

Multiple displacement amplification (MDA) of total genomic DNA from *Meloidogyne* spp. and comparison to crude DNA extracts in PCR of ITS1, 28S D2-D3 rDNA and Hsp90

Andrea M. SKANTAR* and Lynn K. CARTA

USDA-ARS Nematology Laboratory, 10300 Baltimore Avenue, Beltsville, MD 20705, USA

Received: 4 January 2005; revised: 10 March 2005

Accepted for publication: 10 March 2005

Summary – Because the quantity of nematode specimens available for molecular analysis is often limited, the number of analyses possible is constrained by the availability of DNA. Multiple displacement amplification (MDA) was assessed for whole genome amplification of crude DNA from several *Meloidogyne* species. MDA produced microgram quantities of template that resulted in successful amplification of the ribosomal internal transcribed spacer (ITS1) and 28S D2-D3 expansion regions, producing PCR results that were comparable to template generated by the single nematode smash method. MDA greatly improved degenerate primer PCR of single-copy Hsp90, a gene which is more sensitive than multi-copy ribosomal genes to limited DNA template. MDA should expand the number of molecular analyses possible for single nematodes. MDA will also be useful for archiving DNA from valuable specimens and provide a way for laboratories to share identical genetic material for nematode diagnosis.

Keywords – diagnosis, *Meloidogyne arenaria*, *M. chitwoodi*, *M. floridensis*, *M. hapla*, *M. incognita*, *M. javanica*, molecular identification, phylogenetics.

With international trade on the increase and the heightened awareness of global pathogen spread, there comes an urgent need for ways to monitor quickly and accurately the movement of nematodes of agricultural concern. Root-knot nematodes (*Meloidogyne* species) attack a wide range of crops and are known to cause serious economic damage throughout the world. The absence of accurate diagnostic tests sensitive enough to distinguish root-knot nematodes from one another hinders efforts to control the spread of highly damaging or invasive species.

Morphological identification of nematodes (see review by Eisenback and Triantaphyllou, 1991) provides a critical line of defence in preventing the introduction of new nematode pests, but a great deal of skill and expertise is required for effective interpretation of the subtle variations that often occur among individuals in a population. Molecular methods useful for distinguishing *Meloidogyne* spp. include isozyme analysis (Esbenshade & Triantaphyllou, 1985), RAPDs (Cenis, 1993; Baum *et al.*, 1994; Blok *et al.*, 1997a), PCR-RFLPs (Harris *et al.*, 1990; Powers & Harris, 1993; Hugall *et al.*, 1994) and sequence differ-

ences within ribosomal (Zijlstra *et al.*, 1995, 1997; Blok *et al.*, 1997b; Wishart *et al.*, 2002; Chen *et al.*, 2003) or mitochondrial DNA (Blok *et al.*, 2002). Some molecular diagnostic tests are straightforward to perform but limited in capacity to discriminate species; others are specific but more time consuming to perform. While new assays based upon single copy genes would be welcome additions to plant-parasitic nematode diagnostics and phylogeny, only a few have been introduced, including RNA polymerase II (Baldwin *et al.*, 1997), major sperm protein (MSP) (Setterquist *et al.*, 1996) and Hsp70 (Beckenbach *et al.*, 1992; Hashmi *et al.*, 1997). A technical barrier to such developments is imposed by the small size of many plant-parasitic nematode juveniles, which severely limits the amount of genetic material available for testing. DNA extracted from the popular ‘single nematode smash’ procedure (Powers & Harris, 1993) is often consumed entirely by a single PCR reaction, preventing the replication of results or the ability to perform more than one assay upon an individual. Using pools of nematodes alleviates the limit on available DNA and allows PCR reactions to be replicated, but con-

* Corresponding author, e-mail: skantara@ba.ars.usda.gov

strains the ability to address questions involving intraspecific variation.

Within the past decade, a number of methods have been developed for whole genome amplification (WGA) from limited amounts of starting material (reviewed in Lasken and Egholm, 2003). These advances promise, by producing vast quantities of genomic DNA from minimal starting material, to revolutionise the development of novel molecular diagnostics for rare biological specimens. Multiple displacement amplification (MDA) is a non-PCR method developed by Dean *et al.* (2001, 2002) which includes the bacteriophage ϕ 29 DNA polymerase and exonuclease-resistant, thiophosphate-modified, degenerate hexamers to amplify genomic DNA from crude or pure sources. Unlike PCR methods that employ thermostable DNA polymerases, ϕ 29 DNA polymerase replicates DNA at a constant temperature of 30°C. The major advantages attributed to MDA compared to PCR-based methods include more complete genome coverage and unbiased amplification (Dean *et al.*, 2002). The high fidelity and accuracy of MDA has been established in genotyping and SSCP mutation assays of human DNA samples that varied in quality and GC content (Yan *et al.*, 2004). Evaluation of single nucleotide polymorphisms (SNPs) performed on MDA from clinical specimens revealed 99.82% complete genome representation and a direct sequencing error rate similar to that of unamplified genomic DNA (Paez *et al.*, 2004). While MDA has recently been applied to other microbes (Gorrochotegui-Escalante & Black, 2003; Jeyaprakash & Hoy, 2004), it has not yet been tested for nematodes.

Recently, we introduced Hsp90 as a new molecule for nematode phylogeny (Skantar & Carta, 2005). Because Hsp90 is single copy in nematodes (Birnbay *et al.*, 2000; Skantar & Carta, 2005; Scholl & Bird, pers. comm.), successful amplification of this gene from uncharacterised specimens is highly dependent upon the quantity and quality of DNA template. The aim of this study was to facilitate Hsp90 amplification from a limited number of specimens available for nematode molecular diagnostics. Our first objective was to assess the PCR performance of MDA DNA template relative to DNA obtained by a crude extraction method widely used in plant-parasitic nematology (Powers & Harris, 1993). The second objective was to employ MDA DNA for the improvement of Hsp90 amplification from root-knot nematodes.

Materials and methods

NEMATODE ISOLATES

The present study consisted of seven root-knot nematode species that had previously been verified by morphology, morphometrics and molecular characters. The origin of nematodes were: *Meloidogyne arenaria* (North Carolina State population 156), Eastern VA, USA; *M. incognita*, Salisbury, MD, USA; *M. javanica*, Tifton, GA, USA; *M. hapla*, Maui, Hawaii; *M. chitwoodi*, Parma, ID, USA; *M. floridensis*, Gainesville, FL, USA; and *M. mayaguensis*, Palm Beach County, FL, USA. Groups of nine nematode juveniles were mechanically disrupted in 50 μ l; two juveniles or single *Meloidogyne chitwoodi* females were disrupted in 20 μ l extraction buffer as described in Thomas *et al.* (1997), and then stored in PCR tubes at -80°C. Extracts were prepared from thawed pools by incubating the tubes at 60°C for 60 min, followed by 95°C for 15 min to deactivate the proteinase K. Extracts were stored at -20°C when not in use.

MULTIPLE DISPLACEMENT AMPLIFICATIONS

The Genomiphi (MDA) kit (Amersham, Piscataway, NJ, USA) was used for multiple displacement amplification of nematode DNA according to the manufacturer's instructions. Genomiphi reactions were performed using crude extract (CE) from single or pooled nematodes as described above. In each case, reactions containing 1 μ l nematode CE was combined with 9 μ l sample buffer, heated to 95°C for 3 min, then chilled at 4°C. To each tube, 9 μ l of reaction buffer and 1 μ l Genomiphi enzyme mix were added and the reactions incubated at 30°C for 16 h. The amplified genomic DNA was ethanol precipitated according to the kit manufacturer's recommendations and resuspended in 20 μ l 10 mM Tris, pH 8.0. DNA preparations were stored at -20°C and used for PCR reactions as described below.

PCR REACTIONS

All PCR reactions contained Eppendorf HotMasterTaq (Brinkmann, Westbury, NY, USA) and the buffer supplied by the manufacturer; all other components were added in the proportions described in the protocols for each gene. DNA templates for each experiment included 5, 2, 1, 0.5 or 0.1 μ l CE or 2, 1, 0.5, 0.1 or 0.01 μ l MDA DNA, with volumes less than 1 μ l generated by serial dilution. In addition, no-template control reactions were routinely performed. All PCR primers were synthesised by

Sigma-Genosys (The Woodlands, TX, USA). The nuclear ribosomal internal transcribed spacer (ITS1) segment was amplified with the primers rDNA2 5'-TTGATTACGTC-CCTGCCCTTT-3' (Vrain *et al.*, 1992) and rDNA1.58S 5'-ACGAGCCGAGTGATCCACCG-3' as described previously (Cherry *et al.*, 1997). The ribosomal LSU D2-D3 expansion segment was amplified with primers D2A 5'-ACAAGTACCGTGAGGGAAAGTTG-3' and D3B 5'-TCGGAAGGAACCAGCTACTA-3' (Courtright *et al.*, 2000) as previously described (Al-Banna *et al.*, 1997). The Hsp90 primers M3F 5'-GCGTCGYATTCGTGAA-GTGG-3' and M2R 5'-ATGGGCTTTGTCTTGTMMAR-CTCTT-3' were based upon an alignment of root-knot nematode Hsp90 sequences (Skantar, unpubl.). Each 25 μ l Hsp90 PCR reaction contained 500 μ M of primers M3F and M2R, 200 μ M each dNTP, plus 1 unit Taq polymerase and the indicated amount of template DNA. PCR reactions were incubated at 94°C for 2 min, followed by 45 cycles of 94°C for 20 s, 58°C for 30 s, and 68°C for 30 s, with a final step of 68°C for 5 min. The second set of Hsp90 primers, U831B 5'-AAYAARACMAAGCCNA-TYTGGAC-3' and L1110B 5'-TCGCARTTCTCCATR-ATCAA-3', were similar to primers described previously (Skantar & Carta, 2000), which were based upon an alignment of *Caenorhabditis elegans*, *Brugia pahangi*, zebrafish, salmon, mouse, and human sequences. Each 25 μ l PCR reaction contained 500 μ M of each of U831B and L1110B, 200 μ M dNTPs, 1 unit Taq polymerase, and the indicated amount of template DNA. PCR reactions were incubated using a modified ramped annealing PCR (Skantar & Carta, 2000): 94°C for 2 min, followed by 45 cycles of 94°C for 20 s, 65°C for 5 s, 60°C for 5 s, 55°C for 5 s, 50°C for 5 s and 68°C for 1 min, and finishing with a 15 min step at 68°C. For all genes examined, 12.5 μ l of each PCR reaction was separated on 1% agarose gels in Tris-Acetate-EDTA (TAE), stained with ethidium bromide, and visualised with UV illumination.

Results

The initial goal was to assess the PCR performance of MDA DNA compared to DNA prepared by the 'nematode smash' method that is commonly used for identification and phylogenetic studies. Successful amplification of Hsp90 is more sensitive to the amount of input genomic DNA than amplification of the ribosomal genes, which are present in the genome in multiple copies. Therefore, it was necessary to use extracts from pools of nematodes in order to raise the level of Hsp90 target template so

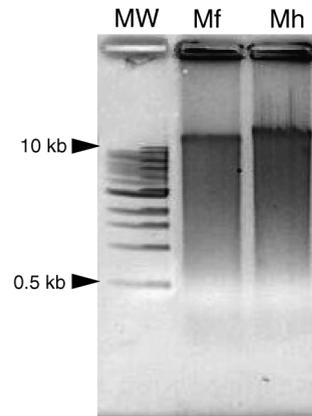


Fig. 1. Size of typical multiple displacement amplification products from nematodes. The 1 kb ladder (MW) was from New England Biolabs (Beverly, MA, USA) and includes fragments of 10, 8, 6, 5, 4, 3, 2, 1.5, 1.0, and 0.5 kb. MDA reactions contained 1 μ l (1/50th volume) of a crude extract from a pool of nine nematodes. Each MDA lane contained 5 μ l out of the 20 μ l MDA reaction mixed with 1 μ l electrophoresis sample buffer. MDA products ranged in size from approximately 0.5 kb to 12 kb. Mf = *Meloidogyne floridensis*; Mh = *Meloidogyne hapla*.

that side-by-side comparisons CE and MDA PCR results could be performed. To simplify the overall analysis and comparisons of results with different genes, pooled extracts of nine J2 nematodes were employed for all of the genes tested.

One μ l of the pooled extract was subjected to multiple displacement amplification and then purified by ethanol precipitation. MDA typically produced microgram quantities of genomic DNA, ranging in size from 0.5-12 kb (Fig. 1). A wide range of template concentrations was examined in order to compare the quantity and quality of PCR products produced. CE DNA was tested over a 50-fold range of DNA concentrations and MDA DNA was tested over a 200-fold range (Fig. 2). For *M. arenaria*, *M. incognita*, and *M. javanica*, the expected *ca* 750 bp ITS1 PCR products were observed at or above 0.5 μ l CE template and at all levels of MDA DNA. For *M. hapla*, 1-5 μ l CE gave detectable PCR products, but lower levels did not. The *M. hapla* MDA reactions gave rise to the expected product at all DNA amounts through 0.1 μ l, except for the lowest amount, 0.01 μ l. However, two other larger products were also observed.

Another experiment using the same template preparations involved amplification of the LSU D2-D3 expansion segments of rDNA (Fig. 3). For *M. arenaria*, *M. incognita* and *M. javanica*, the expected *ca* 800 bp product was generated at most CE levels except the lowest, although

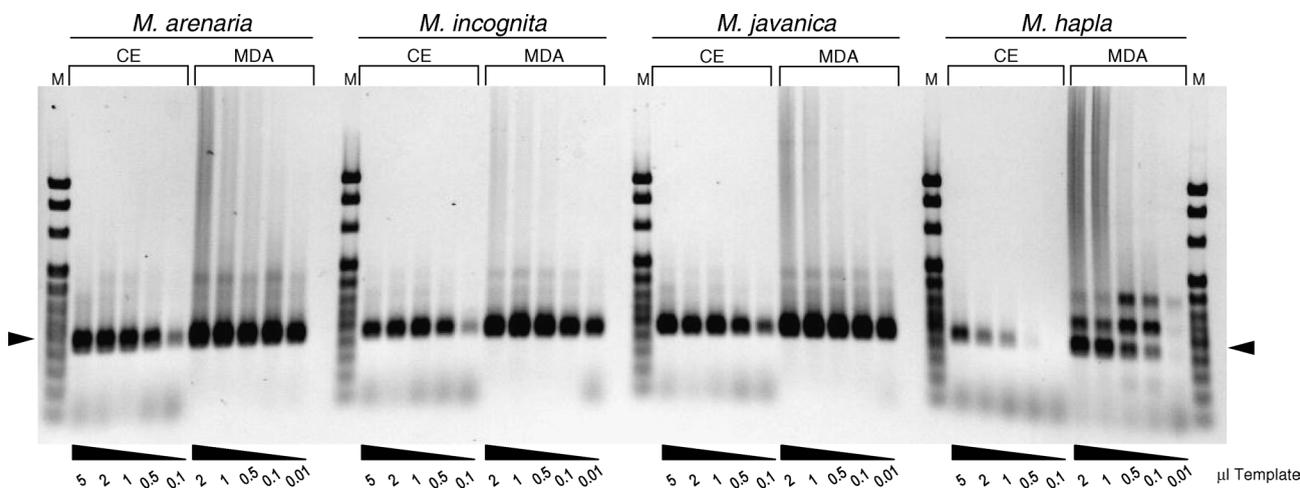


Fig. 2. Comparison of ribosomal ITS1 region amplified from four different root-knot nematodes. DNA templates were either crude extracts (CE) from pools of nine nematodes or MDA DNA generated from 1 μ l (1/50th volume) of the same pooled CE. Agarose gel electrophoresis of PCR products produced from *Meloidogyne arenaria*, *M. incognita*, *M. javanica* and *M. hapla*. Numbers below represent the amount of template DNA in μ l added to 25 μ l PCR reactions. Arrows denote position of expected products. M = 100 bp ladder (New England Biolabs).

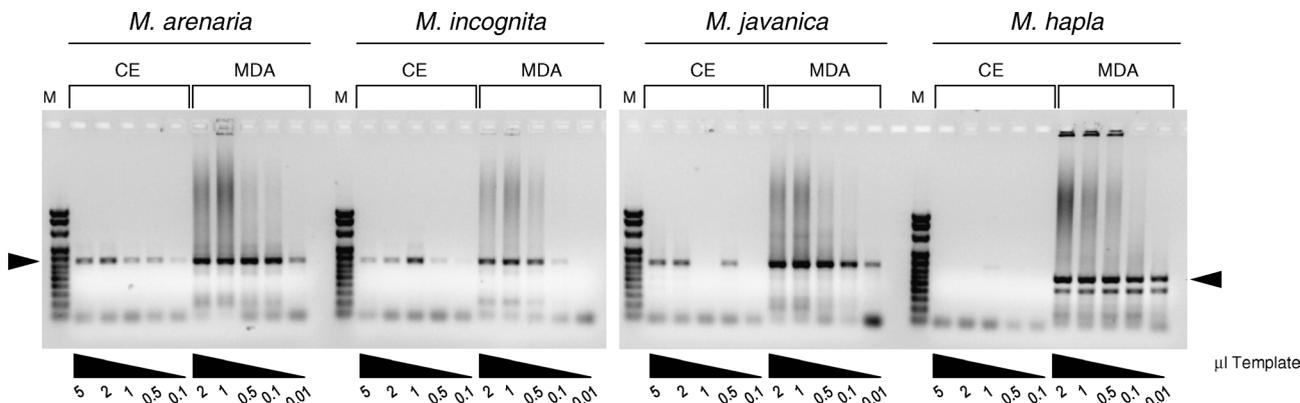


Fig. 3. Comparison of ribosomal 28S D2-D3 expansion segments amplified from four different root-knot nematodes. DNA templates were either crude extracts (CE) from pools of nine nematodes or MDA DNA generated from 1 μ l (1/50th volume) of the same pooled CE. Agarose gel electrophoresis of PCR products produced from *Meloidogyne arenaria*, *M. incognita*, *M. javanica* and *M. hapla*. Numbers below represent the amount of template DNA added to 25 μ l PCR reactions. Arrows denote position of expected PCR products. M = 100 bp ladder; with fragment sizes 1517, 1200, 1000, 900, 800, 700, 600, 517/500 (comigrating bands), 400, 300, 200, and 100 bp (New England Biolabs).

the reactions at each DNA concentration did not generate as much D2-D3 product as ITS1 product. The MDA reactions were stronger over the range of DNA concentrations tested than those containing CE. For *M. hapla*, the CE reactions failed to generate any products. The major MDA D2-D3 PCR product was smaller than for the other root-knot nematodes (*ca* 650 bp); an additional band at *ca* 400 bp was also observed.

A similar side-by-side comparison of CE and MDA dilution series using the U831/L1110 primer pair was not possible due to the poor amplification of Hsp90 with less than 10 μ l CE (not shown). Thus, the root-knot nematode-specific Hsp90 primers, M3F and M2R, were used for this purpose (Fig. 4). With these primers, CE template from most of the root-knot nematodes showed strong amplification of the expected *ca* 300 bp Hsp90

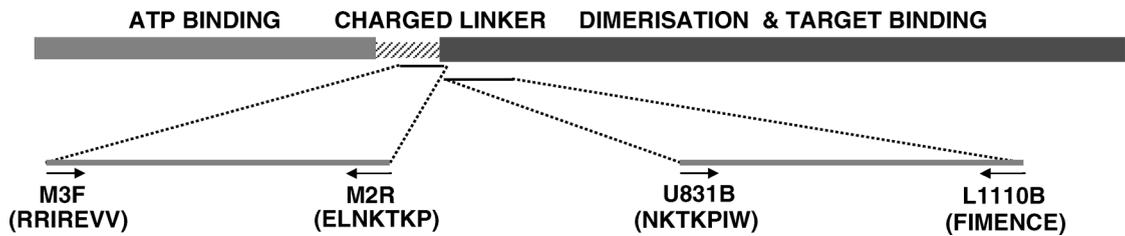


Fig. 4. Schematic diagram of *Hsp90*, with positions of PCR primers M3F, M2R, U831B, and L1110B marked by arrows. The conserved amino acid domains corresponding to the primer sequences are indicated in parentheses.

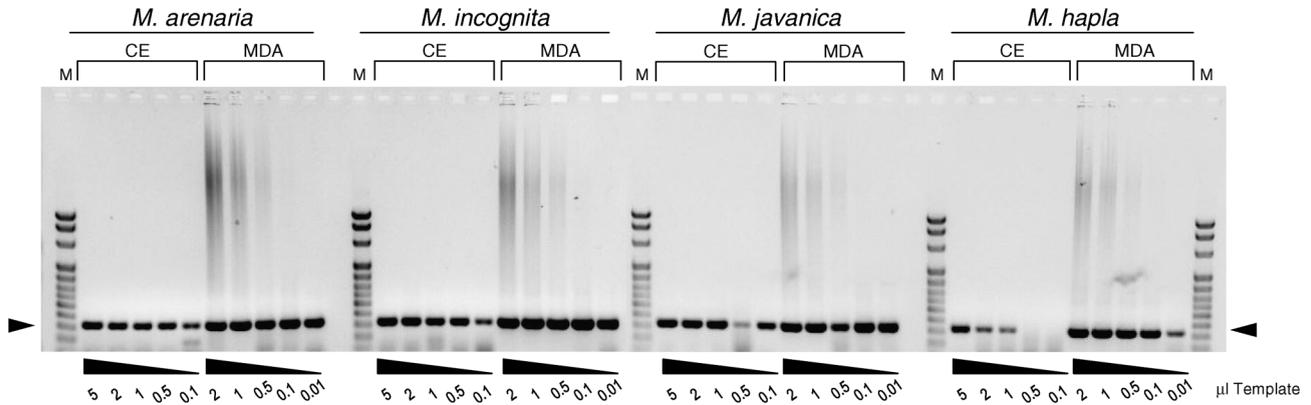


Fig. 5. Comparison of *Hsp90* amplified from four different root-knot nematodes. PCR reactions of 25 μ l included primers M3F and M2R and DNA template from either crude nematode extracts (CE) or multiple displacement amplification (MDA). Agarose gel electrophoresis of PCR products produced from *Meloidogyne arenaria*, *M. incognita*, *M. javanica* and *M. hapla*. Numbers below represent the amount of template DNA added to 25 μ l PCR reactions. Arrows denote position of expected PCR products. M = 100 bp ladder (New England Biolabs).

PCR products. However, the *M. hapla* CE reactions were relatively weaker than the other species reactions at low levels of DNA (Fig. 5). MDA DNA from all four species produced strong PCR bands over the entire 200-fold range of template DNA added.

The next experiment involved an examination of *Hsp90* fragments produced with MDA DNA and the U831B and L1110B primers. MDA DNA was generated from CE prepared from one, two, or nine J2 of several root-knot nematode species. The number of nematodes in a given species pool was purely incidental, reflecting the sometimes limited material obtained as interceptions. *Meloidogyne arenaria*, *M. incognita*, *M. javanica* and *M. hapla* were all included because of their worldwide distribution and importance to agriculture. Three other species of regulatory importance, *M. mayaguensis*, *M. floridensis* and *M. chitwoodi*, were also tested. Strong *Hsp90* amplification was observed in all of the MDA reactions (Fig. 6). Two *Hsp90* PCR products of slightly different size were detected for

M. incognita, but single bands were visible for all other species, with small differences in migration distance apparent for some of them.

Discussion

We have demonstrated that MDA is an effective method of WGA for several species of root-knot nematode, which further suggests that this method will have broad applicability in nematode molecular systematics and identification. MDA produced robust amounts of genomic DNA regardless of the number of nematodes in the crude extract, and worked equally well with mechanically disrupted juveniles and females. MDA from single *M. chitwoodi* females produced micrograms of genomic DNA from as little as 5% of the crude extracts and similar yields were obtained from pools of two to nine juveniles. Because successful PCR was achieved with as low as 0.01 μ l MDA DNA, we estimate that crude extracts subjected to MDA

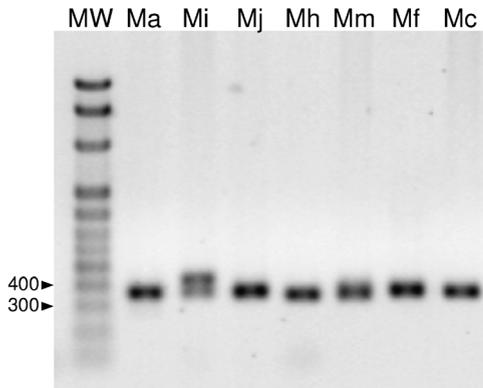


Fig. 6. Agarose gel electrophoresis of *Hsp90* PCR products produced with degenerate primers U831B and L1110B and MDA DNA generated from 1 μ l of 'nematode smash' crude extracts. Extracts from Ma, Mi, Mj and Mh were prepared from pools of nine J2; Mm and Mh were from pools of two J2; Mc was from a single female. Ma = *Meloidogyne arenaria*; Mi = *M. incognita*; Mj = *M. javanica*; Mh = *M. hapla*; Mm = *M. mayaguensis*; Mf = *M. floricidensis*; Mc = *M. chitwoodi*. MW = 100 bp ladder (New England Biolabs).

can produce sufficient template for as many as 10 000 to 40 000 PCR reactions, depending upon the number, size, and stage of nematodes in the starting material, and the gene being amplified. We did not attempt MDA with less than 1 μ l CE, but it is possible that using lower amounts of input CE could extend this estimate even further. The presence of non-DNA contaminants within crude nematode extracts prevented accurate measurements of their DNA concentrations, so it was not possible to directly equate concentrations of CE and MDA genomic DNA. Nevertheless, these experiments provide a practical comparison of PCR results using MDA-produced genomic DNA and the nematode CE volumes typically employed.

The ribosomal internal transcribed spacer ITS1 and the LSU D2-D3 expansion regions are two readily amplified ribosomal genes that have been previously examined from root-knot nematodes (Powers & Harris, 1993; Chen *et al.*, 2003), thus providing an established experimental context in which to evaluate template produced by MDA. The sizes of ITS1 PCR products amplified from MDA DNA were consistent with those reported previously (Powers *et al.*, 1997). For most root-knot species, both CE and MDA templates produced strong bands at all DNA concentrations tested (Fig. 2). As low as 0.01 μ l MDA DNA produced strong ITS1 products that were comparable to or greater than the signals resulting from 0.5 μ l nematode CE. The exception was *M. hapla*, which produced a relatively strong ITS1 signal from 5 μ l CE

DNA that gradually disappeared at lower levels of CE. While high levels of MDA DNA from *M. hapla* produced strong bands at the expected size (ca 450 bp), lower amounts of template produced additional bands at ca 600 and ca 800 bp. The identity of these products is unclear; they could be an artefact of MDA amplification, result from mispriming in the rDNA, or may indicate that the crude DNA preparation used for MDA of *M. hapla* was of lesser quality than for the other nematodes.

The results from the D2-D3 comparison generally gave similar results to the ITS1, although the signal strengths of the D2-D3 products were lower overall (Fig. 3). Some of the CE PCR reactions were higher or lower than expected for serial dilutions of the DNA. It is likely these data points resulted from pipetting errors (Fig. 3; *M. incognita* and *M. javanica*, 1 μ l CE). Again, the *M. hapla* CE and MDA results were not as robust as those from the other nematodes. CE reactions gave no products and MDA templates produced a smaller band in addition to the ca 600 bp D2-D3 band. These reactions were performed with the same CE and MDA DNA as for ITS1, indicating that both genes in *M. hapla* were affected similarly. As with any method involving DNA polymerase activity that replicates input DNA without bias, MDA results can be affected by contamination. Therefore, nematodes used for MDA should be prepared with care to ensure they are free from microbial contamination. The precise reason for the lower quality PCR results with *M. hapla* is not clear but, as a cautionary measure, fresh nematode CE and MDA DNA were prepared for the next set of experiments.

The M3F/M2R *Hsp90* reactions were successful for all concentrations of CE and MDA for all nematodes (Fig. 5), with the exception of the lowest CE levels for *M. hapla*, and the 0.5 μ l CE point for *M. javanica*. M3F (two-fold degenerate) and M2R (four-fold) were designed to amplify *Hsp90* within the genus *Meloidogyne*, with the initial aim of developing this fragment as a diagnostic marker. While the PCR procedure was straightforward, we discovered no apparent size variation in this fragment among the root-knot nematodes (Fig. 5) and a low level of sequence variation among the mitotic parthenogens (data not shown), thus limiting its diagnostic potential for the genus. However, increasing the degeneracy of primers M3F and M2R may yet prove useful for examining variation across a broader range of nematode taxa.

The U831B/L1110B *Hsp90* fragment was of interest because significant sequence variation in this region had been previously detected among several root-knot nematode species (Skantar & Carta, 2005). This variability is

apparent in the slightly different sizes of PCR products (Fig. 6), such as the slower-migrating band for *M. incognita* and the slightly faster migrating bands for *M. arenaria* and *M. hapla*. U831B and L1110B are less degenerate modifications (24-fold and four-fold, respectively) of a previous primer set (Skantar & Carta, 2000). However, even with increased specificity, these primers sometimes required two cycles of amplification to produce strong PCR products from the extracts of single nematodes (Skantar & Carta, 2005). The benefit of MDA for amplification of Hsp90 with these degenerate primers was reflected in the strong products obtained from a single round of PCR. It is likely that adding more genomic DNA increased the number of productive interactions between the degenerate primers and the target gene, leading to higher product yield. Raising the level of crude nematode extracts in Hsp90 PCR reactions generally does not achieve the same advantage (data not shown), as high levels of impure extract may contribute an excess of PCR-inhibiting contaminants. While genomic DNA preparations from bulk quantities of nematodes also give strong Hsp90 PCR products (data not shown), preparing them requires significantly more nematodes, time, and labour than MDA.

The technical improvements shown here should significantly broaden the appeal of Hsp90 and open the door for analysis of other single-copy genes for nematode identification and phylogenetics. The combination of MDA and degenerate primers will facilitate future surveys of Hsp90 across the phylum Nematoda. On a finer scale, with MDA it will be possible to address questions about Hsp90 allelic variation within and among individuals in a population. Whether Hsp90 sequences can be used to develop reliable diagnostics for the major root-knot nematodes or for species of regulatory importance remains to be validated through the examination of multiple populations of these species.

The potential applications of MDA to nematode identification and systematics are far reaching. For instance, with MDA it should be possible to archive the genetic material from individual nematodes, thereby eliminating the need for more labour-intensive culture methods, such as propagating single egg-mass cultures (Hugall *et al.*, 1999). MDA could facilitate the development and production of DNA 'type specimens' that may be shared among scientists. Thus, it would be possible to generate a set of DNA standards that could be widely distributed, providing a practical tool for greater uniformity in nematode molecular diagnostics. Another possible advance result-

ing from MDA is the ability to archive large quantities of genetic material from rare, difficult-to-maintain, or historically valuable specimens. It will now be feasible to perform morphological analysis on a specimen, catalogue its unique features in a digital archive (De Ley & Bert, 2002), and with MDA produce a corresponding genetic archive. Also, MDA provides an attractive alternative to the international or intrastate exchange of live specimens, a practice that presents a number of regulatory challenges and restrictions.

Acknowledgements

We thank Maria Hult, Sharon Ochs and Donna Ellington for technical assistance. We thank the following people for providing nematode isolates: Saad Hafez, *M. chitwoodi*; Janete Brito, *M. mayaguensis*; Andy Nyzcepir, *M. floridensis*; Don Schmitt, *M. hapla*.

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References

- AL-BANNA, L., WILLIAMSON, V. & GARDNER, S.L. (1997). Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. *Molecular Phylogenetics and Evolution* 7, 94-102.
- BALDWIN, J.G., FRISSE, L.M., VIDA, J.T., EDDLEMAN, C.D. & THOMAS, W.K. (1997). An evolutionary framework for the study of developmental evolution in a set of nematodes related to *Caenorhabditis elegans*. *Molecular Phylogenetics and Evolution* 8, 249-259.
- BAUM, T.J., GRESSHOFF, P.M., LEWIS, S.A. & DEAN, R.A. (1994). Characterization and phylogenetic analysis of four root-knot nematode species using DNA amplification fingerprinting and automated polyacrylamide gel electrophoresis. *Molecular Plant-Microbe Interactions* 7, 39-47.
- BECKENBACH, K., SMITH, M.J. & WEBSTER, J.M. (1992). Taxonomic affinities and intra- and interspecific variation in *Bursaphelenchus* spp. as determined by polymerase chain reaction. *Journal of Nematology* 24, 140-147.
- BIRNBY, D.A., LINK, E.M., VOWELS, J.J., TIAN, H., COLACURCIO, P.L. & THOMAS, J.H. (2000). A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in *Caenorhabditis elegans*. *Genetics* 155, 85-104.
- BLOK, V.C., PHILLIPS, M.S., MCNICOL, J.W. & FARGETTE, M. (1997a). Genetic variation in tropical *Meloidogyne* spp.

- as shown by RAPD-PCR. *Fundamental and Applied Nematology* 20, 127-133.
- BLOK, V.C., PHILLIPS, M.S. & FARGETTE, M. (1997b). Comparison of sequences from the ribosomal DNA intergenic region of *Meloidogyne mayaguensis* and other major root-knot nematodes. *Journal of Nematology* 29, 16-22.
- BLOK, V.C., WISHART, J., FARGETTE, M., BERTHIER, K. & PHILLIPS, M.S. (2002). Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology* 4, 773-781.
- CENIS, J.L. (1993). Identification of four major *Meloidogyne* spp. as shown by random amplified polymorphic DNA (RAPD-PCR). *Phytopathology* 83, 76-78.
- CHEN, P., ROBERTS, P.A., METCALF, A.E. & HYMAN, B.C. (2003). Nucleotide substitution patterning within the *Meloidogyne* rDNA D3 region and its evolutionary implications. *Journal of Nematology* 35, 404-410.
- CHERRY, T., SZALANSKI, A.L., TODD, T.C. & POWERS, T.O. (1997). The internal transcribed spacer region of *Belonolaimus* (Nemata: Belonolaimidae). *Journal of Nematology* 29, 23-29.
- COURTRIGHT, E.M., WALL, D.H., VIRGINIA, R.A., FRISSE, L.M., VIDA, J.T. & THOMAS, W.K. (2000). Nuclear and mitochondrial DNA sequence diversity in the Antarctic nematode *Scottinema lindsayae*. *Journal of Nematology* 32, 143-153.
- DEAN, F.B., NELSON, J.R., GIESLER, T.L. & LASKEN, R.S. (2001). Rapid amplification of plasmid and phage DNA using phi29 DNA polymerase and multiply-primed rolling circle amplification. *Genome Research* 11, 1095-1099.
- DEAN, F.B., HOSONO, S., FANG, L., WU, X., FARUQI, A.F., BRAY-WARD, P., SUN, Z., ZONG, Q., DU, Y., DU, J., DRISCOLL, M., SONG, W., KINGSMORE, S.F. & EGHOLM, M. (2002). Comprehensive human genome amplification using multiple displacement amplification. *Proceedings of the National Academy of Sciences, USA* 99, 5261-5266.
- DE LEY, P. & BERT, W. (2002). Video capture and editing as a tool for the storage, distribution, and illustration of morphological characters of nematodes. *Journal of Nematology* 34, 296-302.
- EISENBACK, J.D. & TRIANTAPHYLLOU, H.H. (1991). Root-knot nematodes: *Meloidogyne* species and races. In: Nickle, W.R. (Ed.). *Manual of agricultural nematology*. New York, USA, Marcel Dekker, pp. 191-274.
- ESBENSHADE, P.R. & TRIANTAPHYLLOU, A.C. (1985). Use of enzyme phenotypes for identification of *Meloidogyne* species (Nematoda: Tylenchida). *Journal of Nematology* 17, 6-20.
- GORROCHOTEGUI-ESCALANTE, N. & BLACK IV, W.C. (2003). Amplifying whole insect genomes with multiple displacement amplification. *Insect Molecular Biology* 12, 195-200.
- HARRIS, T.S., SANDALL, L.J. & POWERS, T.O. (1990). Identification of single *Meloidogyne* juveniles by polymerase chain reaction amplification of mitochondrial DNA. *Journal of Nematology* 22, 518-524.
- HASHMI, G., HASHMI, S., SELVAN, S., GREWAL, P.S. & GAUGLER, R. (1997). Polymorphism in heat shock protein gene (*hsp70*) in entomopathogenic nematodes (Rhabditida). *Journal of Thermal Biology* 22, 143-149.
- HUGALL, A., MORITZ, C., STANTON, J. & WOLSTENHOLME, D.R. (1994). Low, but strongly structured mitochondrial DNA diversity in root-knot nematodes (*Meloidogyne*). *Genetics* 136, 903-912.
- HUGALL, A., STANTON, J. & MORITZ, C. (1999). Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. *Molecular Biology and Evolution* 16, 157-164.
- JEYAPRAKASH, A. & HOY, M.A. (2004). Multiple displacement amplification in combination with high-fidelity PCR improves detection of bacteria from single females or eggs of *Metaseiulus occidentalis* (Nesbitt) (Acari: Phytoseiidae). *Journal of Invertebrate Pathology* 86, 111-116.
- LASKEN, R.S. & EGHOLM, M. (2003). Whole genome amplification: abundant supplies of DNA from precious samples or clinical specimens. *Trends in Biotechnology* 21, 531-535.
- PAEZ, J.G., LIN, M., BEROUKHIM, R., LEE, J.C., ZHAO, X., RICHTER, D.J., GABRIEL, S., HERMAN, P., SASAKI, H., ALTSCHULER, D., LI, C., MEYERSON, M. & SELLERS, W.R. (2004). Genome coverage and sequence fidelity of phi29 polymerase-based multiple strand displacement whole genome amplification. *Nucleic Acids Research* 32, e71.
- POWERS, T.O. & HARRIS, T.S. (1993). A polymerase chain reaction method for identification of five major *Meloidogyne* species. *Journal of Nematology* 25, 1-6.
- POWERS, T.O., TODD, T.C., BURNELL, A.M., MURRAY, P.C.B., FLEMING, C.C., SZALANSKI, A.L., ADAMS, B.A. & HARRIS, T.S. (1997). The rDNA internal transcribed spacer region as a taxonomic marker for nematodes. *Journal of Nematology* 29, 441-450.
- SETTERQUIST, R.A., SMITH, G.K., JONES, R. & FOX, G.E. (1996). Diagnostic probes targeting the major sperm protein gene that may be useful in the molecular identification of nematodes. *Journal of Nematology* 28, 414-421.
- SKANTAR, A.M. & CARTA, L.K. (2000). Amplification of HSP90 homologs from plant-parasitic nematodes using degenerate primers and ramped annealing PCR. *BioTechniques* 29, 1182-1185.
- SKANTAR, A.M. & CARTA, L.K. (2005). Molecular characterization and phylogenetic evaluation of the Hsp90 gene from selected nematodes. *Journal of Nematology*, in press.
- THOMAS, W.K., VIDA, J.T., FRISSE, L.M., MUNDO, M. & BALDWIN, J.G. (1997). DNA sequences from formalin-fixed nematodes: Integrating molecular and morphological approaches to taxonomy. *Journal of Nematology* 29, 250-254.

- VRAIN, T.C., WAKARCHUK, D.A., LEVESQUE, A.C. & HAMILTON, R.I. (1992). Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15, 563-573.
- WISHART, J., PHILLIPS, M.S. & BLOK, V.C. (2002). Ribosomal intergenic spacer: A polymerase chain reaction diagnostic. *Phytopathology* 92, 884-892.
- YAN, J., FENG, J., HOSONO, S. & SOMMER, S.S. (2004). Assessment of multiple displacement amplification in molecular epidemiology. *BioTechniques* 37, 136-138.
- ZIJLSTRA, C., LEVER, A.E.M., UENK, B.J. & VAN SILFHOUT, C.H. (1995). Differences between ITS regions of isolates of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. *Phytopathology* 85, 1231-1237.
- ZIJLSTRA, C., UENK, B.J. & VAN SILFHOUT, C.H. (1997). A reliable, precise method to differentiate species of root-knot nematode in mixtures on the basis of ITS-RFLPs. *Fundamental and Applied Nematology* 20, 59-63.