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Nucleotide polymorphisms and an improved PCR-based mtDNA diagnostic for parthenogenetic root-knot nematodes (*Meloidogyne* spp.)

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Summary – Sequence analysis of 2212 bp of six closely related mitochondrial DNA (mtDNA) haplotypes that dominate Australian populations of *Meloidogyne* (Hugall *et al.*, 1994) revealed twelve polymorphic nucleotide sites and one deletion. Despite this low diversity, there are enough variable restriction enzyme sites among these sequences to provide diagnostic tests. Using a selection of these sites, we have developed a multiplexed PCR-based diagnostic that simultaneously amplifies two small regions of the mitochondrial genome, and then digests the product with HinfI or MnlI. The diagnostic test identifies the haplotypes, even in mixtures, found in *M. arenaria, M. incognita, M. javanica,* and *M. hispanica* and also *M. hapla* and *M. chitwoodi.* This is an improvement over previous mtDNA PCR tests for *Meloidogyne* in that it discriminates between more species and races (*e.g., M. arenaria* races from *M. javanica*) and, because smaller products are amplified, it should be more robust. We also developed primers to amplify a region of 63 bp variable number tandem repeats. The resulting DNA banding pattern may differentiate isolates within restriction enzyme haplotypes and, potentially, can be used to verify the identity of nematode isolates maintained in culture.

Résumé – ***Polymorphisme des nucléotides et amélioration du diagnostic par amplification en chaîne par polymérase de l'ADN mitochondrial appliquée aux espèces parthénogénétiques de*** **Meloidogyne** – L'analyse de séquences de 2212 paires de bases provenant de six ADN mitochondriaux haplotypes très voisins de *Meloidogyne* (Hugall *et al.*, 1994) a permis de mettre en évidence douze sites nucléotidiens polymorphiques et une délétion. En dépit de cette faible diversité, il existe assez de sites différents d'enzymes de restriction parmi ces séquences pour fournir des tests de diagnostic. En utilisant un choix de ces sites, il a été mis au point un test diagnostique multiplex fondé sur l'amplification en chaîne par polymérase (PCR), test amplifiant simultanément deux étroites régions du génome mitochondrial, et digérant ensuite le produit par HinfI ou MnlI. Ce test diagnostique permet d'identifier les haplotypes – même en mélange – présents chez *M. arenaria, M. incognita, M. javanica* et *M. hispanica,* ainsi que chez *M. hapla* et *M. chitwoodi.* Ce nouveau test représente une amélioration par rapport aux tests PCR sur DNA mitochondrial appliqués aux *Meloidogyne* en ce sens qu'il permet la discrimination entre un plus grand nombre d'espèces et de races (par ex. : races de *M. arenaria* vis-à-vis de *M. javanica*); de plus, des produits plus réduits étant amplifiés, ce test est de ce fait plus robuste. Ont été également mis au point des amorces amplifiant une région de 63 paires de bases en nombre variable de répétitions en tandem. Les schémas de zonation qui en résultent permettent de différencier les isolats à l'intérieur des haplotypes d'enzymes de restriction et peuvent être utilisés pour vérifier l'identité des isolats de nématodes maintenus en élevage.

Key-words : *Meloidogyne* spp., mitochondrial DNA, molecular diagnosis, PCR.

Root-knot nematodes (*Meloidogyne* spp.) are major pests of a wide range of crops. More than 40 species have been described. Most of these have different host ranges and some have more than one host race so that controlling root-knot nematodes by the use of resistance or crop rotation requires accurate identification and prediction of host range. Current methods of identification based on morphology (Jepson, 1987), cytogenetics (Triantaphyllou, 1985) and a differential host range test (Taylor & Sasser, 1978) are inaccurate, unreliable, and/or time-consuming. Esterase phenotype is considered to be a useful taxonomic character (Esbenshade & Triantaphyllou, 1990), but requires adult females at a specific developmental stage. Accordingly, recent emphasis has been on developing DNA-based diagnostics, particularly those that employ gene amplification (PCR) methods. A number of studies have examined nuclear sequences to develop species and strain specific markers, using in particular satellite and other repeated sequences because the high copy-number should enhance the sensitivity of a PCR diagnostic (*e.g.,* Castagnone-Sereno *et al.*, 1995*a, b*).

Other studies (Harris *et al.*, 1990; Powers & Harris, 1993; Hugall *et al.*, 1994) have concentrated on mitochondrial DNA (mtDNA) because of its large copy number, small genome, ease of extraction and rapid evolutionary rate in other taxa (Moritz *et al.*, 1987). Using RFLP analysis of whole genomes, Hugall *et al.* (1994) identified ten haplotypes in 98 Australian isolates. These variants fell into two genetically divergent groups. The first group included six mtDNA haplotypes; one each corresponding to *M. incognita* and

M. javanica, and three to *M. arenaria* race 2, while the sixth, haplotype G, had an esterase phenotype (S2-M1) corresponding to that of *M. hispanica* and atypical populations of *M. arenaria* (Ebsenshade & Triantaphyllou, 1985). This haplotype has been identified subsequently as *M. hispanica* based on its reaction (unpubl.) in the standard host range test (Taylor & Sasser, 1978). The second group of mtDNA variants consisted of four relatively divergent haplotypes from *M. hapla,* a detailed analysis of which is presented in Hugall *et al.* (1996). These variants of *M. hapla* are more than 20 % different from the mtDNAs found in other species, making their diagnosis relatively simple (*e.g.,* Powers *et al.,* 1986).

It happens that the species of greatest agricultural significance (*M. javanica, M. arenaria, M. incognita*) are also those with the most closely related mtDNA, making the design of a diagnostic test both important and challenging. As these species reproduce by mitotic parthenogenesis, mtDNA haplotype should reflect total genotype and so track genetic lineages. This is indicated by the perfect correlation between diagnostic esterase phenotype and mtDNA haplotype (Hugall *et al.,* 1994). Therefore, mtDNA haplotypes have a high potential as diagnostics for species and races of *Meloidogyne* which reproduce by mitotic parthenogenesis.

Powers and Harris (1993) reported a test to differentiate between five mtDNA haplotypes from the species *M. incognita, M. javanica, M. arenaria, M. hapla,* and *M. chitwoodi.* The mtDNA haplotypes they identified in the first three species were equivalent to haplotypes B, D, and A in Hugall *et al.* (1994). Their test required amplification between the COII and l-rRNA genes and subsequent digestion with DraI and HinfI of a PCR fragment up to 1.7 kb long. The PCR diagnostic, which could be applied to juveniles and adult females, represented a significant advance in DNA-based diagnostics for *Meloidogyne* but had some significant limitations. First and foremost, it was unable to distinguish between haplotype C of *M. arenaria* and haplotype D of *M. javanica.* Second, it relied on amplification of relatively large fragments, which could restrict its application for degraded or contaminated DNA extracts.

In the present study, we obtained mtDNA sequences representing each haplotype identified by whole-mtDNA RFLP studies of *M. javanica, M. arenaria* and *M. incognita* (*i.e.,* A, B, C, D, G and H; Hugall *et al.,* 1994) and compared these to homologous sequences from *M. hapla* (Hugall *et al.,* 1997). The segment sequenced was from the 3′ end of the open reading frame (ORF) (Okimoto *et al.,* 1991) into the 5′ end of the cytochrome b gene (Fig. 1) and encompasses the 1.7 kb region examined by Powers and Harris (1993). From these, we identified sufficient polymorphic restriction sites (and a length variant) to discriminate all haplotypes previously identified from whole-genome RFLPs. To develop a robust and practical diagnostic, we designed primers that simultaneously amplify (" multiplex ") two relatively short (< 750 bp) regions spanning the necessary sites.

Another practical concern with nematode research is the potential for cross-contamination of cultured stocks, as appears to have contributed to confusion over mtDNA variation within *M. hapla* (*e.g.,* Hyman & Powers, 1991). Means of distinguishing individual isolates via DNA fingerprinting could be of considerable use in monitoring culture collections. Our previous study demonstrated considerable variation in copy-number of 63 bp and 102 bp tandem repeats (Figs 2, 6 *in* Hugall *et al.,* 1994) that constitute a large part of the overall length of the *Meloidogyne* mtDNA molecule (Okimoto *et al.,* 1991). Many nematode isolates have multiple bands, probably due to heteroplasmy, and most isolates have a distinct pattern, even those within haplotype groups. This leads to the possiblity of using these patterns as DNA fingerprints to verify the identity of laboratory-reared isolates. Here, we design primers that amplify the 63 bp repeat cluster, selected because of its suitable size range for PCR (four to fourteen copies; Fig. 6 *in* Hugall *et al.,* 1994).

Materials and methods

Sample preparation

Isolates representing *Meloidogyne* from throughout Australia (Hugall *et al.,* 1994) were maintained as single eggmass cultures on tomato cv. Tiny Tim. Eggs were removed from roots in 1 % sodium hypochlorite (McClure *et al.,* 1973). Females were dissected from roots and placed in 30 µl of 2 % Triton X-100 in 1.5 ml Eppendorf® tubes, macerated with disposable plastic pestles and stored at – 70 °C. Five microlitres was then adjusted to 5 % Chelex (Bio-Rad) and the supernatant used for PCR template. *M. chitwoodi* (DNA supplied by T. Powers and T. Harris) and *Heterodera trifolii* (cysts supplied by C. Mercer and J. Grant) were used as controls.

Sequencing

Total DNA was extracted from eggs and females (Dowling *et al.,* 1990). Representatives of haplotypes A (*M. arenaria,* isolates NQ1 and 15), B (*M. incognita,* J and 35), C (*M. arenaria,* NQ7), H (*M. arenaria,* 77), D (*M. javanica,* 60 and 78), and G (*M. hispanica,* 12) were sequenced for 2212 bp, spanning the region from the ORF to the cytb gene between primers MORF and MMT2 (Fig. 1) using the primers listed in Table 1. The four *M. hapla* types in Hugall *et al.* (1994) have also been sequenced and analysed elsewhere (Hugall *et al.,* 1997). Cycle sequencing protocols using the BRL® kit are the same as described by Hugall *et al.* (1994). Amplified PCR products were sequenced directly without cloning. Sequence data for *M. javanica* (haplotype D population 60) and *M. hapla* (haplotype E population 113) are lodged in the Genome Services Data Base (ac-

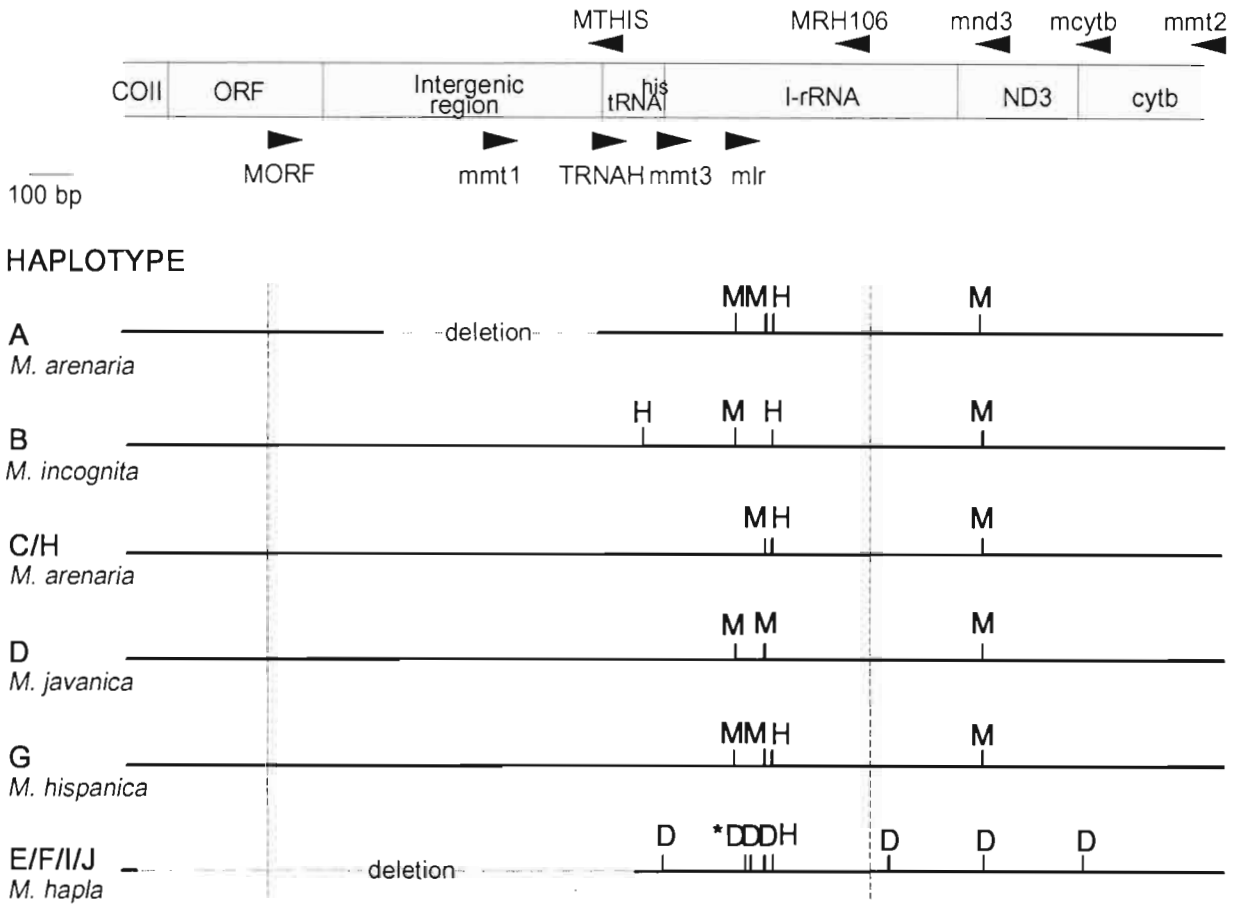

Fig. 1. *Alignment map showing relative positions of mtDNA genes of* Meloidogyne *spp., primers used in this study and diagnostic restriction sites. Primers in upper case were used in the multiplex test and for sequencing. (Primers in lower case were used for sequencing only; bold letters in restriction map show diagnostic sites; D : DraI; H : HinfI; M : MnlI; the asterix marks a polymorphic DraI site among* M. hapla *isolates.)*

Table 1. *Primers used in this study.*

Primer name	Sequence	Gene	3′ position	Reference
63VNL	5′-GAAATTGCTTTATTGTTACTAAG-3′	intergenic	53 bp 5′ to 63 bp tandem repeats*	this study
63VTH	5′-TAGCCACAGCAAAATAGTTTTC-3′	$tRNA^{Ser}$	76*	this study
MORF	5′-ATCGGGGTTTAATAATGGG-3′	ORF	19	Hugall *et al.*, 1994
MMT1	5′-TAAATCAATCTGTTAGTGAA-3′	intergenic	530	Harris *et al.*, 1990
MTHIS	5′-AAATTCAATTGAAATTAATAGC-3′	$tRNA^{His}$	722	Hugall *et al.*, 1994
TRNAH	5′-TGAATTTTTTATTGTGATTAA-3′	$tRNA^{His}$	757	this study
MMT3	5′-GAAAAATAAAAAAATTTTGTT-3′	l-rRNA	915	T. O. Powers (pers. comm.)
MLR	5′-ATGATTTTTTGTGTCTGCTCA-3′	l-rRNA	1116	this study
MRH106	5′-AATTTCTAAAGACTTTTCTTAGT-3′	l-rRNA	1271	this study
MND3	5′-TTTCCCAACCTATTAAAACCTCT-3′	ND3	1674	this study
MCYTB	5′-AATCTGCTCCATTTAACT-3′	cyt b	1917	this study
MMT2	5′-ATAAACCAGTATTTCAAACT-3′	cyt b	2194	Harris *et al.*, 1990

* Numbers refer to position in the entire sequence of mtDNA of *M. javanica* (Okimoto *et al.*, 1991).
All other position numbers refer to the sequence of mtDNA of *M. javanica* (Gene Services Data Base accession no. L76262).

cession nos. L76261 and L76262, respectively) and these provide nucleotide position numbers and gene designations.

Amplification conditions

Amplification reactions contained 0.4 µM of each primer (Table 1), 50 µM of each dNTP, 1 unit of Taq DNA polymerase I (Gibco BRL) and 2 µM $MgCl_2$ in a final volume of 25 µl. Amplification occurred during 33 cycles of 30 s denaturation at 94 °C, 30 s annealing at 50 °C and 70 s extension at 72 °C. Primers MORF, TRNAH, MTHIS and MRH106 (Fig. 1, Table 1) were designed for the multiplex diagnostic assay of females and eggs. In this test, MTHIS concentration was halved to 0.2 µM to reduce amplification of a larger product from MORF to MRH106. Restriction enzyme and buffer were added directly to aliquots of amplification products.

To determine the ability of the diagnostic test to differentiate components of mixtures, extracts of two *M. arenaria* variants, haplotypes B (isolate H) and C (isolate NQ7) were mixed and the combined template was amplified with four multiplexed primers as described above.

To amplify the tandem array of 63 bp repeats, primers 63VTL and 63VTH were designed from sequence presented by Okimoto *et al.* (1991). One primer is located in $tRNA^{Ser}$ and the other in a noncoding region. These primers were used to amplify the 63 bp VNTR of DNA extracted from pooled egg masses and individual adults.

Results

Sequence analysis

Complete sequences of 2212 bp from the ORF to cytb were obtained for nine isolates of six haplotypes. The maximum sequence divergence among these six types was only 0.6 %. This region covers 35 bp of the ORF, the complete intergenic region, tRNAHis, 16SrRNA and ND3 genes, and 339 bp from the 5′ end of cytochrome b gene (Fig. 1). This revealed twelve polymorphic sites among five sequence types A, B, D and G, with haplotypes C and H being identical over this region (Table 2). Haplotype A is also distinguished by a 529 bp deletion in the intergenic region (see also Powers & Harris, 1993; Hugall *et al.*, 1994). An unrooted tree based on these differences (Fig. 2) indicates that haplotype B (*M. incognita*) is relatively divergent from the others (mean divergence of 0.34 % *vs* 0.14 % among the others), whereas the two *M. arenaria* haplotypes A and C are the most similar. The deletion in haplotype A includes several informative sites, but analyses that exclude these sites produce the same topology (Fig. 3 *in* Hugall *et al.*, 1996).

Diagnostic test

Six of the twelve nucleotide polymorphisms affect restriction enzyme recognition sites (Table 2) and these, together with the length variation unique to haplotype A, form the basis of the diagnostic test. Four primers (MORF, MTHIS, TRNAH and MRH106) can be combined to amplify simultaneously two adjacent regions containing the diagnostic restriction sites and deletion. The primers are located in regions conserved among *M. javanica, M. incognita,* and *M. arenaria* and the TRNAH and MRH106 sequences are also conserved in *M. hapla.* The sequence from MORF to MRH106 for *M. incognita* (haplotype B) is shown in Fig. 3.

Amplification with TRNAH and MRH106 produced fragments of 557 bp (553 to 554 among *M. hapla* isolates due to insertions and/or deletions) which contain

Table 2. *Polymorphisms among five mtDNA haplotypes of* Meloidogyne *within the 2212 bp from MORF to MCYTB primers and restriction enzymes diagnostic for these polymorphisms.*

Position* / Haplotype	110	113	187	237	474	624	790	1086	1163	1182	1375****	1959****
B	A	T	A	G	C	C	C	G	A	G	G	A
A	G	G	–**	–	–	–	A	G	G	G	A	A
C/H	G	G	A	A	C	C	A	A	G	G	A	A
D	G	G	A	A	T	T	A	G	G	A	A	A
G	G	G	T	G	C	C	A	G	G	G	A	T
Diagnostic restriction enzyme					MaeII	AflIII	HinfI	MnlI	MnlI	EcoRI/HinfI		
Affected haplotype***					– D	– D	+ B	– C	– B	+ D/– D		

* Numbers refer to position in the *M. javanica* sequence accession no. L76262; ** –, missing due to 529 bp deletion; *** +, –; gain and loss of restriction site, respectively; **** these two sites are outside the fragments amplified in the multiplex test.

four diagnostic restriction sites that together distinguish haplotypes A/G, B, C/H, D, and *M. hapla*. Primer MRH106 is sufficiently downstream from the HinfI polymorphism at position 1182 to give a fragment length difference which can be resolved on 2 % agarose gels. Digestion with HinfI distinguished haplotype B through a site gain and haplotype D by a site loss (Fig. 4 top; Tables 2, 3). This digestion did not differentiate between haplotypes A, C/H and G or between *M. hapla* haplotypes. However, digestion of this PCR product with MnlI differentiated haplotypes C/H because of a unique MnlI site loss in haplotypes C and H (Fig. 4 bottom, Tables 2, 3).

Based on the HinfI/MnlI digestion of the TRNAH-MRH106 fragment, all types can be distinguished except haplotypes A (*M. arenaria*) and G (*M. hispanica*). To separate these, we incorporated simultaneous amplification of the adjacent MORF-MTHIS fragment which is 743 bp in haplotypes B, C/H, D and G, but only 214 bp in A because of a 529 bp deletion (Fig. 1, Table 3). Because of the absence of the region containing the MORF primer site, there can be no amplification between MORF and MTHIS in *M. hapla* or *M. chitwoodi*.

In general, simultaneous amplification of the two regions was reliable. However, in some cases, " read

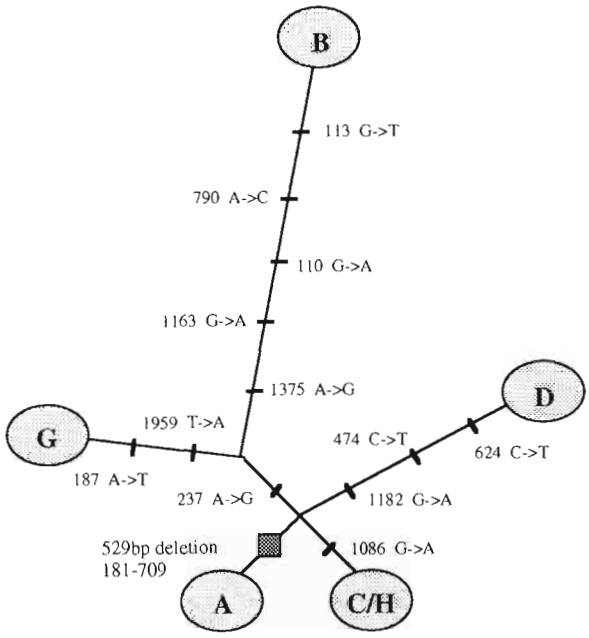

Fig. 2. *Most parsimonious unrooted tree of differences among haplotypes. (Haplotypes A and C/H correspond to* M. arenaria, *B to* M. incognita, *D to* M. javanica *and G to* M. hispanica; *these are all the differences in the 2212 bp sequenced from primers MORF to MMT2; the arrows refer to changes from base to tip of the branches, which are in proportion to the number of differences; note that the deletion in A spans four variable sites.)*

Table 3. *Expected fragment sizes following amplification of* Meloidogyne *mtDNA with primers MORF, MTHIS, TRNAH and MRH106 and digestion with HinfI or MnlI.*

Haplotype Primers	Fragment sizes (bp) MORF-MTHIS	Fragment sizes (bp) HinfI TRNAH-MRH106	Fragment sizes (bp) MnlI TRNAH-MRH106
A	214	445, 112	346, 134, 77
B	743	396, 112, 49	346, 211
C	743	445, 112	423, 134
D	743	557	346, 134, 77
G	743	445, 112	346, 134, 77
M. hapla	-	442-444*, 111	553-555*

* Variation between isolates due to insertions and/or deletions.

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        10         20         30         40         50         60         70         80         90         100
<-------MORF-------->    ORF          >><<                intergenic region
ATCGGGGTTT AATAATGGGT TCTTTATTGT GTTAATTATT AAAATATTAT TTTGGTTTTT TAGTGAAATT TTTTATTTAA TTTATTTTTA TACGAAAAAT
TATTAGGGAA AATATTTTGA TTTTTTGTTA TTTAGAGTTT AAGTTCTTTA TAGAACAAAA TTTTTTATCT TTTTAGTATT CTATTGAAAT AGAAGAATTA     200
TAAATTAGTT AATTCTAATT TTATTTATTT TATATGGATT ATATAAAATT TTAAATTTTT TGATTTTAGT TTATTTACGT ATTTAGTTGA TTTTTTTAAT     300
AGATTTAGTT CATCTGTTGA TGTAACACAG AGTGGCTCTT TAGTTAATTT ACCTGAAGTT AAAAATGGTC AATTATATGA TATAACTTTT GGTTATCATC     400
AAGAATATTT CAAGGAAATT AAGGTGCTCA TCCTGATAAA GATCGTAATT TTTATAGTTG TGATATTGTT ATACGTCAAT CACAAGCTTT AGAAGAATAT     500
CATAATGAAT TAAATCAATC TGTTAGTGAA AAAATAGTTA TTTTTATTAA TAGAGATATA AGGAGATTTA ATTTTTAAGT TAAATCCAAT TCGTAATGTT     600
TGGAATTTAC CAAGGTAGAA TTACACGTTA AATTTAGAAG AATTGTTGAA AAGAATGAAT TCTTAATGGA AACAGTAAGG ATATTTTTTA TATAATTATT     700

                    >> <<                        tRNA^His                          >><<        1-rRNA
                        <---------MTHIS-------->
TTTAATAATA TTAAAAATAA AGCTATTAAT TTCAATTGAA TTTTTTATTG TGATTAAAAA AGTTTTTGGC TAAATTATTT TTTTAGATTC TTTTTTATTG     800
                                  <---------TRNAH-------->

TTGAAAAAAT TAAAAACAAA TTGTTTTTTA CAATAATTAA AATTTATAAT ATTTCAATTT TTAATTTTTA GTTTTAAATA AAAATTACAA ATATGAAAAA     900
TAAAAAAATT TTGTTATAAA TTAAATTAAT TTATTTATAT TAAAAATATT TTTTATAATT TTTATTTTTT TTATTTTTTT AAAAAAAAAA TAATTTAATT    1000
ATAATTATTT ATTTAAATTT AATAATTAAA TATAAAATTT TTTATTAAAT AAATTTAATA ATAAATGTTT TTTAAATTCT TTGAGGTTTT GATTTTTGAT    1100
TTTTTGTTTC TGCTCATTGT TAAAGAAAAG CACTTTTAGC GTGATTGGTC AAAGGTAGCG AGATAATTTG TTTTTTTATT GGATTCTAGT ATGAATGGAT    1200
TTTTTGTTAT TAAATATATT GATTTTAAAA AGAATTTTAA ATTTATTTAA AAAATAATAA ATAAAATAAA ACTAAGAAAA GTCTTTAGAA ATT            1293
                                                                                <---------MRH106-------->
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Fig. 3. *Nucleotide sequence of the region containing the defining restriction enzyme site polymorphisms used in the multiplex test. (Sequence of* M. incognita *(haplotype B) from primers MORF to MRH106; gene boundaries are marked by << >>, polymorphic sites are underlined.)*

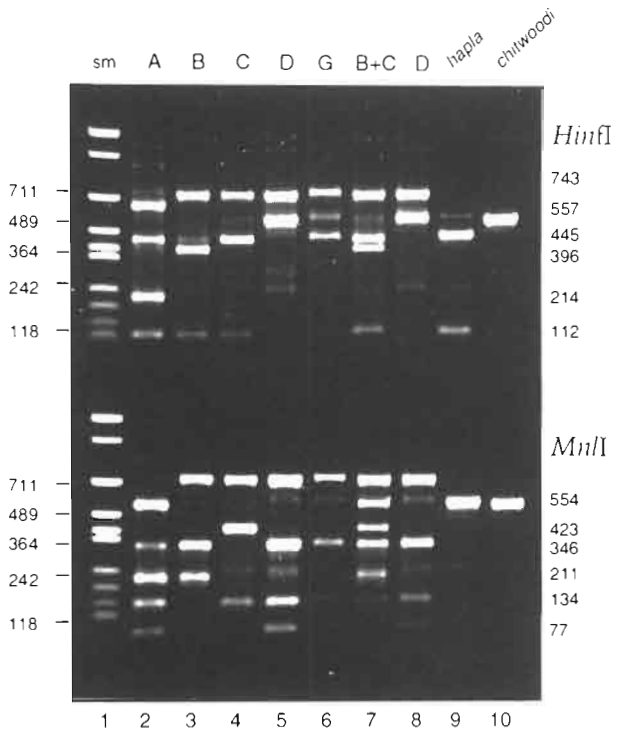

Fig. 4. *Multiplexed PCR products of amplification of* Meloidogyne *mtDNA with primers MORF, MTHIS, TRNAH and MRH106 and digestion with HinfI or MnlI. (Lanes 1-10 : size marker (with band sizes on the left), population NQ1 (haplotype A), Z (B), 68 (C), NQ2 (D), 12 (G), mixture of haplotypes B and C, 42 (D), 113 (E) and* M. chitwoodi; *band sizes (bp) of digested PCR products are shown on the right; for MnlI, 554 bp refers to uncut size in* M. hapla*).*

through " amplification of larger fragments occurred between MORF and MRH106. For example, lane 2 of Fig. 4 shows bands of 659 and 550 bp, respectively for HinfI and MnlI digests, corresponding to digestion of a 771 bp amplification product between MORF and MRH106.

The consistency of these markers was verified by application of the test to 50 isolates of *Meloidogyne* (six of haplotype A, seven B, four C, 29 D, and four *M. hapla*) previously characterised by Hugall *et al.* (1994). The diagnostic HinfI sites and the deletion in haplotype A were already known from the earlier whole-mtDNA RFLP studies. This additional survey confirmed the presence of the diagnostic MnlI sites across the larger series.

For *M. hapla,* there are no MnlI sites and just one invariant HinfI site within the TRNAH-MRH106 fragment (Figs. 1, 4) and the MORF-MTHIS fragment does not amplify.

Where there may be a mixture of more than one haplotype in a sample, it is necessary to distinguish a real mixture from artefacts due to incomplete digestion by the restriction enzyme. In this case, digestion with one of the restriction enzymes will identify one haplotype component clearly but the presence of the other haplotype may not be easily distinguished from partial cutting. However, digestion with the second restriction enzyme will identify this other haplotype component. For example, the fragment pattern produced by HinfI digestion of the mixture of haplotypes B and C in lane 7 of Fig. 4 is consistent with either a mixture of haplotypes B and C/H, or a pure sample of haplotype B with partial digestion. However, the 423 and 131 bp bands resulting from digestion with MnlI indicated the presence of haplotype C/H. Under these circumstances the multiplex amplification using MORF and MTHIS is needed to identify mixtures of A and D.

Amplification of 63 bp tandem array

Amplification using the primers flanking the 63 bp tandem repeat revealed fragment patterns that varied among samples (Fig. 5A) with several samples having multi-banded (*i.e.,* heteroplasmic) patterns. The fragment patterns also varied within haplotypes, as shown by isolates 42, 71, 70, and X of haplotype D. The fragment patterns produced in this PCR test were consistent with those observed in end-labelled MboI digests of whole mtDNA (*e.g.,* NQ7 and 70 in Fig. 2 in Hugall *et al.,* 1994), indicating that this pattern can be faithfully reproduced by PCR.

To investigate the stability of these patterns among individuals of an isolate within and between generations derived successively by single egg mass culture, we have amplified DNA extracted from cultures harvested over a period of one year. Fig. 5B shows four isolates B, Z, 68, and 77 with two or three different harvests, one of them from one to six adults and the rest from pooled egg-masses. In each case the pattern is maintained.

Discussion

Restriction enzyme haplotype test

The multiplexed PCR test differentiates four parthenogenetic types (*M. arenaria,* haplotypes A, C/H; *M. incognita,* haplotype B; *M. javanica,* haplotype D) that represent more than 95 % of *Meloidogyne* found in Australia, as well as *M. hispanica* (haplotype G) and *M. hapla.* This was achieved by sequence analysis which identified sufficient polymorphic restriction sites to discriminate between all of the relevant mtDNA variants found in our earlier study of whole-genome RFLPs (Hugall *et al.,* 1994). The protocol developed in this study complements and extends that of Powers and Harris (1993) which did not distinguish beyond the species level. In particular, we used amplification products which included a region next to (but outside) the sequence used in their study to distinguish additional haplotypes, *viz.* haplotypes C and H. The distinction be-

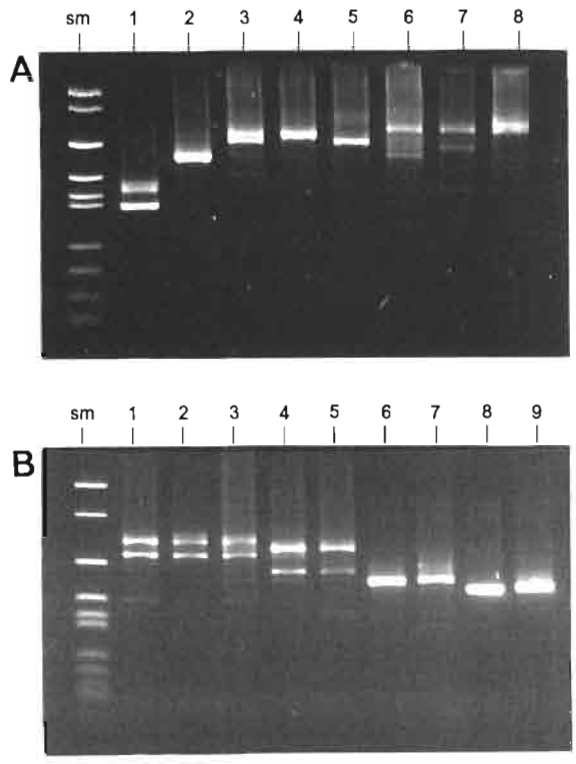

Fig. 5. *Amplification of the 63 bp tandem repeat region of* Meloidogyne *mtDNA. A : Lanes 1-8 : population NQ7 (haplotype C), NQ1 (A), 42 (D), 35 (B), 71 (D), 70 (D), Z (B), X (D) respectively. All DNA was extracted from egg masses; B : Lanes 1-3, population B (haplotype D), lanes 4-5 Z (B), lanes 6-7 68 (C) and lanes 8-9 77 (C/H) respectively. (In each case the first lane of an isolate is from chelex-extracted DNA of one to six individual adult females with the remaining lanes from pooled DNA extracted from egg masses; each sample is from successive greenhouse cultures spanning up to one year; the population Z in Fig. 5A represents a third sample of this isolate; band sizes [bp] for the size marker [sm] shown on the left are the same as in Fig. 4.)*

tween haplotypes A and C/H of *M. arenaria* is important for nematode management as they have different host ranges (Stanton *et al.*, 1992) and are equally common in Australian agriculture (Hugall *et al.*, 1994). In addition, we can identify haplotype G (*M. hispanica*) and differentiate all of these from *M. chitwoodi* and *M. hapla.*

By explicitly identifying the necessary polymorphisms the PCR test can be built around smaller fragments (< 750 bp) than those in previous tests (*e.g.*, up to 1.7 kb; Powers & Harris, 1993), which should make it more sensitive and reliable and therefore able to be applied to a broader range of assays.

Amplification of non-target DNA in other nematodes is unlikely because the primers appear to be specific to the target taxa. The primers did not amplify *Heterodera trifolii* (data not shown). Primer MHR106 is in a reasonably conserved region among nematodes, but MTHIS is not. Moreover, non-specific amplification would be apparent because the test relies on the production of RFLPs with fragments of a particular size.

Given the very low sequence divergence and distribution of polymorphisms, it may be difficult to develop haplotype-specific primers, *e.g.*, for dot-blot assays against specific oligonucleotides (*e.g.* Chacon *et al.*, 1991). Therefore, the ability of the test to distinguish components of mixtures of haplotypes would be an advantage. In principle, the combination of HinfI and MnlI patterns will do this and we demonstrated this for a mixture of haplotypes B and C as distinguished from partial digestions. However, further testing is required to assess sensitivity to different proportions in field isolates.

As expected, the effectiveness of PCR for diagnosis of *Meloidogyne* appears to be independent of life stage. Harris *et al.* (1990) and Powers and Harris (1993) identified single second-stage juveniles. In this study, we diagnosed adult females and eggs. In addition, we have consistently diagnosed single adult females (unpubl.). Further improvements involve development for routine use with root and soil samples and single second-stage juveniles and also to reduce the amount of partial digestion.

DNA BANDING PATTERNS FROM 63 bp REPEAT

The diversity of fragment patterns produced by amplifying the 63 bp tandem repeats, together with the observation that, in a few isolates, these patterns are stable, suggest that this offers a useful approach to confirming the purity of isolates. Arrays of short tandem repeats in mtDNA are typically hypervariable (Moritz *et al.*, 1987) as a result of a high mutation rate balanced by genetic drift within and among individuals (Birky *et al.*, 1989). Despite the high mutability for copy-number, several studies have found the resulting genotypes to be relatively stable among generations (*e.g.* crickets, Rand & Harrison, 1986, 1989; frogs, Monnerot *et al.*, 1984; *Drosophila*, Hale & Singh, 1986). In the case of *Meloidogyne,* a further indication of stability is the clustering of repeat numbers within RFLP-defined haplotypes (Fig. 6 *in* Hugall *et al.*, 1994). However, more comprehensive analyses of 63 bp repeat genotypes in isolates across generations is necessary to confirm their stability and utility as DNA " fingerprints ".

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