*Fundam. appl. Nematol.*, 1998, **21** (3), 265-271

# Mitochondrial DNA length variation in *Meloidogyne incognita* isolates of established genetic relationships: utility for nematode population studies

Lawrence E. WHIPPLE, David H. LUNT and Bradley C. HYMAN

*Department of Biology, University of California, Riverside, CA 92521, USA.*

Accepted for publication 17 September 1997.

**Summary** – Six *Meloidogyne incognita* isolates with previously characterized genetic relationships were used to test the utility of a 63 base-pair mitochondrial Variable Number Tandem Repeat (VNTR) as a marker for population studies. The polymerase chain reaction (PCR) was used to amplify this locus and to measure copy number and allele frequencies of the 63 bp VNTR. Individual nematodes were typically heteroplasmic and maintained mitochondrial DNA (mtDNA) molecules containing up to thirteen distinguishable VNTR size classes. Each allele was composed of one to 21 repeat copies. Hierarchical statistics revealed that diversity was low (7%) among the isolates whereas 60% of the total genetic diversity measured for these six isolates resides within individuals. Likelihood ratio tests revealed that diversity indices were independent of genetic relatedness and race designation, limiting the utility of this locus for studies of population differentiation. As *M. incognita* is an obligate parthenogen, paternal contribution to heteroplasmy is excluded and diversity within individual nematodes at this mtDNA locus is primarily a consequence of mutation to different repeat copy numbers. © Orstom/Elsevier, Paris

**Résumé** – ***Variabilité de longueur de l'ADN mitochondrial chez des isolats de* Meloidogyne incognita *ayant des relations génétiques établies : utilité pour l'étude des populations de nématodes*** – Six isolats de *Meloidogyne incognita*, dont les relations génétiques ont été précédemment établies, sont utilisés pour tester l'utilité d'une « répétition de tandems en nombre variable » (VNTR) d'une valeur de 63 paires de bases mitochondriales comme marqueurs en vue d'étude de populations. La réaction en chaîne de polymérase (PCR) est utilisée pour amplifier ce locus et mesurer le nombre de copies et la fréquence des allèles des VNTR à 63 paires de bases. Les individus du nématode sont typiquement hétéroplasmiques et conservent des molécules d'ADN mitochondrial (mtDNA) contenant jusqu'à treize classes de VNTR ayant des tailles distinctes. Chaque allèle est composé d'une à 21 copies répétées. La statistique hiérarchique révèle que la diversité entre isolats est faible (7 %), tandis que 60 % de la diversité génétique totale mesurée pour ces six isolats provient de la diversité entre individus. Des tests de rapports aléatoires montrent que les indices de diversité sont indépendants de la parenté génétique et de la race, limitant ainsi l'utilité de ce locus pour la différenciation des populations. *M. incognita* étant un organisme à parthénogenèse obligée, une contribution paternelle à l'hétéroplasmie est exclue ; la diversité entre individus concernant ce locus du mtDNA est donc en premier la conséquence de mutations conduisant à des nombres différents de copies répétées. © Orstom/Elsevier, Paris

**Keywords:** mitochondrial DNA, *Meloidogyne incognita*, molecular biology, nematodes, PCR, population genetics, root-knot, VNTR.

The genetic structure of populations is typically defined in quantitative terms by measuring how allele frequencies are distributed among individual genomes (Wright, 1922). Mitochondrial DNA (mtDNA) polymorphism has been successfully employed as a marker for population and phylogenetic studies (Avise *et al.*, 1987; Harrison, 1989), in part due to its rapid evolution, matrilineal inheritance patterns, apparent absence of recombination (but see Lunt & Hyman, 1997) and an effective population size ($N_e$) one-fourth that of nuclear alleles. One type of polymorphism exhibited by mtDNA molecules is size variation, and previous studies have demonstrated that mitochondrial genome size can differ within and among individuals in populations (Rand, 1993). Variation of this type is often the consequence of short repeated DNA segments, termed Variable Number Tandem Repeats (VNTRs), duplicated to different copy numbers within each mtDNA molecule. How mitochondrial genomes carrying different sized VNTR alleles become distributed among organismal hierarchies (within individuals, among individuals in a population, and between populations), can be analyzed statistically (Birky *et al.*, 1989) to draw inferences regarding population structure and gene flow (Rand & Harrison, 1989).

Hierarchical analysis using mitochondrial VNTRs has now been applied to insect (Rand & Harrison, 1989), fish (Árnason & Rand, 1992; Cesaroni *et al.*, 1997) and mammalian (Wilkinson & Chapman, 1991) isolates, resulting in a variety of conclusions regarding structuring of these populations. However,

these quantitative descriptions of population differentiation were generated without a prior knowledge of the genetic relationships between population samples nor among the individuals that comprise them. When employing a VNTR marker for the first time, it is important to assess if analysis of that locus using hierarchical statistics of allele frequencies is capable of resolving known relationships. Given the rapid mutation rate of VNTRs to different allele sizes (Rand & Harrison, 1989) and the attendant concerns of homoplasy, it would be useful to apply this methodology to a genetically defined model system and determine whether accurate descriptions of population differentiation can be realized using isolates of known relationships.

The mitochondrial genome of *Meloidogyne* is a particularly suitable model for this purpose. Root-knot nematode mtDNA molecules carry three sets of VNTRs, unrelated in nucleotide sequence, with repeat units that are eight, 63 and 102 base pairs (bp) in length, respectively (Okimoto *et al.*, 1991). Each represents a polymorphic locus for which an independent statistical treatment of mtDNA diversity can be conducted. In this report, we exploit the development of a PCR assay (Hyman & Whipple, 1996) to investigate the diversity of the 63 bp mitochondrial VNTR in six *M. incognita* isolates that represent two different host-races. Unlike previous studies involving other organisms, the relationships among these six nematode isolates are well-established (Roberts, 1995; Roberts *et al.*, 1995), and provide a suitable model system to rigorously test the predictive value of this methodology for studying nematode population differentiation.

## Materials and methods

### NEMATODES

Six *M. incognita* isolates of Californian origin, identified herein as A-F, were kindly provided by Dr P. Roberts (Department of Nematology, UC-Riverside, CA, USA) and maintained on cowpea (*Vigna unguiculata* [L.] Walp.) cv. CB46 (carrying the resistance gene *Rk*), susceptible cv. CB3 (lacking *Rk*), or susceptible tomato (*Lycopersicum esculentum* [L.] Mill.) cv. Tropic (lacking gene *Mi*) in the greenhouse at 26°C. Isolates A (Indio, Riverside County) and C (Patterson, Western Stanislaus County) are race 1; isolate F (Poplar, Tulare County) is race 3. Isolates E (Roberts *et al.*, 1995) was descended from an original field population sub-sampled from a resistant cowpea plot (Denair, Eastern Stanislaus County) and is also race 1. All race characterizations were based on the NCSU host differential test.

Isolate E was propagated in the greenhouse on susceptible tomato for over 4 years. During this time virulence on resistant cowpea diminished, resulting in a less virulent isolate (D) (Roberts, 1995). When D was returned to resistant cowpea and continuously maintained, virulence returned to levels approaching that of the original field subsample; this renewed virulent isolate is termed B (M. Petrillo & P. Roberts, unpubl.). Virulence was measured by comparing production of egg masses on resistant (*Rk+*) plants to susceptible plants.

To obtain the most unbiased survey of each isolate, individual nematodes were randomly picked from pooled J2 preparations that had been propagated on multiple host plants. Possible genetic bottlenecks imposed by analysis of siblings within single egg masses were avoided by this sampling regime.

### DNA EXTRACTION AND PCR AMPLIFICATION

DNA was obtained from individual J2 by maceration in chilled 0.5 ml centrifuge tubes containing 10 µl of sterile water, and with the exception of some control experiments (below) the entire sample used as template for PCR amplification. The sequences of the forward and reverse primers were 5′-CTATTTTAA-AGTTATCGACTG-3′ and 5′-CCTAAAGACTTT-TTATCCTAAC-3′, respectively. The forward primer specifically anneals with to a noncoding region fifteen bp upstream of the 63 bp repeating unit array while the reverse primer binds five bp downstream of the VNTR within the mitochondrial $tRNA_{met}$ gene (Hyman & Whipple, 1996). PCR products were electrophoretically fractionated on Tris-Acetate-EDTA buffered (Maniatis *et al.*, 1982) 2% (w/v) MetaPhor agarose gels (FMC, Rockland, MD, USA). DNA was visualized by ethidium bromide staining (1 µg/ml) and illumination with a Fotodyne 3-3000 UV transilluminator.

Fidelity and reproducibility of the PCR amplifications have been addressed in an exhaustive series of control experiments (Whipple, 1997). Duplicate reactions using aliquots of the same extract produced identical amplification products. A systematic variation of template concentration and cycle number also indicated that our results are robust to any potential sources of artifact.

### DENSITOMETRY AND STATISTICAL ANALYSIS

Experimental and statistical methodologies were described previously in an expanded format (Hyman & Whipple, 1996). In summary, digitized images of ethidium bromide stained MetaPhor gels were captured electronically with a GAS 4000 Gel Documentation System (EverGene, Taiwan). Intensities of DNA bands were measured using ImageQuaNT 4.1 densitometry software (Molecular Dynamics, Sunnyvale, CA, USA). Hierarchical statistics were used to analyze band frequencies according to previously

described methods (Birky *et al.*, 1989; Rand & Harrison, 1989). *K* statistics provide a measure of genetic diversity within an individual ($K_b$) and among individuals within a population ($K_c$); *C* statistics predict at which hierarchical level ($C_i$, within an individual; $C_{ip}$, among individuals within a population; $C_{pt}$, between populations) genetic diversity is most likely to occur.

## Results

### PCR AMPLIFICATION OF THE 63 BP MTDNA VNTR

PCR amplification of the 63 bp mtDNA VNTR locus using DNA template derived from individual *M. incognita* J2-stage nematodes generated multiple products; examples for isolate D are shown in Fig. 1. When sized by gel electrophoresis, each band differed in size from its adjacent neighbor by 63 bp. The large collection of PCR products amplified in each reaction indicated that individual nematodes are heteroplasmic and maintain a complex collection of 63 bp VNTR alleles.

### HIERARCHICAL ANALYSIS OF ALLELE FREQUENCY DISTRIBUTION

Allele frequencies were measured from individual J2-stage nematodes comprising isolates A-F. Variation of allele copy number ranges and frequencies were observed among all six isolates (Fig. 2). Table 1 presents a quantitative treatment of these allele frequency data, including measurements of heteroplasmy and apportionment of diversity at the 63 bp VNTR locus into genetic hierarchies. Among these six isolates, the 63 bp VNTR locus contained as few as one repeat copy (isolates B, C, D, F) to as many as 21 tandem repeats (isolate C). The frequency of homoplasmic individuals (f[h]), varies considerably (0 to 33%) among these six isolates.

Using calculated mean $K_b$ and $K_c$ values, hierarchical structure (*C*-statistics) across all six isolates of *M. incognita* was assessed. Most of the genetic diver-

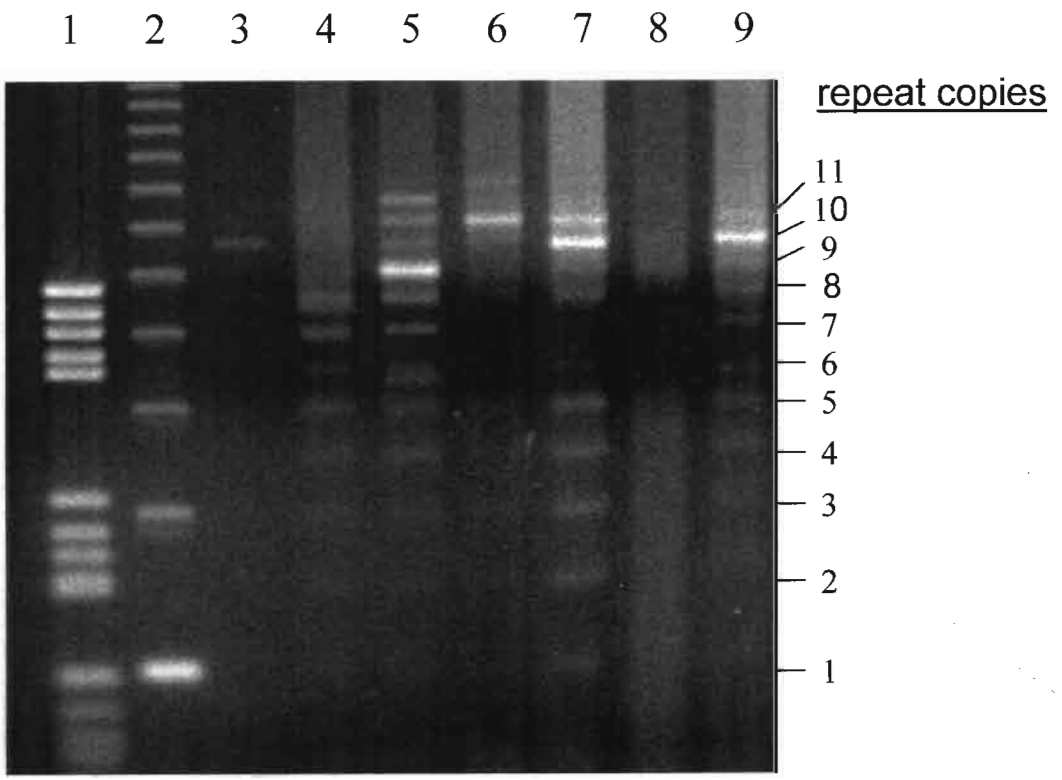


***Fig. 1.*** *Genetic diversity of the 63 bp mtDNA VNTR within* Meloidogyne incognita *individuals. Cellular DNA extracted from seven different* M. incognita *(isolate D) individuals was used as template in PCR reactions to amplify the 63 bp VNTR. Amplification products were fractionated on a 2% MetaPhor agarose gel. Lane 1, pBR322/Hae III molecular size standard; lane 2, 123 bp ladder molecular size standard; lanes 3-9, PCR amplification products from individual nematodes. VNTR copy numbers are denoted along the right column.*

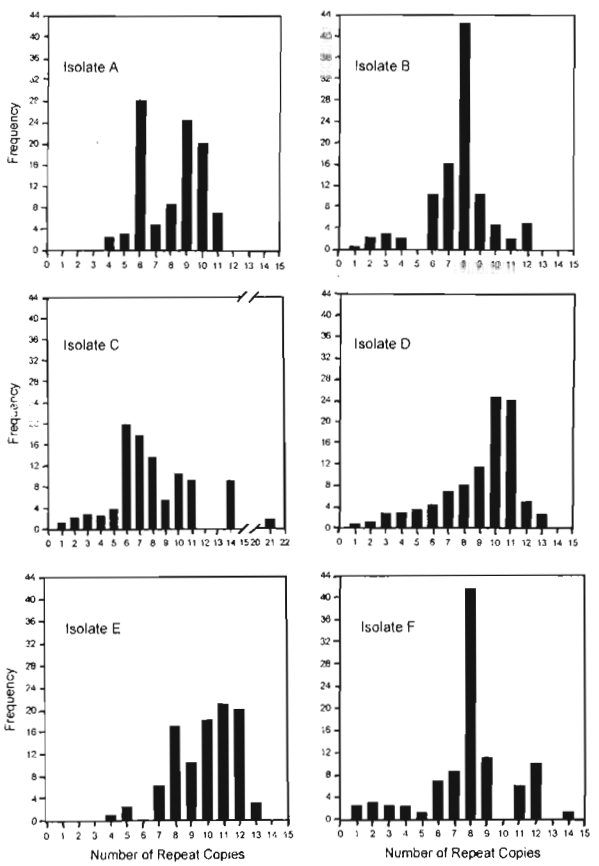


***Fig. 2.*** *Comparison of 63 bp allele frequencies among six representative* Meloidogyne incognita *isolates (Abcissa, number of repeat copies; ordinate; frequency of allele in each isolate as measured by quantitative densitometry of PCR amplification products).*

sity found in the grand pool is associated with individual nematodes ($C_i$ = 0.58), whereas within-isolate diversity ($C_{ip}$ = 0.35) contributes one-third of the total genetic diversity to this data set (Table 1). A modest diversity level ($C_{pt}$ = 0.07) can be attributed to divergence between the six different *M. incognita* isolates.

### Genetic relatedness does not influence allele frequency variation

A statistical comparison employing the likelihood ratio test, or *G*-test (Sokal & Rohlf, 1987), was used to test if differences in allele frequency distributions among these six isolates were statistically significant. The first comparison was among all six *M. incognita* isolates, A-F, with the *G*-test revealing a significant difference in the frequency distributions of VNTR alleles within this data set ($G$ = 343.298, *d.f.* = 70, $P < 0.001$). Consistent with simple inspection of the allele frequency distributions (Fig. 2), quantitative treatment of this data reveals that this group of six isolates is not homogenous.

To compare the effects of genetic relatedness on allele frequency distribution, the six isolates were split into two groups of three. One group consisted of samples A, C and F which originated from field isolates of wide biogeographic separation. These samples represent two different host races, and are therefore of distant shared ancestry. The second group was composed of isolates B, D and E which are characterized by documented recent shared ancestry and are of very close genetic relationship. These two groups were then compared after averaging the frequency distributions of the isolates within groups. Differences in allele frequencies between the genetically related and less-related groupings were not statistically significant ($G$ = 22.18, *d.f.* = 14, $0.05 < P < 0.10$). This result indicates that both sets of isolates carried a similar collection of 63 bp VNTR alleles maintained at comparable frequencies, and that sub-sampling of this sort does not introduce a bias into our analyses.

A critical test of the VNTR methodology is to independently measure allele frequency distributions *among* the genetically related and *among* the more distant isolates. A prediction of this analysis is that distantly related isolates would reveal significant differences in allele frequency distributions, whereas the very closely related isolates would not. For this purpose, a *G* test was carried out to compare frequency distributions among isolates within each group. The three distantly related isolates (A, C, F) showed a significant difference in their frequency distributions ($G$ = 147.623, *d.f.* = 26, $P < 0.001$). The group of genetically related isolates B, D, and E unexpectedly revealed a level of heterogeneity ($G$ = 120.272, *d.f.* = 24, $P < 0.001$) nearly equivalent to that of the distantly related isolates.

## Discussion

### Extensive mtDNA heteroplasmy in *M. incognita*

Heteroplasmy is the presence of multiple mtDNA forms within individuals. With the application of PCR to mtDNA analysis, heteroplasmy resulting from the presence of short mtDNA VNTRs now appears to be a common occurrence (Rand, 1993). Surveys of mtDNA VNTRs among populations of organisms representing wide taxonomic distances, including bats (Wilkinson & Chapman, 1991), crickets (Rand & Harrison, 1989), sturgeon (Brown *et al.*, 1992), cod (Árnason & Rand, 1992) and sea bass (Cesaroni *et al.*, 1997) reveal frequencies of heteroplasmic individuals ranging from 42-100%. In this study, we have found

***Table 1.*** *Hierarchical analysis of 63 base pair mtDNA VNTRs in six* Meloidogyne incognita *isolates.*

| Isolate | Race | n[a] | Allele size classes[b] | f(h)[c] | $K_b$ range | Mean $K_b$ | $K_c$ |
|---|---|---|---|---|---|---|---|
| A | 1 | 8 | 4-11 | 0.25 | 0-0.86 | 0.45 | 0.78 |
| B | 1 | 12 | 1-12 | 0.08 | 0-0.81 | 0.53 | 0.72 |
| C | 1 | 12 | 1-21 | 0.33 | 0-0.85 | 0.37 | 0.84 |
| D | 1 | 7 | 1-13 | 0.00 | 0.44-0.83 | 0.69 | 0.85 |
| E | 1 | 11 | 4-13 | 0.18 | 0-0.77 | 0.47 | 0.75 |
| F | 3 | 12 | 1-14 | 0.17 | 0-0.90 | 0.47 | 0.69 |
| mean $K_b$ = 0.48 | | | | $C_i$ = 0.58 | | | |
| mean $K_c$ = 0.77 | | | | $C_{ip}$ = 0.35 | | | |
| $K_t$ = 0.83[d] | | | | $C_{pt}$ = 0.07 | | | |

[a] Number of individuals sampled.

[b] 63 bp repeat copy number.

[c] Frequency of homoplasmic individuals.

that over 80% of the root-knot nematodes analyzed are heteroplasmic. It has been suggested that maintenance of mtDNA heteroplasmy in animal cells is a consequence of paternal leakage and mutation to different VNTR copy numbers (Rand & Harrison, 1989; Brown *et al.,* 1992). *M. incognita* is the first obligate parthenogen to be analyzed in this fashion, and paternal contribution can be excluded from this system. As a consequence, mutation to different 63 bp repeat copies is the primary mechanism by which size-variable heteroplasmy is generated and maintained in root-knot nematodes.

We have observed a complex set of VNTRs in all isolates of root-knot nematodes analyzed using our PCR assay (Hyman & Whipple, 1996; this study). These results are in conflict with those of Okimoto *et al.* (1991), who reported a considerably reduced spectrum of 63 bp VNTR alleles using DNA hybridization. In this earlier study, when bulked DNA samples prepared from several *M. incognita* and *M. javanica* populations were tested, a single band was often observed. This suggested apparent homoplasmy of a specific 63 bp allele in these root-knot nematode isolates. The sensitivity of detection available by PCR, hybridization conditions, or other technical factors may have contributed to these competing observations.

## Population biology considerations

Several molecular approaches (reviewed in Caswell-Chen *et al.,* 1993; Hyman & Whipple, 1996) including RAPD methodology, nuclear and mtDNA RFLP analysis, and nuclear satellite DNA characterization, have recently been evaluated to examine the population stucture of phytonematodes. To study genetic diversity from the level of the individual and upwards, analysis of single nematodes is necessary. This requires the use of PCR as an alternative molecular approach, where the small amount of DNA present in an individual J2 is limiting. It has been previously demonstrated (Hyman & Whipple, 1996) that PCR results obtained from individual nematodes are consistent with amplification profiles obtained from pooled nematodes of the same isolate.

Analysis of representative individuals from each isolate, selected from a pool derived from multiple plant hosts, is a necessary feature of our sampling regime. Previous studies have revealed that the mean mtDNA diversity at the 63 bp VNTR among siblings within an egg mass ($K_c$ = 0.44; Whipple, 1997) is dramatically reduced when compared to the same measure ($K_c$ = 0.77) obtained using the broader sampling employed here (Table 1). This result is consistent with an anticipated genetic bottleneck in a mother-offspring pair, and indicates that a substantial bias in our data would have been introduced if egg masses were exclusively analyzed.

*K*-statistics describe the probability of sampling two different alleles from a mtDNA pool, and provide a useful measure of genetic diversity at all hierarchical levels: within individuals, among individuals within a population, and among populations. This type of statistical analysis has been successfully applied to mtDNA VNTRs amplified by PCR. Average within-individual diversity ($K_b$) levels are observed to be relatively low for bats (Wilkinson & Chapman, 1991), crickets (Rand & Harrison, 1989), and sturgeon (Brown *et al.,* 1992), with values ranging from 0.13 to 0.18. In contrast, $K_b$ estimates are elevated in cod (0.51; Árnason & Rand, 1992) and the root-knot nematodes (0.50) studied here (Table 1). Moreover, total diversity ($K_t$) is measured to be 0.83 (Table 1)

for these same *M. incognita* isolates, a level higher than that published for any other organism (0.47-0.76). These elevated diversity levels are likely to be a consequence of the complex spectrum of mtDNA size classes maintained within an individual nematode (Fig. 1). Root-knot nematodes analyzed in the present study can carry up to thirteen easily detectable 63 bp VNTR alleles (Fig. 1, lane 7), most probably a result of high mutation rates to different copy numbers at the 63 bp VNTR locus.

Maintenance of diversity within individuals is thought to occur by rapid changes in VNTR copy number that overcome processes which tend to homogenize mtDNA populations. These include preferential replication of selected mtDNA haplotypes and drift during vegetative germ line cell divisions, which if not countered, would ultimately result in fixation of different mtDNA forms within individuals (Birky *et al.*, 1989). While the present data cannot discriminate among the relative combination of these factors, the maintenance of extensive heteroplasmy indicates that the rate of mutation to different 63 bp repeat copies exceeds the effects of mechanisms that drive homoplasmy.

Likelihood ratio tests (Sokal & Rohlf, 1987) reveal differences in allele frequency distributions that are significant among the six *M. incognita* isolates. This variation can be explained in part by a diminished ability to found new lineages within geographically separate isolates of sedentary endoparasites, where migration is negligible and dispersal is reliant on stochastic mechanical strategies. *C*-statistics (Table 1) apportion diversity into hierarchical levels, and provide a quantitative measure as to which population level (within individuals, among individuals within a population, or among populations) genetic variation occurs. For the 63 bp mtDNA VNTR, this method reveals that 58% of the diversity lies within *M. incognita* individuals ($C_i$), while 35% resides among individuals within an isolate ($C_{ip}$). $C_{pt}$ is useful in determining the relative amounts of variation among populations and contributes only 7% (Table 1) to the total mtDNA diversity. This value for $C_{pt}$ is entirely consistent with the RFLP study of Hugall *et al.* (1994), where low population diversity was demonstrated among Australian *Meloidogyne* populations using mtDNA RFLP techniques. With the addition of this data set to those now available (Hugall *et al.*, 1994; Cesaroni *et al.*, 1997), low but quantitatively significant differences in mtDNA VNTR allele frequency distributions observed among populations appears to be an emergent trend.

Our results now permit a useful consideration of hierarchical statistics as a methodology to examine root-knot nematode population differentiation. The experimental design of this study employed some isolates of known genetic relatedness. Unexpectedly, likelihood analysis revealed that in the most crucial tests, which involved pairings among isolates with greater or with lesser relatedness, allele frequency distributions generated differences that were significant. This result was not anticipated, in that isolates of recent shared ancestry (B, E, D) might be expected to reveal a more similar range of allele sizes and frequency distributions relative to distantly related populations (A, C, F). That equivalent levels of repeat copy number divergence was involved in pooled data sets incorporating isolates B, E and D and independently A, C, and F calls into question the utility of hierarchical methodology at the 63 bp VNTR locus for examining *Meloidogyne* population structure. A similar conclusion was independently drawn in a recent study (Cesaroni *et al.*, 1997) that used mtDNA VNTRs to examine differentiation among European sea bass populations, but without the advantage of supportive information on genetic relationships that the present work provides.

Hyman and Whipple (1996) first evaluated the utility of a PCR-based assay targeting the 63 bp mtDNA VNTR to analyse root-knot nematode population differentiation. Stanton *et al.* (1997) employed an identical strategy to examine a small number of Australian *Meloidogyne* isolates, and concluded that while the variability at this mtDNA region looked promising, a more comprehensive study is necessary to confirm the stability of this locus as a useful genetic marker. The more detailed analysis contributed by the present work suggests the 63 bp VNTR alleles mutate to different copy numbers at a rapid rate. As such, homoplasy may obscure our ability to interpret data of this sort in the context of population structure. This locus, however, is one of three VNTRs found in the *Meloidogyne* mitochondrial genome, and preliminary evidence indicates the 102 bp VNTR is mutating to different sized alleles at a rate two- to three-fold less than that of the 63 bp repeat array (D.H. Lunt & B.C. Hyman, unpubl.). We are currently evaluating this locus as a suitable marker for inferring the structure of root-knot nematode populations.

**References**

ÁRNASON, E. & RAND, D.M. (1992). Heteroplasmy of short tandem repeats in mitochondrial DNA of Atlantic Cod, *Gadus morhua*. *Genetics*, 132: 211-220.

AVISE, J.C., ARNOLD, J., BALL, R. M., BERMINGHAM, E., LAMB, T., REEB, C. A. & SAUNDERS, N.C. (1987). Intraspecific phylogeography: The link between population genetics and systematics. *A. Rev. ecol. Syst.*, 18: 489-522.

BIRKY, C. W., FUERST, P. & MARUYAMA, T. (1989). Organelle gene diversity under migration, mutation and drift: equilibrium expectations, approach to equilibrium,

effects of heteroplasmic cells and comparison to nuclear genes. *Genetics*, 121: 613-627.

BROWN, J. R., BECKENBACH, A. T. & SMITH, M. J. (1992). Mitochondrial DNA length variation and heteroplasmy in populations of white sturgeon (*Acipenser transmontanus*). *Genetics*, 132: 221-228.

CASWELL-CHEN, E. P., WILLIAMSON, V. M. & WESTERDAHL, B. B. (1993). Applied biotechnology in nematology. *Suppl. J. Nematol.*, 25: 719-730.

CESARONI, D., VENANZETTI, F., ALLEGRUCCI, G. & SBORDONI, V. (1997). Mitochondrial DNA length variation and heteroplasmy in natural populations of the european sea bass, *Dicentrarchus labrax*. *Mol. Biol. Evol.*, 14: 560-568.

HARRISON, R. G. (1989). Animal mitochondrial DNA as a genetic marker in population and evolutionary biology. *Trends ecol. Evol.*, 4: 6-11.

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.

HYMAN, B. C. & WHIPPLE, L. E. (1996). Application of mitochondrial DNA polymorphism to *Meloidogyne* molecular population biology. *J. Nematol.*, 28: 268-276.

LUNT, D. H. & HYMAN, B. C. (1997). Animal mitochondrial DNA recombination. *Nature*, 387: 247.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular cloning: a laboratory manual.* New York, USA, Cold Spring Harbor Laboratory Press, x + 545 p.

OKIMOTO, R., CHAMBERLAIN, H. M., MACFARLANE, J. L. & WOLSTENHOLME, D. R. (1991). Repeated sequence sets in mitochondrial DNA molecules of root knot nematodes (*Meloidogyne*): nucleotide sequences, genome location and potential for host-race identification. *Nucleic Acids Res.*, 19: 1619-1626.

RAND, D. M. (1993). Endotherms, ectotherms and mitochondrial genome size variation. *J. mol. Evol.*, 37: 281-295.

RAND, D. M. & HARRISON, R. G. (1989). Molecular population genetics of mtDNA size variation in crickets. *Genetics*, 121: 551-569.

ROBERTS, P. A. (1995). Conceptual and practical aspects of variability in root-knot nematodes related to host plant resistance. *A. Rev. Phytopath.*, 33: 199-221.

ROBERTS, P. A., FRATE, C. A., MATTHEWS, W. C. & OSTERLI, P. P. (1995). Interactions of virulent *Meloidogyne incognita* and *Fusarium* wilt on resistant cowpea genotypes. *Phytopathology*, 85: 1288-1295.

SOKAL, R. R. & ROHLF, F. J. (1987). *Introduction to biostatistics.* New York, USA, W. H. Freeman & Co., xii + 363 p.

STANTON, J., HUGALL, A. & MORITZ, C. (1997). Nucleotide polymorphisms and an improved PCR-based mtDNA diagnostic for parthenogenetic root-knot nematodes (*Meloidogyne* spp.). *Fundam. appl. Nematol.*, 20: 261-268.

WHIPPLE, L. E. (1997). *Mitochondrial DNA length diversity among* Meloidogyne *isolates of established genetic relationships.* Masters Thesis, University of California, Riverside, CA, USA, 75 p.

WILKINSON, G. S. & CHAPMAN, A. M. (1991). Length and sequence variation in evening bat D-loop mtDNA. *Genetics*, 128: 607-617.

WRIGHT, S. (1922). Coefficient of inbreeding and relationship. *Am. Naturalist*, 56: 330-338.