Australasian Plant Pathology (1998) 27: 112-115

Non-manual lysis of second-stage *Meloidogyne* juveniles for identification of pure and mixed samples based on the polymerase chain reaction

J.M. Stanton, C.D. McNicol and V. Steele

Plant Protection Unit, Department of Primary Industries, Meiers Road, Indooroopilly, Queensland 4068 Australia and Co-operative Research Centre for Tropical Plant Pathology, The University of Queensland, Queensland 4072 Australia

Corresponding author: J.M. Stanton (Email stantoj@dpi.qld.gov.au)

Abstract

Non-manual methods of lysing single second-stage juveniles (J2s) of *Meloidogyne* and direct squashing of nematodes were assessed for consistency by the success of subsequent amplification of mitochondrial DNA by polymerase chain reaction. Microwave heating and boiling resulted in amplification of DNA from only 10% of J2s; treatment with proteinase K, 20%; direct squashing, 50%; and 24 h incubation in NaOH, 81%. Components of mixtures of mtDNA types could be detected consistently by DNA amplification only if they constituted at least 30% of the mixture.

Additional keywords: root-knot nematodes, diagnostics

Introduction

Root-knot nematode (*Meloidogyne*) is a major pest of most horticultural crops grown in Australia and worldwide. It causes an estimated 12% yield loss even with control strategies which often include routine use of chemical nematicides (Sasser and Freckman 1987). With the trend away from chemical pesticides comes a need to identify nematodes in order to apply species- and race-specific methods of non-chemical control, including cultivar resistance, crop rotation and biological control.

Hugall *et al.* (1994) described ten mtDNA types in Australian populations of *Meloidogyne*. A test based on the polymerase chain reaction (PCR) was developed (Stanton *et al.* 1997) to distinguish between seven of these mtDNA types; these groups correspond to *M. arenaria* (mtDNA types A and C/H), *M. incognita* (B), *M. javanica* (D), *M. hispanica* (G) and two groups of *M. hapla* (E/F and I/J). This test involves simultaneous amplification of mtDNA with four primers, followed by digestion of the two PCR products with either *Hin*fI or *Mnl*I to produce diagnostic fragments. The fragments produced for the most common species are shown in Table 1.

This test now allows rapid and accurate identification of all agriculturally important *Meloidogyne* species found in Australia (Hugall *et al.* 1994). However, this and comparable tests (e.g. Power and Harris 1993) require lysis of nematodes to release DNA before amplification. Adult female *Meloidogyne* are readily lysed by squashing with disposable pestles. However, second-stage juveniles (J2s), which is the stage found in soil, have proven more difficult to lyse. The most successful lysis methods involve manual disruption of individuals, which is time-consuming, tedious and inconsistent. Harris *et al.* (1990) found that squashing J2s with micro-pipette tips resulted in amplification from fewer than 50% of J2s and this was not improved by subsequent boiling for 10 min, incubation in proteinase K or freezing and thawing. V. Williamson (personal communication) described a technique which allowed consistent lysis in the PCR tube but also required manual disruption of the juvenile for best results. A non-manual method will be necessary in developing a routine PCR-based technique to detect nematodes in soil or to quantify soilborne or extracted nematodes.

We have tested a range of methods for their ability to lyse *Meloidogyne* J2s for PCR amplification of DNA quickly, consistently and with little manipulation. We tested several methods initially under a range of conditions for their potential. We then tested the most promising method more intensively for its consistency.

Meloidogyne frequently occurs in mixtures of species in a population (e.g. Stanton *et al.* 1992). It has been suggested that components of mixtures could be elucidated using the PCR-based mtDNA diagnostic test (Stanton *et al.* 1997) but this has not been tested extensively. In this study, we used the most consistent lysis method to assess the sensitivity of the diagnostic test in determining components of mixtures in various proportions.

Methods

Nematode culture Nematode populations were maintained as single eggmass cultures on tomato (*Lycopersicon esculentum* L.) cv. Tiny Tim in the glasshouse. For all experiments, freshly-hatched second-stage juveniles (J2s) were picked by hand from a suspension and dispensed into 1.5 mL Eppendorf tubes.

Lysis The following methods were tested for their ability to lyse second-stage juveniles.

(a) One or ten J2s were added to 30 µL 2% Triton X–100 and heated for 10, 20, 30, 60, 120, 300 or 600 sec at 20, 50, 70 or 100% power in a 650 W Sanyo microwave oven. A total of 187 nematodes was tested.

(b) Six single J2s were each added to 30 µL 2% Triton X–100 and heated in a water bath to 50, 70 or 100°C for 2 or 5 min, while two tubes containing ten J2s each were boiled for 2 or 10 min.

(c) A method described by V. Williamson (personal communication) was modified by omitting manual disruption of juveniles. Single J2s were added to 2.5 µL lysis buffer (1 x PCR buffer (Gibco-BRL), 60 µg/mL proteinase K) in 0.2 mL Eppendorf tubes. They were kept at –70°C for 10 min and then overlaid with oil. Tubes were heated in a thermocycler at 60°C for 1 h, then 95°C for 15 min. The rest of the PCR reagents (22 µL) were injected below the oil and amplification proceeded as described below (total volume 24.5 µL). A total of 56 nematodes was tested.

(d) Thirty single J2s were placed on a glass slide in 30µL 2% Triton X–100 and squashed with a micropipette tip before transferring the extract to an Eppendorf tube.

(e) This is a modification of a method described by Klimyuk *et al.* (1993). Single J2s, replicated ten times, were incubated separately at 25°C in 20µL 0.25 M NaOH for either 5 min or 24 h. After further incubation in a thermocycler at 99°C for 2 min, 10 µL 0.25 MHCl, 5 µL 0.5 M Tris-HCl, pH 8.0, and 5 µL 2% Triton X–100 were added and incubated for another 2 min. The test with 24 h incubation was repeated another six times to test its consistency.

Lysis was assessed by the success of subsequent amplification of lysed juvenile extract using the mtDNA diagnostic test (Stanton *et al.* 1997).

Amplification conditions Amplification reactions contained 1 µL of lysed juvenile extract, 0.4 µM each

Table 1 Sizes (bp) of mtDNA amplification products of second-stage juveniles of *Meloidogyne* spp. with the primers MORF and MTHIS, and sizes of fragments resulting from digestion with *Hin*fI and *Mnl*I of the 557 bp amplification product with the primers TRNAH and MRH106[A]

Primer pair	MtDNA type				
	A	B	C	D	E
MORF-MTHIS product	214	743	743	743	–[B]
TRNAH-MRH106 product cut with *Hin*fI	445	396	445	557	*ca.* 443[C]
	112	112	112		111
		49			
TRNAH-MRH106 product cut with *Mnl*I	346	346	423	346	*ca.* 554[C]
	134	211	134	134	
	77			77	

[A]Data derived from Stanton *et al.* (1997).

[B]No amplification with these primers.

[C]Amplification product in mtDNA type E is 553–555 bp resulting in *Hin*fI digestion fragments of 442–444 bp.

of primers MORF, TRNAH and MRH106, 0.2 μM of primer MTHIS (Stanton *et al.* 1997), 10 μM of each dNTP, 1 unit of *Taq* DNA polymerase I (Gibco BRL), 50 mM $MgCl_2$ in a final volume of 24.5 μL in the supplied buffer. Amplification occurred during one cycle of 3 min at 94°C then 32 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 52°C and 70 sec extension at 70°C followed by one cycle of 2 min at 72°C. In all cases, appropriate positive and negative controls were used. PCR products were separated by electrophoresis on a 2% agarose gel, stained with 0.5 % ethidium bromide and viewed under UV light.

Detection of components of mixtures Nematode mixtures tested were mtDNA types C (population 104 of *M. arenaria*), D (population 35 of *M. javanica*) and E (population 113 of *M. hapla*) in the following proportions; 1 J2:9 J2s, 3:7, 5:5, 7:3 and 9:1. Appropriate numbers of J2s were placed in each tube and were lysed in NaOH with 24 h incubation as described above in method (e). Following amplification of 1 μL of the lysed mixture, 3 μL of each PCR product was digested with *Hin*fI or *Mnl*I (Biolab New England) following the manufacturer's protocol. The resulting fragments were separated on a 2% agarose gel as above.

Results and Discussion

No lysis method allowed subsequent amplification from all nematode extracts (Table 2). The most consistent amplification followed lysis in NaOH for 24 h (method (e)) with amplification from an average of 81% of individuals (range 50–100% in different experiments, standard deviation = 17.5). In this laboratory, we have also found that similar amplification resulted from incubation for 1–5 days in NaOH but incubation for more than 7 days resulted in no amplification (unpublished data). No amplification followed lysis with only 5 min incubation in NaOH.

Lysis method (c) requires manual disruption of the nematode for best results (V. Williamson, personal communication). However, such treatment involves manipulation of individual nematodes which is time-consuming and so was not used further in our study. Without squashing the nematodes, only 20% were lysed sufficiently for amplification (Table 2).

In the mixtures tested (Table 3), mtDNA types were detected only if they were present as at least 30% of the mixture, i.e. the minor components of the 1:9 mixtures were not detected except for C in the D:C mixture. For several reasons, a diagnosis of mixture components was not always possible. Firstly, the *ca.* 557 bp bands may have resulted from incomplete digestion of the 557 bp PCR product. Secondly, it was not possible to distinguish between bands of 442–444 bp in mtDNA type E and 445 bp in mtDNA type C or between 553–555 in mtDNA type E and 557 bp in mtDNA types C and D. Improved resolution on polyacrylamide gels may solve this problem. Thirdly, there was no diagnostic band for mtDNA type E in mixtures with mtDNA type C so its presence could not be confirmed.

The NaOH lysis method (e) was 81% successful for single J2s (Table 2). Because mixtures often J2s were lysed together, each J2 was present in the same concentration as when a single J2 was lysed separately so a similar per cent lysis was expected. Therefore, when a mtDNA type was present as 50, 30 or 10% of the mixture, there was about 100, 99 and 81% chance, respectively, of at least one J2 of the minor or equal component of the mixtures being

Table 2 DNA amplification with polymerase chain reaction following lysis of single second-stage juveniles of *Meloidogyne javanica*

Lysis method[A]	n	Per cent J2s amplified
(a) Microwaving (30 sec at 100% power)[B]	11	10
(b) Boiling	26	10
(c) Proteinase K incubation	56	20
(d) Squashing	30	50
(e) Sodium hydroxide (24 h incubation)[B]	70	81

[A]Methods are described in text.
[B]Results of the best treatment conditions only are shown. These conditions were the most consistent of those tested. In these cases, n is the number of nematodes tested under these conditions.

Table 3 Detection of components of three mixtures of mtDNA types of *Meloidogyne* spp. by amplification of second-stage juveniles lysed with sodium hydroxide followed by digestion with *Hin*fI or *Mn*lI

MtDNA type mixture[A]	Mixture ratio 9:1	7:3	5:5	3:7	1:9
D:C	D,C[B]	D	D,C	C	C
D:E	D	D,E	D,E	E	E
C:E	C	C	–	C	–

[A]Mixture ratios indicate the number of second-stage juveniles of each mtDNA type added to the lysis reaction.
[B]Components detected.

lysed sufficiently for detection. However, as minor or equal components were not detected in about 30% of cases, lack of detection is not likely to be due solely to poor lysis. Perhaps this situation may be explained by competition between components during amplification.

Because of the poor ability to detect the mtDNA type of a nematode in mixtures when in low concentration, it would be more sensitive to amplify DNA from a number of single females or J2s separately.

Acknowledgements

This work was funded by Rural Industries Research and Development Corporation and Co-operative Research Centre for Tropical Plant Pathology.

References

Harris, T.S., Sandall, L.J. and Powers, T.O. (1990) – Identification of single *Meloidogyne* juveniles by polymerase chain reaction amplification of mitochondrial DNA. *Journal of Nematology* **22**: 518-524.

Hugall, A., Moritz, C., Stanton, J. and Wolstenholme, D. (1994) – Low, but strongly structured mitochondrial DNA diversity in root knot nematodes (*Meloidogyne*). *Genetics* **136**: 903-912.

Klimyuk, V.I., Carroll, B.J., Thomas, C.M. and Jones, J.D.G. (1993) – Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *The Plant Journal* **3**: 493-494.

Powers, T.O. and Harris, T.S. (1993) – A polymerase chain reaction method for identification of five major *Meloidogyne* species. *Journal of Nematology* **25**: 1-6.

Sasser, J.N. and Freckman, D.W. (1987) – A world perspective on nematology: the role of the Society In *Vistas on Nematology* (Eds J.A. Veech and D.W. Dickson), pp. 7-14. Society of Nematologists Inc. Hyattsville.

Stanton, J., Hugall, A. and Moritz, C. (1997) – Nucleotide polymorphisms and an improved PCR-based mtDNA diagnostic for parthenogenetic root-knot nematodes (*Meloidogyne* spp.). *Fundamental and Applied Nematology* **20**: 261-268.

Stanton, J.M., O'Brien, P.C. and Schipke, L.G. (1992) – Species of root-knot nematode (*Meloidogyne* spp.) affecting tobacco in north Queensland, including two new host races of *M. arenaria*. *Australasian Plant Pathology* **21**: 150-157.

Manuscript received 22 July 1997, accepted 19 March 1998.