

# USE OF MOLECULAR BIOLOGICAL METHODS TO IDENTIFY PLANT PARASITIC NEMATODES ASSOCIATED WITH SUGARCANE IN SOUTH AFRICA

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## Abstract

Plant parasitic nematode genera commonly found associated with sugarcane were collected from trial sites in various regions of the sugar industry. Nucleic acids were extracted and the rDNA internal transcribed spacer 1 (ITS1) region amplified. Identification of the important genera was possible by size differentiation of the amplification products. Sequencing of the ITS1 region was used to confirm the identity of the isolates, by comparison with sequences in GenBank.

*Keywords:* sugarcane, nematodes, PCR, rDNA, ITS1 region

## Introduction

Plant parasitic nematodes are an important growth constraint to sugarcane. Current methods of identifying and enumerating nematodes to study their biodiversity in soil and roots are based on the use of morphological characteristics. However, these methods are labour-intensive, require experienced personnel and sophisticated microscopes. With the recent advances in molecular biology, powerful new methods, such as the polymerase chain reaction (PCR), have been developed. These techniques have been widely used for the diagnosis of viral, bacterial and fungal pathogens. The aim of this work was to develop a DNA extraction and PCR method for identifying plant parasitic nematodes associated with sugarcane.

## Materials and methods

### *Biological material*

Nematode genera commonly found associated with sugarcane were collected from nematology trial sites in various regions of the sugar industry (Mpumalanga, North Coast, Midlands).

### *DNA extraction methods*

Six extraction buffers (Appendix 1) frequently used for extracting nucleic acid from nematodes were tested. Genomic DNA was extracted from 10 juveniles of *Meloidogyne* by crushing in 100 ul buffer, adding 200 ug/ml proteinase K (Roche), freezing at -80°C for 10 min, heating at 65°C for 1 hour then 94°C for 10 min, high speed centrifugation and then collecting the supernatant. An alternative method using Chelex-100™ was initially tested on 10 juveniles each of *Helicotylenchus* and *Xiphinema*. DNA was extracted by crushing nematodes in 40 ul 1x TE pH 8.0, adding 10 ul of 20% Chelex-100™ (Bio-Rad), vortexing,

heating to 95°C for 5 min and collecting the supernatant after high-speed centrifugation. Based on the results obtained, the Chelex-100™ was the method of choice for further DNA extractions.

#### PCR

A 10 ul aliquot of extracted DNA (~20 ng) was used, and the Internal Transcribed Spacer 1 (ITS1) region of rDNA amplified. The primers (18S: 5' TTGATTACGTCCCTGCCCTTT 3' (Vrain *et al.*, 1992) and 5.8S: 5' ACGAGCCGAGTGATC CACCG 3' (Cherry *et al.*, 1997)) amplify all organisms in the phylum Nematoda. The PCR cycling conditions were: 94°C for 3 min (1 cycle), 94°C denaturing for 1 min, 57°C annealing for 1 min, 72°C extension for 1 min (30 cycles) followed by a final extension at 72°C for 10 minutes. The amplification products were electrophoresed on 1% agarose gels and viewed using a UV transilluminator.

#### Sequencing of nematode genera

Amplification products were extracted from agarose gels, purified using the QIAquick Gel Extraction Kit (Qiagen Ltd) and prepared for sequencing using the Big-Dye Sequencing Terminator Kit (Applied Biosystems). Sequences obtained were compared to sequences in GenBank using the BLAST search protocol ([www.ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)).

### Results and discussion

#### DNA extraction methods

Two DNA extraction buffers (3 and 6) yielded no DNA, two (2 and 4) yielded DNA <100 ng/ul, and two (1 and 5) yielded DNA ~300 ng/ul. When tested on a number of other nematode genera, buffer 1 produced >1000 ng/ul, buffer 4 >300 ng/ul and buffer 2 <100 ng/ul (Table 1).

**Table 1. Quantity of DNA extracted from six nematode genera using three different DNA extraction buffers.**

Genera	Buffer	DNA Concentration (ng/ul)
<i>Pratylenchus</i>	Solution 1	1263
	Solution 2	84
	Solution 4	362
<i>Helicotylenchus</i>	Solution 1	1203
	Solution 2	86
	Solution 4	366
<i>Xiphinema</i>	Solution 1	1141
	Solution 2	67
	Solution 4	312
<i>Paratrichodorus</i>	Solution 1	1489
	Solution 2	90
	Solution 4	463
<i>Criconemella</i>	Solution 1	1217
	Solution 2	87
	Solution 4	572
<i>Tylenchorhynchus</i>	Solution 1	1481
	Solution 2	280
	Solution 4	568

Interestingly, the amount of DNA extracted by each of the three buffers was relatively constant, irrespective of nematode size or genus. The Chelex-100™ method extracted considerably less DNA (2-4 ng/ul) than that of the abovementioned methods. However, 20 ng was sufficient for amplification, and the resulting amplification products were much cleaner (suggesting less non-specificity). This method is also faster, simpler and cheaper, and was adopted as the preferred method for all further molecular work.

#### *PCR and sequence analysis*

Amplification at an annealing temperature of 55°C produced many non-specific bands. However, increasing the temperature to 57°C reduced non-specific amplification. Observable ITS1 size variation existed between the different nematode genera (Table 2). The size of the amplification products for genera from sugarcane are similar to those reported in the literature for the same genera from other crops. Of interest is that certain genera such as *Pratylenchus* and *Paratrichodorus* often produced two amplification products per reaction. Sequence analysis of the 500 and 700 bp fragments for *Pratylenchus* showed that both bands were of nematode origin and were most similar to that of *Pratylenchus penetrans* from Easter Lillies in the USA (AY286308). Similarly for *Paratrichodorus*, both bands (900 and 1200 bp) were of nematode origin and were most closely related to *Paratrichodorus anemonae* (AF036600). Comparing sequences of genera from sugarcane to reference isolates in GenBank was difficult, as few sequences are available in the database for many of the genera. Currently, the total number of sequences available in GenBank, for all gene regions of all nematodes are: *Pratylenchus* (2013), *Helicotylenchus* (0), *Meloidogyne* (66482), *Xiphinema* (54), *Paratrichodorus* (13) and *Criconebella* (0). This number is reduced even further when searching for sequences specific to the ITS1 region of nematodes (72).

**Table 2. ITS1 size variation between different plant parasitic nematode genera.**

Genera	Size (bp)*	Size (bp)**
<i>Pratylenchus</i>	500/700	650 (spp dependent)
<i>Helicotylenchus</i>	900	800
<i>Meloidogyne</i>	400	410
<i>Xiphinema</i>	>1000	850-1100
<i>Paratrichodorus</i>	900/1200	1200
<i>Criconebella</i>	700	600
<i>Tylenchorhynchus</i>	700	700 (spp dependent)
<i>Longidorus</i>	>1000	1200
<i>Scutellonema</i>	900	900
<i>Hoplolaimus</i>	900	750
<i>Hemicycliophora</i>	650	?

\*Experimental data \*\*Literature data (Powers *et al.*, 1997)

The work reported here represents the initial stages of the project, to characterise nematode genera by means of their ITS1 region. Future work will involve in-depth analysis of the available sequence data. With that information, genera-specific primers will be designed for the development of a diagnostic method for the identification and eventual quantification of nematodes in soil and root samples.

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## Appendix 1

Buffer 1: Waeyenberge *et al.*, 2000.

Buffer 2: Nguyen *et al.*, 2001.

Buffer 3: Li *et al.*, 1996.

Buffer 4: Subbotin *et al.*, 2000.

Buffer 5: Iwahori *et al.*, 1997.

Buffer 6: Fullando *et al.*, 1997.

