1988

Estimation of Genetic Divergence in *Meloidogyne* Mitochondrial DNA

Thomas O. Powers
University of Nebraska-Lincoln, tpowers1@unl.edu

Laurie J. Sandall
University of Nebraska-Lincoln

JOURNAL OF NEMATOLOGY

VOLUME 20 OCTOBER 1988 NUMBER 4

Journal of Nematology 20(4):505–511. 1988.

Estimation of Genetic Divergence in *Meloidogyne* Mitochondrial DNA

THOMAS O. POWERS AND LAURIE J. SANDALL

Abstract: Restriction fragments from purified mitochondrial DNA can be readily detected following rapid end-labeling with [α-^{32}P]nucleoside triphosphates and separation by gel electrophoresis. Mitochondrial DNA from 12 populations of *Meloidogyne* species was digested with 12 restriction enzymes producing more than 60 restriction fragments for each species. The mitochondrial genome of *M. arenaria* is the most genetically distinct of the four species compared. *M. arenaria* shows approximately 2.1–3.1% nucleotide sequence divergence from the mitochondrial genomes of *M. javanica, M. incognita,* and *M. hapla.* Among the latter three species, interspecific estimates of sequence divergence range from 0.7 to 2.3%. Relatively high intraspecific variation in mitochondrial restriction fragment patterns was observed in *M. hapla.* Intraspecific variation in *M. incognita* resulted in sequence divergence estimates of 0.5–1.0%. Such polymorphisms can serve as genetic markers for discerning mitochondrial DNA genotypes in nematode populations in the same way that allozymes have been used to discern nuclear DNA genotypes.

Key words: Meloidogyne spp., mitochondrial DNA, molecular evolution, nucleotide sequence divergence.

The ability to directly probe an organism's genome greatly simplifies diagnosis and creates the potential to distinguish individuals and populations within a species (6,14). It also permits the empirical measurement and estimation of genetic divergence among species. Restriction endonucleases are the fundamental tools for such analyses. These enzymes cut DNA at specific recognition sequences, most commonly stretches of 4–6 nucleotides. The DNA fragments generated by restriction endonuclease digestion are separated on the basis of size by electrophoresis and can be visualized in several ways. End-labeling of DNA with radioactive nucleotides is an effective means for visualizing fragments in large-scale diagnostic surveys (2). Differences in restriction patterns among organisms are referred to as restriction fragment length polymorphisms (RFLP) and indicate genetic differentiation.

Numerous genetic and systematic studies have focused on mitochondrial DNA (mtDNA) (1,3,13). This discrete piece of extrachromosomal DNA is easily extracted and purified. Its high rate of evolution permits comparisons of specific or subspecific taxa (12,13). Furthermore, its uniparental pattern of inheritance and apparent lack of recombination make mtDNA an ideal molecule for examining maternal dispersal and establishing matriarchal lineages.

Techniques for purification and rapid detection of *Meloidogyne* mtDNA have been presented (8). The objectives of this study were to further characterize mtDNA from *Meloidogyne* populations and to estimate the percentage of nucleotide sequence divergence among mitochondrial genomes from these populations.

Received for publication 15 September 1987.

Journal Series No. 8454, Agricultural Research Division, University of Nebraska.

Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583-0722.

We thank Mischell Partridge and Cynthia Stryker for help in culturing nematodes and Dr. Garry Duncan for valuable discussions. We also thank Drs. J. Sasser, P. Roberts, V. R. Ferris, G. Griffin and J. Smolik for providing *Meloidogyne* isolates.

MATERIALS AND METHODS

Nematode isolates: All nematodes were maintained on greenhouse-grown tomato (*Lycopersicon esculentum* L. cv. Rutgers). Original isolations are presented in Table 1.

Preparation of mtDNA: Mitochondrial DNA was extracted from ca. 0.35 ml packed nematode eggs obtained by the method of Hussey (5) and purified by CsCl density gradient centrifugation (8).

Restriction endonuclease digestion: Restriction enzymes were obtained from Bethesda Research Laboratories or Boehringer Mannheim Laboratories and used according to manufacturer's recommendations. The genetically informative enzymes and their recognition sequences are EcoRI (GAATTC), MboI (GATC), HindIII (AAGCTT), HinfI (GANTC), TaqI (TCGA), DdeI (CTNAG), and ClaI (ATCGAT).

Labeling, electrophoresis, and autoradiography of DNA: The sensitivity of autoradiography coupled with end-labeling permits the visualization of small amounts (10–20 ng) of DNA. Ten nanograms of DNA were digested to completion with a restriction endonuclease in a total volume of 10 µl. The DNA fragments were then radioactively end-labeled (2) with the large fragment of *E. coli* DNA polymerase I (Klenow) and the appropriate [α-^{32}P]dNTP (0.5 units Klenow, 2 µCi[^{32}P]dNTP) for 20 minutes at 0 C. The choice of dNTP was dictated by the base sequence of the enzymatic recognition site. The fragments were separated electrophoretically in 0.7%, 1.0%, or 1.2% agarose gels with 0.09 M Tris, 0.09 M borate, 2.0 mM EDTA buffer, pH 8.0, for 3.5 hours at 100 V. The HindIII fragments of bacteriophage λ DNA were included as size standards. Gels were dried on Whatman 3 MM paper using a heated vacuum gel drying apparatus and autoradiographed without intensifying screens with Kodak AR film for 12 hours.

Restriction data analysis: The total proportion of shared fragments (F) between two mitochondrial genomes for seven restriction digestions was calculated by the formula,

$$F = \frac{2Nxy}{(Nx + Ny)}$$

Nx = number of fragments in genotype x, Ny = number of fragments in genotype y, Nxy = number of common fragments. F values were used to calculate estimates of nucleotide sequence divergence, p, by the method of Nei and Li (7):

$$p = 1 - \left[\frac{-F + (F^2 + 8F)^{1/2}}{2}\right]^{1/n}$$

where n is the number of base pairs recognized per cleavage site. p values calculated separately for enzymes with four and six base pair recognition sequences were combined by a simple weighted average of p values.

Results

Purified mtDNA was isolated from 12 populations of *Meloidogyne* spp. (Table 1). The DNA was digested by 12 restriction enzymes. Four enzymes—BamHI, XhoI, SstI, and KpnI—did not recognize any restriction sites in mtDNA from the 12 populations. AvaI recognized a single restriction site in all genomes. The remaining enzymes produced more than 60 total restriction fragments in each population (Table 2). Representative mtDNA restriction patterns generated by digestion with three of these enzymes—EcoRI, MboI, and HindIII—are presented in Figures 1–3.

Figure 1 is an autoradiograph comparing *Meloidogyne* mtDNA digested with EcoRI and end-labeled with [^{32}P]dATP. Race 1 and race 2 of *M. arenaria* (lanes A and B) display identical patterns but are different from other *Meloidogyne* species. The most notable difference is the presence of an approximately 3.0-kilobase (kb) fragment in *M. arenaria* which is absent in the other species. *M. incognita* (lanes D–H) and *M. hapla* (lanes I, K, L) share 2.2-kb and 1.0-kb fragments that are absent in *M. arenaria*, and only the 2.2-kb fragment is shared by *M. javanica*. *M. javanica* (lanes C, J) is further distinguished by a unique 0.5-kb fragment. The largest pair of fragments in Figure 1 displays slight differences in mobility among all four *Meloidogyne* species and within *M. incognita* populations. These differences appear to arise from size variation in the mitochondrial genomes rather than changes in restriction sites.

TABLE 1. Origins of *Meloidogyne* isolates used for restriction fragment analysis.

Lane	Species	Race†	Origin
A	*M. arenaria*	1	Florida (NCSU #351)
B	*M. arenaria*	2	North Carolina (NCSU #64)
C	*M. javanica*		Indio, California
D	*M. incognita*	2	California (NCSU E1135)
E	*M. incognita*	3	Alabama (NCSU #108)
F	*M. incognita*	4	(NCSU)
G	*M. incognita*		Indiana
H	*M. incognita*	1	Riverside, California
I	*M. hapla*		San Bernadino, California
J	*M. javanica*		Riverside, California
K	*M. hapla*		Nevada
L	*M. hapla*		Yankton, South Dakota
M	λ/Hind standard with fragment sizes 23.0, 9.4, 6.6, 4.0, 2.2, 2.0, 0.50, and 0.10 kb.		

† Numbers refer to host-specific races (11).

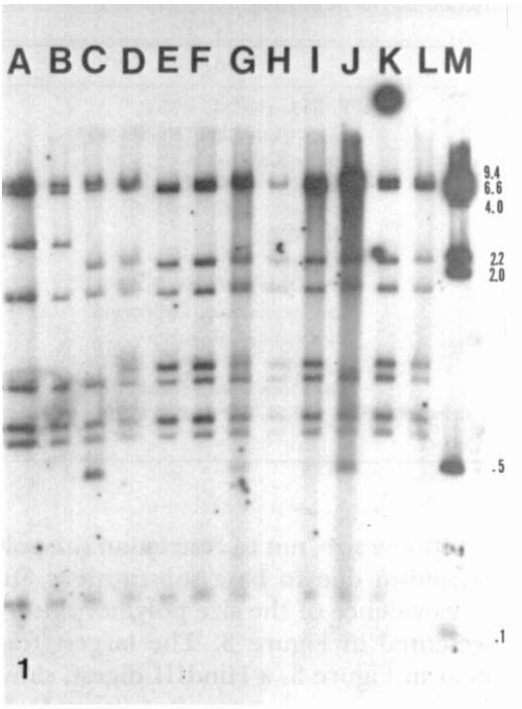
Figure 2 compares the mtDNA of the same *Meloidogyne* populations cut with MboI. In all the mitochondrial genomes, this enzyme produces at least 15 fragments. Both races of *M. arenaria* are identical for the MboI fragments, but they can be discriminated easily from the other three species. *M. javanica, M. incognita,* and *M. hapla* populations have similar MboI patterns; however, mobility of the three largest fragments varies among populations. Races 2–4 of *M. incognita* (lanes D–F) vary in the size of the second mitochondrial fragment. In race 2 the second fragment is approximately 0.5 kb smaller than the 4.0-kb first fragment. In race 3 the first and second fragments are approximately the same size, and in race 4 the first fragment is close to 4.5 kb in size. This polymorphism is most likely due to a difference in genome size, not to restriction site polymorphism due to base substitution. Further evidence of the size polymorphism is presented in Figure 3. The largest fragment in Figure 3, a HindIII digest, shows the size increase in races 2–4 (lanes D–F). In this figure the size increase is not as dramatic because the fragment is larger and the increase is therefore proportionally smaller, but nonetheless it illustrates the nature of this intraspecific mtDNA polymorphism.

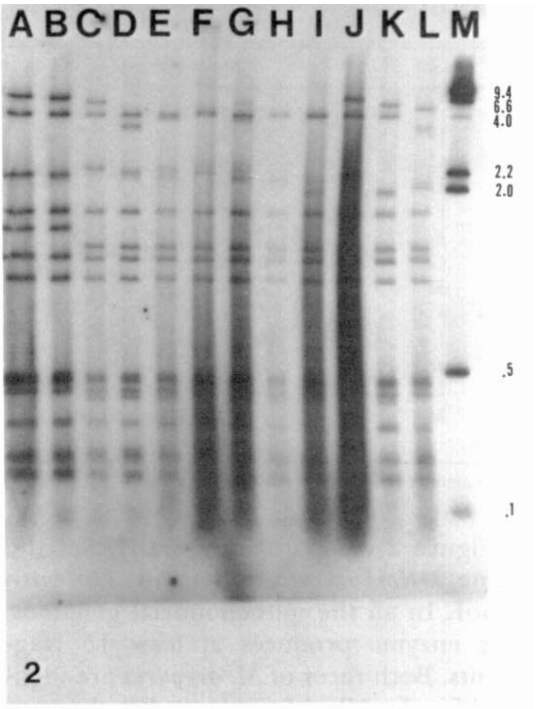
Figure 3 reveals two other features of the genomes under comparison. The *M. hapla* population originating from South Dakota (lane L) has a two-fragment pattern as in *M. arenaria,* whereas the other genomes representative of *M. hapla* (lanes I, K) have a pattern similar to *M. incognita* and *M. javanica.* This intraspecific poly-

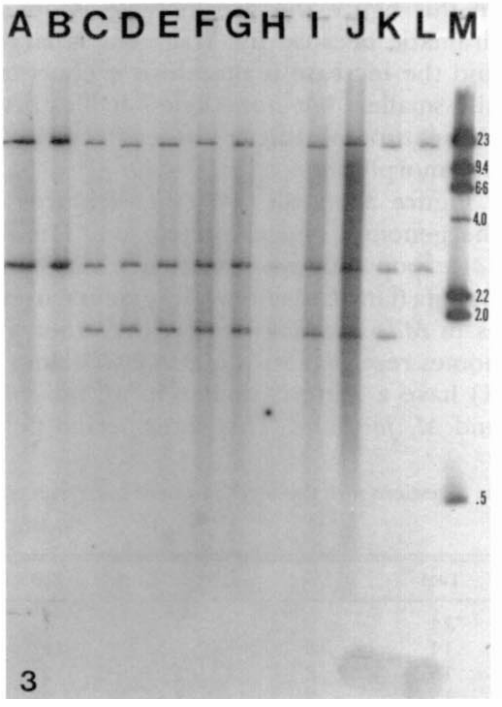
TABLE 2. Number of scorable mtDNA fragments from digestions with the seven restriction enzymes used for calculating estimates of nucleotide divergence.

MtDNA†	MboI	EcoRI	HinfI	TaqI	DdeI	HindIII	ClaI
A, B	13	8	14	14	9	2	2
C	15	9	10	14	8	3	2
D	15	9	12	15	8	3	2
E	16	9	12	15	7	3	2
F	12	9	12	15	8	3	2
G	18	10	14	—	8	3	2
H	13	9	10	15	8	3	2
I	15	9	12	13	8	3	2
J	11	9	11	9	8	3	2
K	14	9	13	15	8	3	2
L	13	9	13	14	8	2	2

† Letters refer to isolates described in Table 1.







FIGS. 1–3. Mitochondrial DNA from *Meloidogyne* populations A–L (Table 1) digested with restriction endonucleases and end-labeled with [^{32}P] nucleoside triphosphates. Lane M is phage λ DNA digested with HindIII. Fragment size is presented in Table 1. 1) EcoR1 digestion end-labeled with [^{32}P]dATP. Arrow indicates 3.0-kb fragment in lanes A and B. 2) MboI digestion end-labeled with [^{32}P]dGTP. 3) HindIII digestion end-labeled with [^{32}P]dATP. Figures 1–3 are 1.0%, 1.2%, and 0.7% agarose gels, respectively.

TABLE 3. Estimates of nucleotide sequence divergence, p, between mitochondrial genomes of the *Meloidogyne* species listed in Table 1.

MtDNA compared	A, B	C	D	E	F	G	H	I	J	K	L
A, B	—	0.029	0.022	0.021	0.022	0.025	0.022	0.025	0.028	0.024	0.031
C	0.69	—	0.013	0.012	0.013	0.008	0.014	0.008	0.011	0.015	0.016
D	0.75	0.84	—	0.008	0.005	0.010	0.008	0.007	0.021	0.014	0.011
E	0.76	0.87	0.90	—	0.008	0.009	0.008	0.007	0.021	0.016	0.017
F	0.75	0.84	0.94	0.90	—	0.017	0.007	0.009	0.017	0.015	0.012
G	0.66	0.89	0.87	0.89	0.80	—	0.007	0.010	0.015	0.014	0.022
H	0.75	0.85	0.89	0.89	0.92	0.85	—	0.010	0.011	0.016	0.012
I	0.74	0.90	0.91	0.91	0.89	0.87	0.88	—	0.018	0.015	0.013
J	0.70	0.87	0.83	0.76	0.80	0.82	0.86	0.79	—	0.023	0.022
K	0.73	0.82	0.83	0.82	0.82	0.83	0.81	0.82	0.74	—	0.018
L	0.75	0.82	0.86	0.80	0.85	0.75	0.86	0.85	0.75	0.81	—

Values above the diagonal are pairwise estimates of nucleotide sequence divergence, p, as calculated in Nei and Li, 1979. Values below the diagonal are calculations of total proportion of shared fragments, F, of seven restriction digestions.

morphism underscores the relatively high degree of variation found within *M. hapla*. Figure 3 also displays evidence of a mixture of genotypes in population G, a *M. incognita* isolate from Indiana. The largest fragment in lane G actually contains two fragments of similar size. Digestion with other enzymes indicates that in addition to a prominent *M. incognita*-like genome in the population, a small percentage of individuals possess a genotype similar to that extracted from the two *M. javanica* populations. Population G was not raised from a single egg mass; therefore there is a greater likelihood of it being a genetically mixed population.

Estimates of nucleotide sequence divergence, p, were calculated from restriction fragment data; Table 3 presents a matrix of genetic distances for all pairwise sample comparisons. The greatest sequence divergence found between mitochondrial genotypes was p = 0.031 (or 3.1%) between *M. arenaria* and the *M. hapla* isolate from South Dakota. All sequence divergence estimates for interspecific comparisons with *M. arenaria* exceeded 2.0%. This estimate is in sharp contrast to the complete lack of measurable divergence between the two *M. arenaria* mitochondrial genomes. Intraspecific divergence was found in all other species. Comparisons among *M. incognita* ranged from 0.5 to 1.0%, and estimates of 1.3–1.8% were found among the three *M. hapla* isolates.

A phenogram (Fig. 4) was constructed from p values in Table 3 according to the unweighted pair-group method (UPGMA) (10). Two major genotypic clusters distinguished by 2.5% sequence divergence separate *M. arenaria* and the other species. The two *M. javanica* genomes form a subcluster branching at 1.5% divergence, in

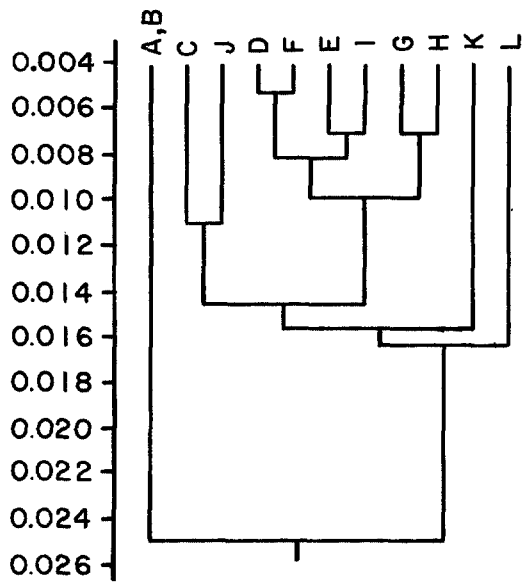


FIG. 4. Phenogram calculated from p values by the unweighted pair group method, UPGMA. A–L represent mitochondrial genomes from the *Meloidogyne* isolates in Table 1.

contrast to the *M. hapla* genomes, which are characterized by a relatively high degree of intraspecific variation and do not form a distinct cluster in the phenogram. The *M. hapla* isolate from California is more similar in its mitochondrial genotype to *M. incognita* isolates than it is to the other *M. hapla* genomes.

Discussion

Previous studies have indicated that analysis of *Meloidogyne* mitochondrial DNA may be useful for species identification (8). The current study has used a technique of higher resolution, [^{32}P] end-labeling of restriction fragments, to further characterize the *Meloidogyne* mitochondrial genome and to provide estimates of nucleotide sequence divergence among genomes.

Before restriction patterns of mtDNA can be used for species identification, it is important to consider some theoretical concerns about the relationship of a maternally inherited genome and species boundaries. The degree of mitochondrial divergence between two species is a function of mutation rate and time, since the species shared an ancestral genome. Assuming the mutation rate is constant for nematode mtDNA and there is no mtDNA recombination or interspecies transfer, mitochondrial restriction patterns may reliably indicate the amount of genetic divergence occurring since speciation. Generally, the more recent the speciation event, the more similar the restriction pattern. Mitochondrial divergence, however, does not begin or cease with speciation. If sufficient time has passed to allow for genetic divergence, two conspecific individuals in the same population may possess different mitochondrial genomes. Therefore, it is important to consider intraspecific variation when using mitochondrial restriction patterns as species-specific markers.

Two other causes of concern about possible discordance between biological species and mtDNA genotype are interspecies transfer of mtDNA and differential extinction of maternal lineages. The parthenogenetic mode of reproduction of the *Meloidogyne* species examined in this study makes interspecies transfer of mtDNA unlikely. In amphimictic organisms there are few unambiguous cases of mtDNA transfer across species boundaries. Wilson et al. (13) have evaluated all putative cases and concluded there is no evidence that "mtDNA is more capable of moving across a hybrid zone than is nuclear DNA."

The theoretical basis for discordance due to stochastic extinction of maternal lineages has been demonstrated by computer simulations (1). An important finding of these simulations is that theoretically the phylogenetic arrangement of matriarchal lineages can lack concordance with species boundaries, especially when speciations are very recent. To date there are too few species-level mitochondrial phylogenies to evaluate the extent and effect of lineage extinctions.

Our survey of *Meloidogyne* mtDNA restriction patterns indicates that in most cases extensive genetic divergence has not obscured species boundaries. *M. arenaria*, the most divergent of the four species analyzed, still shares approximately 75% of its restriction fragments with the other species. No mitochondrial divergence was detected between the two *M. arenaria* host races; yet chromosomal and enzymatic analyses have shown this species to be highly variable (4). The intraspecific homogeneity we observed may be due to a small sample size.

Pairwise species comparisons among *M. javanica*, *M. incognita*, and *M. hapla* show that these species share 80–90% of their mitochondrial restriction fragments. This degree of divergence contrasts with the considerable divergence (less than 25% shared restriction fragments) found between two sibling species of cyst nematodes, *Heterodera glycines* and *H. schachtii* (9). The relative similarity within *Meloidogyne* species may reflect more recent speciation events in these parthenogenetic nematodes. Surprisingly the species relationships indicated by the mitochondrial data are not in agreement with those derived from enzymatic data (4). Specifically,

M. hapla does not appear distantly related to the other obligatory mitotic parthenogenetic species and the mitochondrial data does not support a close relationship between *M. javanica* and *M. arenaria*. However, an assessment of phylogenetic relationships was not the objective of this study. A phylogenetic analysis of *Meloidogyne* mitochondrial genomes will be more informative when comparative restriction site data (comparative restriction maps) rather than restriction fragment data are available. This study does suggest that there is sufficient genetic divergence among populations and species of *Meloidogyne* to conduct phylogenetic studies. Furthermore, it is evident that mitochondrial restriction patterns can serve as population-specific genetic markers and that strategies for species identification based on mitochondrial RFLP can be developed.

Literature Cited

1. Avise, J. C. 1986. Mitochondrial DNA and the evolutionary genetics of higher animals. Philosophical Transactions of the Royal Society of London B 312:325–342.

2. Brown, W. M. 1980. Polymorphism in mitochondrial DNA of humans as revealed by restriction endonuclease analysis. Proceedings of the National Academy of Sciences USA. 77:3605–3609.

3. Brown, W. M. 1983. Evolution of animal mitochondrial DNA. Pp. 62–88 *in* M. Nei and R. K. Koehn, eds. Evolution of genes and proteins. Sunderland, MA: Sinauer.

4. Esbenshade, P. R., and A. C. Triantaphyllou. 1987. Enzymatic relationships and evolution in the genus *Meloidogyne* (Nematoda: Tylenchida). Journal of Nematology 19:8–18.

5. Hussey, R. S. 1971. A technique for obtaining quantities of living *Meloidogyne* females. Journal of Nematology 3:99–100.

6. Jeffreys, A. J., V. Wilson, and S. L. Thein. 1985. Individual-specific "fingerprints" of human DNA. Nature 316:76–79.

7. Nei, M., and W.-H. Li. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences USA 76:5269–5273.

8. Powers, T. O., E. G. Platzer, and B. C. Hyman. 1986. Species-specific restriction site polymorphism in root-knot nematode mitochondrial DNA. Journal of Nematology 18:288–293.

9. Radice, A. D., T. O. Powers, L. J. Sandall, and R. D. Riggs. 1988. Comparisons of mitochondrial DNA from the sibling species *Heterodera glycines* and *H. schachtii*. Journal of Nematology 20:443–450.

10. Sneath, P. H. A., and R. R. Sokal. 1973. Numerical taxonomy. San Francisco: W. H. Freeman and Co.

11. Taylor, A. L., and J. N. Sasser. 1978. Biology, identification and control of root-knot nematodes (*Meloidogyne* species). Raleigh: North Carolina State University Graphics.

12. Vawter, L., and W. M. Brown. 1986. Nuclear and mitochondrial DNA comparisons reveal extreme rate variation in the molecular clock. Science 234: 194–196.

13. Wilson, A. C., R. L. Cann, S. M. Carr, M. George, U. B. Gyllensten, K. M. Helm-Bychowski, R. G. Higuchi, S. R. Palumbi, E. M. Prager, R. D. Sage, and M. Stoneking. 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biological Journal of the Linnean Society 26:375–400.

14. Wirth, D. F., W. O. Rogers, R. Barker, Jr., H. Dourado, L. Suesebang, and B. Albuquerque. 1986. Leishmaniasis and malaria: New tools for epidemiologic analysis. Science 234:975–979.