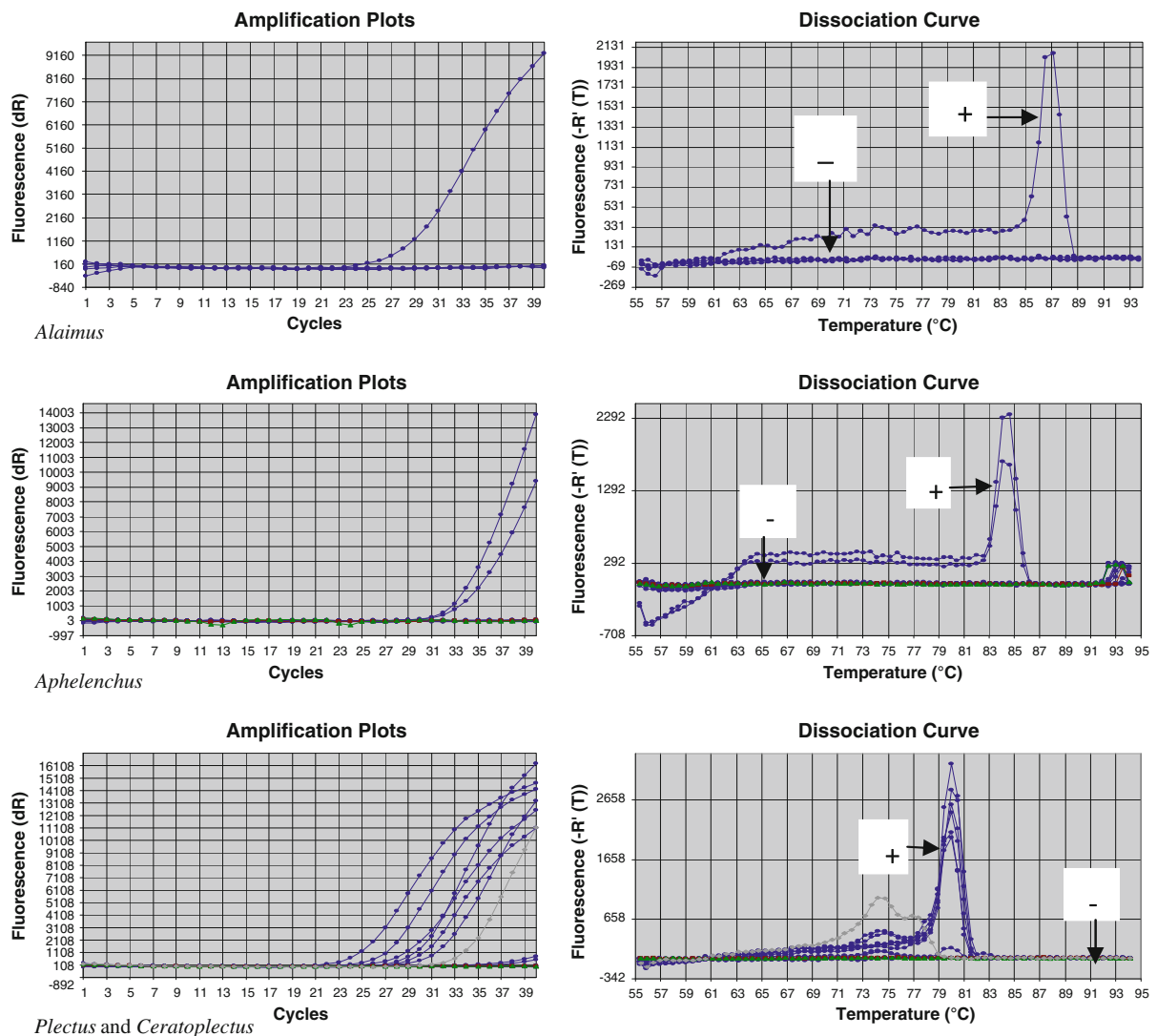


Fig. 2 Quantitative PCR (qPCR) results (amplification plots and dissociation curve) of selected primers designed and tested on pooled nematode DNA of different soil samples and single nematode DNA from a GM banana CFT in Uganda

qPCR primer testing

The cycle threshold (Ct) varied for different primer pairs. It varied from Ct 23 for *Alaimus*, *Plectus* and *Ceratoplectus* to Ct 31 for *Aphelenchus*. The Ct is proportional to the logarithm (base 2) of initial amount of target in a sample. In no case was a Ct value recorded after 40 cycles when primer pairs were used to amplify DNA of any other genus other than the DNA from which the primers were developed. In addition melt curves provided a single peak when used to amplify the DNA from which the primers were

developed and no peak for negative control sample indicating the lack of primer dimers. The melting temperature of each product size varied within the range of 80 °C for *Plectus* and *Ceratoplectus* and 87 °C for *Alaimus* primers (Fig. 2). The melt curve was identical in all cases when amplifying from a single nematode or for the respective genus in a mix of many different nematodes. The linear slope of the Standard curve plot for the 12 specific primers designed ranged between -3.1 and -3.6 and the reaction efficiency was between 90 and 110 %, which is acceptable (Stratagene 2009). This indicated that the primers

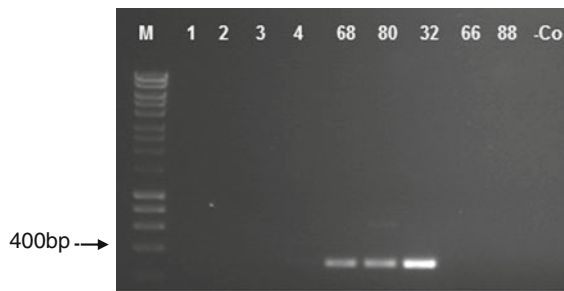


Fig. 3 Agarose gel electrophoresis of PCR products of nematode DNA amplified using primer pairs designed for *Rotylenchulus* sp, lane M is hyper ladder, 1–4 CFT pooled DNA, lane 5–7 are single nematode DNA for *Rotylenchulus* sp, lane 8 is *Rotylenchulus* DNA, lane 9 is *Acrobeloides* DNA, –Co is negative control (water)

designed had a good linear of fit and therefore of high efficiency.

Confirmation molecular barcode with morphological identification

The positive primers from qPCR, were used to prove success of the molecular method developed, this was confirmed by amplification products obtained with the pooled DNA samples and single nematode DNA of the genera from which the primer was designed (Fig. 3). No amplification was obtained with the negative samples (DNA confirmed by sequencing for closer genera in the phylogenetic tree).

Discussion

The objective of this study was to develop an environmental monitoring tool to evaluate the effect of transgenic bananas on unintended species using nematodes as indicators of any change in soil quality. Using the nematode-universal amplification primers, the 18S rRNA gene from soil nematode samples, we were able to obtain PCR fragments and sequences from nematodes for a wide range of diversity in the soil creating a DNA sequence profile for Ugandan soil which could be used to aid identification.

The sequence information gained allowed the design of 12 genus specific primers that identified over 90 % of individuals to either genus or CP value. The variation that existed amongst the 18S rRNA made it possible to design genus specific primers

though some sequences of those nematodes with the same feeding habits and same CP value, had such minimal differences that it was not possible to design a specific primer. The primers cover the different CP groups from the opportunist nematodes that are very sensitive to disturbance in the soil such as *Dorylaimoides* (Table 1). The specific primers for individual genera or closely related nematode genera with the same CP value were reliable with both individual nematodes and a pool of nematode DNA. These primers can now be used to quantify the number of nematodes in the soil sample using one abundant genus in the sample such as *Acrobeloides* sampled from different locations and at different times to generate a reference value. The estimated number of individuals per genus could be used in the standard faunal analysis approach of Ferris et al. (2001). This provides a basis to detect changes in the nematode soil community due to unintended effects of transgenic plants or any other disturbance to the environment. This approach uses weighted indices based on abundance for each CP value from which the extent of soil disturbance, enrichment, the decomposition channels, C:N ratio and food web condition can be inferred.

Our expectation is that the genus specific primers will support rapid assessments of any unintended environment impact of transgenic crops on the soil community by avoiding the need for morphological identification. The approach may have additional value for both extension and regulatory scientists in allowing identifying the presence of important pathogens such as *Radopholus similis* in soil samples even when an experienced nematode taxonomist is unavailable. This would support the development of agriculture in Uganda. A larger database of the 18S rRNA gene barcodes is now being accumulated at the National Agricultural Research Laboratories, Kwanada (NARL) to realize this potential. A key issue is if the benefits of this rapid approach outweigh its higher cost relative to morphologically based identification of nematode genera. The first approach of nematode molecular barcoding in Uganda will be to determine any impact of transgenic banana trials on the soil nematode community and hence soil quality.

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