Natural Selection with Nuclear and Cytoplasmic Transmission. III. Joint Analysis of Segregation and mtDNA in *Drosophila melanogaster*

Andrew G. Clark and Eva M. S. Lyckegaard

Department of Biology, Pennsylvania State University, University Park, Pennsylvania 16802

Manuscript received August 20, 1987
Revised copy accepted November 21, 1987

ABSTRACT

Despite the widespread use of mitochondrial DNA by evolutionary geneticists, relatively little effort has been spent assessing the magnitude of forces maintaining mtDNA sequence diversity. In this study the influence of cytoplasmic variation on viability in Drosophila was examined by analysis of second chromosome segregation. A factorial experiment with balancer chromosomes permitted the effects of cytoplasm and reciprocal crosses to be individually distinguished. The first test used six lines of diverse geographic origin, testing the segregation of all six second chromosomes in all six cytoplasms. The second and third tests were also factorial designs, but used flies from one population in central Pennsylvania. The fourth test was a large chain cross, using 28 lines from the same Pennsylvania population. Only the first test detected a significant nuclear-cytoplasmic effect. Restriction site variation in the mtDNA of all of these lines was assayed by Southern blotting, and statistical tests were performed in an effort to detect an influence of mtDNA type on fitness components. Posterior linear contrasts revealed an effect of mtDNA on segregation only among lines of diverse geographic origin. Within a population, no such influence was detected, even though the experiment was sufficiently large to have revealed statistical significance of a 0.5% segregation difference with a 57% probability.

BY virtue of its generally high level of sequence diversity and unisexual transmission, mitochondrial DNA has proven to be a powerful tool for identification of matrilineal clones and inferences of population structure. The high diversity can be explained in part by the rapid rate of nucleotide substitution (BROWN, GEORGE and WILSON 1979), making mtDNA particularly useful for phylogenetic inference of closely related species and for fine scale biogeography (AVISE 1986; DESALLE, GIDDINGS and KANESHIRO 1986; DESALLE *et al.* 1987; HALE and BECKENBACH 1985; LATORRE, MOYA and AYALA 1986; CANN, STONEKING and WILSON 1987; WILSON *et al.* 1985) and in the analysis of hybrid zones (ASMUSSEN, ARNOLD and AVISE 1987; AVISE and SAUNDERS 1984; CARR *et al.* 1986; HARRISON, RAND and WHEELER 1987). The maternal inheritance of mtDNA has also lead to some novel findings of unidirectional introgression (FERRIS *et al.* 1983; POWELL 1983; SOLIGNAC and MONNEROT 1986). Rapid sequence divergence, and consequent high levels of intrapopulation polymorphism are not, however, properties of mtDNA common to all taxa (POWELL *et al.* 1986; VAWTER and BROWN 1986).

Implicit in much of this work is the assumption that the variation is selectively neutral, but very little effort has been focused on direct experimental measurement of the magnitudes of the forces that maintain the observed levels and patterns of variation. The mathematical theory is well ahead of the experimental work regarding the maintenance of mtDNA variation. A number of theoretical papers have developed expressions for the expected level of variation in a population at steady state with neutral mutations and random drift (TAKAHATA and MARUYAMA 1981; BIRKY, MARUYAMA and FUERST 1983; TAKAHATA 1983; CHAPMAN *et al.* 1982). Direct experimental tests of these models would require estimates of numbers of mitochondria per cell, numbers of cell divisions, numbers of individuals in the population and mutation rates, and the errors in all these estimates would be confounded in making a prediction of the steady state level of polymorphism. The consequences of population subdivision and limited migration have also received theoretical attention (TAKAHATA and SLATKIN 1983, 1984; TAKAHATA 1985; TAKAHATA and PALUMBI 1985). The primary conclusion from theory that incorporates selective effects of mtDNA variants is that polymorphism is not easily maintained by natural selection acting alone (CLARK 1984). Models that admit stable selective polymorphism require precise tuning of parameters, with sexual asymmetry or frequency dependence (GREGORIUS and ROSS 1984).

An observation that cannot be ignored when considering the selective significance of mtDNA variation is the remarkable conservation of gene content and order in the mammalian mitochondrial map (CLAY-

TON 1984; MONNAT and LOEB 1985). Human, mouse and cow mtDNA can be aligned gene-for-gene over nearly the entire genome. Despite the conservation of gene order, and the fact that the mitochondrial genome is very economical (with only 89 of the 16569 bp outside of regions of identified function in the one human mtDNA sequenced by ANDERSON *et al.* 1981) the amount of sequence polymorphism and the rate of nucleotide substitution are high. Although some animals show mtDNA rearrangements (MORITZ and BROWN 1986), plants in general show a greater diversity of mitochondrial genome size and content. Within species, plants show extensive mtDNA rearrangements, some of which are implicated in cytoplasmic male sterility (LEVINGS 1983; DEWEY, LEVINGS and TIMOTHY 1986). These are particularly important from an evolutionary standpoint, since they not only are examples of mtDNA variants with phenotypic expression, but that expression is directly associated with an important component of fitness. Leaf variegation is another well known cytoplasmically transmitted trait in plants, mediated in this case by defective chloroplasts (KIRK and TILNEY-BASSETT 1967). In yeast, the suppressive *petite* mutations and many drug resistant mutations are mapped to mtDNA (BEALE and KNOWLES 1978), but in higher animals the phenotypic expression of mtDNA variation is either very subtle or absent. However, it is possible to recover mammalian cell lines that show drug resistance mediated by mtDNA mutations (WALLACE 1981; SLOTT, SHADE and LANSMAN 1983). Recently there has been a suggestion of an association of mtDNA with maternally transmitted human diseases (MERRIL and HARRINGTON 1985).

It is not necessary to catalog dramatic phenotypic effects of mtDNA variation in order to assess its selective significance. Two alternatives include testing the fit of observed allele frequency distributions to the neutral expectation and direct experimental tests of selection components. WHITTAM *et al.* (1986), using extensive, high resolution data on human mtDNA restriction site variation, tested the fit of these data to the distribution expected under the model of neutral mutations with Ewens-Watterson sampling theory. About 29% of the tests deviated from neutrality in the direction of a deficit of genetic diversity. The weakness of this indirect test is underscored by observing that this departure could be due to several causes, including population substructuring, departure from steady state conditions, or purifying selection, and these cannot be unambiguously distinguished.

Direct tests of selective differences mediated by mtDNA can be done by either directly competing lines that differ in mtDNA restriction patterns (A. F. MACRAE and W. W. ANDERSON, personal communication), or by measuring conditional fitnesses of nuclear genetic variants in different cytoplasmic backgrounds. The latter approach is motivated by the hypothesis that nuclear-cytoplasmic interactions are important in mitochondrial evolution, since the two genomes must be intimately integrated in function and expression. CLARK (1984) showed that, in a simple model with one nuclear locus and two mitochondrial types, viability selection acting alone cannot maintain mitochondrial polymorphism. Only by invoking more complex models of selection involving frequency dependence of fitnesses, or male/female differences in viabilities, can stable mitochondrial polymorphism be maintained. The empirical assessment of mitochondrial effects mediated through nuclear-cytoplasmic interactions therefore has direct bearing on whether the cytoplasmic effects can maintain polymorphism in mtDNA. In the study reported here, the second chromosome segregations of a number of wild isolates of *Drosophila melanogaster* were scored in a series of cytoplasms in a way that allowed partitioning of cytoplasmic and reciprocal cross effects. Posterior statistical tests were then performed to assess the effects of mtDNA restriction site variation on the observed variation in segregation.

MATERIALS AND METHODS

Drosophila strains: Six strains were employed in an assay of variation among geographically diverse populations, described in detail in CLARK (1985). These include isofemale lines AH198S (Apple Hill, California), DV92S (Death Valley, California), Egà-1 (Egà, Denmark), GB8S (Gundlach Bundschu Winery, northern California), R1 (Rothrock State Forest, central Pennsylvania) and St-5 (Stillwater, Minnesota). In addition, another 28 isofemale lines were established in the summer of 1983 from collections made by A. CLARK near the boundary of the Rothrock State Forest. A laboratory stock of *B1 L^2/SM5* was constructed with the P cytotype following a protocol described in CLARK (1985).

Extraction of second chromosomes: Virgin females from each isofemale line were crossed to *B1 L^2/SM5* (P) males. The excellent fertility of F_1 females in all lines suggested that none of them had the M cytotype, and additional test crosses verified this (SCHAEFFER, KIDWELL and FAUSTO-STERLING 1979). Virgin +/*SM5* female offspring were mated individually to *B1 L^2/SM5* males. Resulting +/*SM5* offspring had the same wild second chromosome, and they were sib mated to test for recessive lethality. Recessive lethal chromosomes were not used in the study, and the second chromosomes that were used represent a single randomly chosen non-lethal from each isofemale line. The genetic background of all extracted lines was replaced by backcrossing for ten generations to *B1 L^2/SM5* males. The resulting lines were isogenic for a wild second chromosome, and had the original cytoplasm of the isofemale strain (for any strictly maternally transmitted factors), but the rest of the genome was replaced by that of the *B1 L^2/SM5* (P) line. Background replacement was verified by protein electrophoresis, as described in CLARK (1985). Two different second chromosomes were extracted from each of the isofemale lines R2 through R10, and one of each was used in the factorial crosses (experi-

ments 2 and 3 below), while the other was used in the chain cross.

Cytoplasmic exchange: Letting $+_j/SM5$ (i) represent a line with the jth second chromosome in the ith cytoplasm, stocks such as this were constructed by crossing $+_i/SM5$ (i) females with $+_j/+_j$ (j) males. Curly winged female offspring had the genotype $+_j/SM5$ (i), and when these were mated with $+_j/+_j$ (j) males, the resulting offspring were $+_j/SM5$ (i) and $+_j/+_j$ (i). Once these mixed lines were constructed, they were maintained by mass mating.

Segregation tests: Four experiments were performed to test the segregation of offspring in different cytoplasms. The first three were factorial experiments, testing all combinations of second chromosomes and cytoplasms. The first experiment employed the six lines of diverse geographic origin, the second employed four lines from the Rothrock forest and the third employed five lines from the Rothrock forest. The fourth experiment was a round robin cross, performing a segregation test of the ith chromosome in the $(i - 1)$th, the ith, and the $(i + 1)$th cytoplasm for the set of 28 extracted lines from Rothrock State Forest. For each combination of chromosome i and cytoplasm j, the following two crosses were performed: reciprocal 1 − $+_i/SM5$ (j) × $+_j/+_i$ (j) and reciprocal 2 − $+_j/+_i$ (j) × $+_j/SM5$ (j). In all segregation tests, two virgin females and two males aged 3–4 days were placed in 95-mm shell vials with 5 ml of medium. Mating and egg laying occurred for 4 days, and the adult flies were transferred to fresh vials. These pairs of vials are referred to as duplicates. Flies were removed from the second vial after four more days of mating and egg laying. On the 17th and 18th day after egg laying began, the adult progeny were scored for sex and wing phenotype (*Curly vs.* wild). For each cross in experiments one, two and three, 12 replicate pairs of duplicates, organized approximately in four blocks of three replicates, were scored. Segregation is given as the number of wild progeny divided by one plus the total count of progeny (HALDANE 1956). Eight replicate crosses were followed in the fourth experiment.

mtDNA purification: Mitochondrial DNA was extracted from arbitrary lines of *D. melanogaster* to use as a probe in Southern blots. mtDNA was purified following the procedure of LANSMAN *et al.* (1981) with a few modifications. Fifty grams of third instar larvae were ground in a mortar and pestle, then homogenized in a glass/Teflon tissue homogenizer. Three spins of 5 min at 500 × *g* were done to pellet nuclei and cellular debris. Mitochondria were pelleted by a centrifugation at 13000 rpm for 20 min. A 1.5 M to 1.0 M sucrose step gradient was made to further purify intact mitochondria. Sarkosyl was used to lyse the mitochondria, and a CsCl gradient was prepared with a refractive index between 1.390 and 1.392. This was spun in a Beckman SW40Ti swinging bucket rotor at 38000 rpm for 44 hr. The top band of the gradient was nicked mtDNA with some linear nuclear DNA, and the second band was intact supercoiled circular mtDNA. These bands were individually recovered by puncturing the side of the tube with a syringe needle. After removal of ethidium bromide, probes were prepared by either nick translation (RIGBY *et al.* 1977) or random hexamer labeling (FEINBERG and VOGELSTEIN 1983).

Genomic DNA isolation: Total genomic DNA was isolated from the six geographically diverse lines and the 28 lines used in the chain cross experiment following a variation of the procedure of BENDER, SPIERER and HOGNESS (1983) kindly provided by C. LAURIE. Approximately 100 adult flies were ground in liquid nitrogen, and then homogenized in 2 ml of buffer consisting of 0.08 M NaCl, 0.16 M sucrose, 0.5% SDS, 58 mM EDTA, 124 mM Tris and 20 μl diethylpyrocarbonate, using a glass/Teflon tissue homogenizer. After a 30-min incubation at 65°, 0.3 ml 8 M potassium acetate were added and precipitation was allowed for 1 hr on ice. The sample was then centrifuged at 10,000 rpm in a Sorvall SA600 rotor for 5 min, and DNA was purified from the supernatant following a standard phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. This procedure gave excellent recovery of both nuclear genomic DNA and mitochondrial DNA. RNA was thoroughly removed with DNase-free RNase before electrophoresis.

Southern blot analysis: Genomic DNAs were digested with 19 restriction enzymes following the manufacturers' recommended conditions. Fragments were separated by horizontal agarose electrophoresis (0.9–1.1%), and DNA was transferred to Zetabind nylon filters following a variation of the protocol of SOUTHERN (1975) provided by A.M.F. CUNO. Labeled mtDNA probe was hybridized to the filters at 42° for approximately 20 hr, and washed with moderate stringency. Filters were exposed to Kodak x-ray film with exposures from 6 hr to 3 days. Of the 19 enzymes tried, only the 11 reported in Table 6 gave good digestions with clean transfers. The Bethesda Research Laboratories DNA fragment ladder was used as a length standard on the gels. All autoradiographs were digitized using a Numonics 2200 digitizer, and fragment lengths were estimated by regression, following SCHAFFER and SEDEROFF (1981). Significant length variation was detected by partitioning the error in fragment lengths among effects of gel replication and digitization error using nested analysis of variance.

RESULTS

Segregation analyses: A summary of the designs of the segregation experiments is presented in Table 1. All four experiments were balanced in that the number of replicates was the same for every treatment within an experiment. The experiments were performed in the order given in the table, with approximately 10 months elapsing between the beginning of the first and the end of the last experiment. The raw segregation values and their standard errors appear in Tables 2–4. The segregation values range from 0.301 to 0.626, equating to relative viabilities ranging from 0.60 to 1.25. There is a tendency of the segregation values to exceed the Mendelian value of 0.5, indicating that the chromosomes isolated for the study have some advantage in the isogenic state over the $+/SM5$ flies. Although one expects the average second chromosome isolated from a natural population to be somewhat deleterious when homozygous, the sample analyzed here succeeds in representing a broad range of viabilities. Despite the lack of a particularly stressful (crowded) environment, repeatable deviations from Mendelian ratios were found, and they are interpreted as being due to differences in probability of surviving form zygote to adult. The first three experiments employed 12 replications that were organized in four blocks of three replicates, but this block structure was not strictly maintained. Heterogeneity among blocks was

TABLE 1

Data structures of segregation tests

Classification	Diverse	Rothrock-1	Rothrock-2	Chain
Chromosomes	6	4	5	28
Cytoplasms	6	4	5	28
Reciprocals	2	2	2	2
Replicates	12	12	12	8
Duplicates	2	2	2	2
Vials	1,728	768	1,200	2,688
Flies	154,339	53,080	99,031	227,326

discussed at some length in CLARK (1985), where the segregation among lines of diverse origin was analyzed. In that experiment, there was an increasing trend in the error variance, but no such pattern was seen in any of the other trials. Blocks were in fact homogeneous in the other three studies.

Figure 1 presents the raw segregation data from the chain crosses in a way that shows that chromosomal effects are greater in magnitude than cytoplasmic effects. The first three bars on the left in the figure represent segregation of the R1 chromosome determined by the cross $+/+ \times +/SM5$ (and the reciprocal cross) in the R28, the R1 and the R2 cytoplasms respectively. The small horizontal dashes represent the expected segregation obtained from the linear model whose description follows. Formal tests of the significance of these effects were done using analysis of variance. A partitioning of the components of variance in the segregations was accomplished by fitting the mixed linear model:

$$s_{ijkl} = u + n_i + c_j + r_k + nc_{ij} + nr_{ik} + cr_{jk} + ncr_{ijk} + e_{ijkl}$$

where s_{ijkl} refers to the segregation of the ith chromosome in the jth cytoplasm of the kth reciprocal of the lth replicate. The terms n_i, c_j, and r_k refer to the effects of the nuclear genes (second chromosomes), the cytoplasms and reciprocal crosses, respectively. Note that the design allows orthogonal partitioning of the effects of reciprocal crosses and cytoplasms, a unique and desirable feature. The last term is the error term, assumed to be normally distributed with mean zero, and remaining terms are hierarchical interactions. The nuclear and cytoplasmic effects are treated as random effects, while reciprocal crosses are considered fixed effects, because of the discrete, repeatable difference between the *Cy* × wild and wild × *Cy* crosses. A more complete analysis employing blocks as another random effect was performed, with various other effects nested within blocks. No significant terms involving blocks were revealed by this model, so for simplicity it is not reported here. Similarly, the data were analyzed for both sexes separately and pooled, and results were qualitatively the same (CLARK 1985).

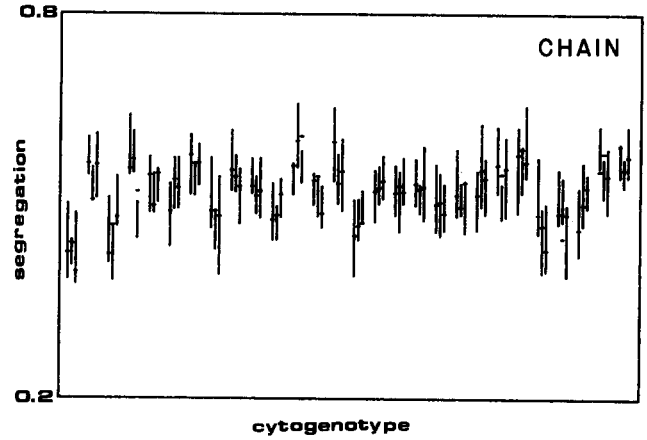

FIGURE 1.—Segregation of the 28 second chromosomes in each of three cytoplasms. Segregation is defined as the fraction of the offspring from a $+/SM5 \times +/+$ cross that are wild in phenotype. The first group of three bars shows ±2 SE, centered on the mean of the segregation of the second chromosome of line R1 within the R28, the R1 and the R2 cytoplasm, respectively. Small horizontal lines are the expected segregation based on the linear model.

The analysis of variance was performed with the help of the GLM procedure (SAS Institute 1985), and results appear in Table 5. The analyses were done on both raw segregation data and on angular transformed data, but since they yielded no qualitative differences, only untransformed results are reported. Means and variances were found to be uncorrelated, and residual errors were independent of predicted segregations. In general, the analysis of residuals revealed no significant departures from the assumptions of the linear model (SEARLE 1971). The results show that there was a significant nuclear effect in all experiments, a nuclear-cytoplasmic interaction in only the test with lines of diverse geographic origin, and a weak direct cytoplasmic effect was seen in the Rothrock-2 experiment. Reciprocal crosses had no effect, supporting the confidence in the interaction found in the first experiment.

Another useful way to examine the results of a linear analysis is to partition the total variance into the components of the random effects. These estimates are obtained by various linear combinations of the observed mean squares, and the linear combinations are determined by the expressions for the model's expected mean squares. Table 5 gives the results of this analysis, and shows that the bulk of the variance falls in the error term. Error in this case includes the inevitable binomial sampling error that is generated in scoring a dichotomous trait. The nuclear effects are generally fairly small, but in the case of the Rothrock-1 experiment, one of the second chromosomes was mildly deleterious, and this results in a much larger nuclear chromosomal component

TABLE 2

Means and standard errors of segregations in Rothrock-1 experiment

	Cytoplasm			
Chromosome	R2	R3	R4	R5
R2	0.319 ± 0.015	0.301 ± 0.010	0.350 ± 0.023	0.307 ± 0.011
R3	0.511 ± 0.013	0.444 ± 0.024	0.465 ± 0.018	0.374 ± 0.024
R4	0.478 ± 0.015	0.490 ± 0.014	0.470 ± 0.008	0.452 ± 0.016
R5	0.432 ± 0.013	0.432 ± 0.012	0.447 ± 0.026	0.424 ± 0.022

TABLE 3

Means and standard errors of segregations in Rothrock-2 experiment

	Cytoplasm				
Chromosome	R6	R7	R8	R9	R10
R6	0.545 ± 0.012	0.584 ± 0.011	0.566 ± 0.016	0.591 ± 0.013	0.581 ± 0.021
R7	0.565 ± 0.012	0.572 ± 0.017	0.572 ± 0.019	0.612 ± 0.028	0.580 ± 0.023
R8	0.518 ± 0.016	0.488 ± 0.023	0.560 ± 0.017	0.535 ± 0.012	0.588 ± 0.020
R9	0.487 ± 0.011	0.550 ± 0.023	0.576 ± 0.025	0.545 ± 0.028	0.546 ± 0.017
R10	0.515 ± 0.014	0.598 ± 0.026	0.626 ± 0.019	0.550 ± 0.026	0.594 ± 0.028

TABLE 4

Means and standard errors of segregations in chain experiment

	Cytoplasm		
Chromosome	I − 1	I	I + 1
R1	0.444 ± 0.029	0.426 ± 0.010	0.433 ± 0.028
R2	0.578 ± 0.015	0.533 ± 0.013	0.562 ± 0.025
R3	0.462 ± 0.026	0.426 ± 0.021	0.508 ± 0.020
R4	0.596 ± 0.023	0.584 ± 0.016	0.477 ± 0.014
R5	0.533 ± 0.022	0.520 ± 0.015	0.532 ± 0.013
R6	0.486 ± 0.025	0.536 ± 0.020	0.536 ± 0.020
R7	0.565 ± 0.023	0.540 ± 0.012	0.564 ± 0.016
R8	0.520 ± 0.028	0.461 ± 0.014	0.469 ± 0.038
R9	0.572 ± 0.023	0.551 ± 0.014	0.515 ± 0.021
R10	0.547 ± 0.014	0.517 ± 0.014	0.528 ± 0.023
R11	0.491 ± 0.022	0.473 ± 0.013	0.513 ± 0.015
R12	0.543 ± 0.012	0.610 ± 0.025	0.562 ± 0.012
R13	0.526 ± 0.012	0.513 ± 0.015	0.499 ± 0.016
R14	0.597 ± 0.028	0.542 ± 0.019	0.549 ± 0.028
R15	0.450 ± 0.030	0.478 ± 0.016	0.500 ± 0.012
R16	0.516 ± 0.020	0.532 ± 0.015	0.545 ± 0.017
R17	0.525 ± 0.020	0.506 ± 0.024	0.539 ± 0.018
R18	0.537 ± 0.018	0.511 ± 0.011	0.535 ± 0.029
R19	0.512 ± 0.026	0.502 ± 0.024	0.498 ± 0.018
R20	0.536 ± 0.026	0.511 ± 0.012	0.498 ± 0.020
R21	0.508 ± 0.023	0.563 ± 0.032	0.530 ± 0.022
R22	0.573 ± 0.026	0.506 ± 0.014	0.545 ± 0.031
R23	0.556 ± 0.033	0.557 ± 0.018	0.600 ± 0.028
R24	0.516 ± 0.030	0.445 ± 0.025	0.450 ± 0.026
R25	0.515 ± 0.021	0.510 ± 0.017	0.446 ± 0.028
R26	0.475 ± 0.026	0.519 ± 0.024	0.523 ± 0.013
R27	0.593 ± 0.016	0.543 ± 0.015	0.540 ± 0.025
R28	0.585 ± 0.019	0.572 ± 0.013	0.552 ± 0.011

(and hence a reduction in the portion of variance due to error).

The analyses to this point have made the implicit assumption that every cytoplasm, isolated as a distinct maternal line from nature, may have distinct cytoplasmically transmitted factors that could affect viability. Many of these may share mitochondrial clones, and hence should not be considered as distinct cytoplasmically, if our intention is to determine the effects of mtDNA variation. By scoring mitochondrial identities using restriction mapping, the relevance of mtDNA variation to the observed segregation variation can be tested.

mtDNA analyses: In addition to variation detected as gains and losses of restriction sites, the mitochondrial DNA of *D. melanogaster* shows considerable variation in total length (REILLY and THOMAS 1980; HALE and SINGH 1986). This type of variation is manifested as quantitative changes in the migration of DNA fragments in agarose gels. In order to assess the confidence in the repeatability of these length estimates, lines with apparently subtle differences were run in replicates, and the error in length estimation attributable to gel-to-gel variation and digitization error was partitioned by nested analysis of variance. This procedure gave statistical confidence to the cases where length variants required subtle distinction of band migration. The confidence in DNA fragment length estimation is greatest for shorter fragments, and generally the fragment show-

TABLE 5

Analysis of variance and magnitudes of variance components from the segregation tests

Source	Diverse		Roth-1		Roth-2		Chain	
	MS	Var	MS	Var	MS	Var	MS	Var
Nuclear	0.061*	3.92	0.445***	35.46	0.051	2.77	0.061***	11.86
Cyto	0.035	1.60	0.042	2.01	0.056*	3.17	0.012	
N × C	0.017**	5.75	0.055	3.66	0.016	2.76	0.012	3.98
Recip	0.038		0.028		0.000		0.060	
N × R	0.013		0.040		0.004		0.008	
R × C	0.005		0.012		0.029		0.009	
N × C × R	0.007		0.012		0.014		0.011	
Error	0.007	88.73	0.007	58.87	0.009	91.29	0.007	84.15

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

MS refers to the mean squares obtained from the mixed model analysis of variance, and Var is the percentage of the variance component, given for the random effects only.

TABLE 6

Kruskal-Wallis tests of the relation between mitochondrial clones and segregation

Diverse lines				
Enzyme	No. of patterns	*K*	Configuration	Tail probability
*Ava*I	2	2.14	5 1	0.333
*Cla*I	2	3.85	3 3	0.108
*Eco*RI	2	3.43	2 4	0.127
*Hae*III	2	0.86	4 2	0.515
*Hin*dIII	1	0.00	6	1.000
*Hpa*II	3	2.50	4 1 1	0.473
*Mbo*I	2	2.14	5 1	0.332
*Pvu*II	2	2.14	5 1	0.318
*Rsa*I	3	1.28	4 1 1	0.674
*Taq*I	1	0.00	6	1.000
*Xba*I	2	0.21	4 2	0.802
All	6	5.00	1 1 1 1 1 1	
*Ava*I	1	0.00	9	1.000
*Cla*I	4	4.42	6 1 1 1	0.157
*Eco*RI	3	2.47	4 1 4	0.339
*Hae*III	3	0.18	5 3 1	0.944
*Hin*dIII	2	0.06	5 4	0.893
*Hpa*II	2	1.37	7 2	0.303
*Mbo*I	1	0.00	9	1.000
*Pvu*II	2	1.35	8 1	0.456
*Rsa*I	4	4.84	5 2 1 1	0.141
*Taq*I	3	0.54	4 3 2	0.828
*Xba*I	2	4.20	7 2	0.059
All	9	8.00	1 1 1 1 1 1 1 1 1	
*Ava*I	2	0.51	26 2	0.487
*Cla*I	5	5.99	17 4 3 1 3	0.193
*Eco*RI	3	1.02	18 7 3	0.626
*Hae*III	4	4.94	21 5 1 1	0.140
*Hin*dIII	3	2.86	20 7 1	0.241
*Hpa*II	3	2.43	25 2 1	0.354
*Mbo*I	3	3.37	26 1 1	0.136
*Pvu*II	2	1.11	27 1	0.334
*Rsa*I	4	2.40	21 3 3 1	0.550
*Taq*I	5	3.72	18 4 4 1 1	0.515
*Xba*I	2	0.28	15 13	0.560
All	24	25.02	3 2 2 1 1 . . .	0.176

ing the length variation was the longest fragment. This meant that for a given length variant, different enzymes would make the difference more or less apparent. The identities of restriction patterns were made with the help of a restriction map inferred from single and double digestions, and by making use of the map constructed by SOLIGNAC, MONNEROT and MOUNOLOU (1986). Three of the lines (R15, R17 and R20) showed clear heteroplasmy, in that there were two mitochondrial classes within samples derived from single females. Heteroplasmy is inferred when the sum of the fragment lengths exceeds 19 kb due to the appearance of an extra band. In all three cases, the extra band was a length variant. This may suggest a high rate of somatic mutations that generate length variation.

Counts of the mitochondrial genotypes identified by the patterns seen from 11 restriction endonucleases are presented in Table 6. Under the infinite alleles model, there is an expected steady state allele frequency distribution, and the goodness-of-fit of observed distributions can be tested using Ewens-Watterson sampling theory (EWENS 1979). Computer simulation gave confidence intervals on the gene identity, common allele frequency and the number of singleton alleles for the configurations revealed by each enzyme in the chain cross, and in no case did the observed data deviate significantly from these neutral expectations. Direct chi square tests of the goodness-of-fit of the observed and expected allele frequency distributions (CLARK 1987) also failed to reject neutrality, but this result should be interpreted with caution: the experiment was not initially designed to make best use of the Ewens-Watterson test, since the test is not very powerful and the number of maternal lineages isolated was small.

In order to assess the significance of the mitochondrial genotype on the phenotypic attributes (segregation and fecundity), canonical correlation and the Kruskal-Wallis test (LEHMANN 1975) were applied.

The data that were analyzed by canonical correlation consisted of tables with the pattern identity for each of the lines along with the segregation and fecundity. Canonical correlation constructs a vector of weights of the patterns in such a way as to maximize their correlation with the segregation. If the mitochondrial genotypes had a sufficiently large effect on viability or fecundity, this would be revealed by a significant canonical correlation. None of the correlations was significant for any of the analyses. The difficulty in interpreting this result is that it is not clear how strong an effect there would have to be in order to be able to detect it. There is no simple formula for the statistical power of the test, and although extensive computer simulation might give empirical estimates of power, the Kruskal-Wallis test provides a much more intuitively satisfying approach to the problem.

The idea behind the Kruskal-Wallis test is that if the mitochondrial genotype has an effect on segregation, then the rank ordering of all of the lines by their segregation coefficients should result in a nonrandom ordering of lines with respect to mtDNA patterns. The analysis can be done for the patterns revealed by single enzymes or for the patterns obtained using the data from all enzymes. Table 6 presents the results of the analysis. For each enzyme, the number of different patterns is given, and the count of lines with each pattern (the sample "configuration") is given. From the ranked segregations, a K statistic is derived based on the sum of the squared ranks (LEHMANN 1975). The critical value of K can be obtained in some cases from tables, but because it is necessary to have a table entry for each configuration, no table could be very complete. It is easiest to construct an empirical distribution of K by sampling over random permutations and generating the distribution of K in a computer (Figure 2). The area under this distribution to the right of the observed value represents the probability of getting a value of K greater than the observed value by chance. This is how the column headed "tail probability" was constructed.

None of the Kruskal-Wallis tests was significant at the 5% level, leading to the conclusion that the mitochondrial genotypes do not have a significant effect on viability measured by second chromosome segregation. The Kruskal-Wallis tests were repeated after stratifying the lines in two different ways. First, mtDNA haplotypes were identified based only on the site gain/loss data, ignoring the length variants. Second, the restriction site data were used to generate maximum parsimony trees, and lines were clustered into groups based on the degree of divergence in lineage (details of this analysis will be reported elsewhere). In neither case was a significant effect of mtDNA type detected by the Kruskal-Wallis test.

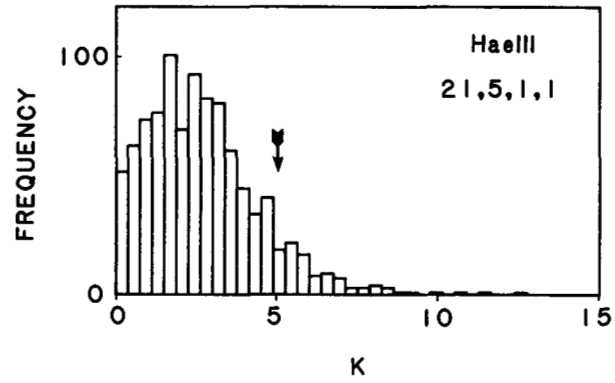

FIGURE 2.—The distribution of K statistics obtained by computer sampling over random permutations of the configuration 21, 5, 1, 1. The *arrow* indicates the actual K statistic from the Kruskal-Wallis test, obtained from the data for the enzyme *Hae*III in the chain cross. The area under the distribution and to the right of the *arrow* represents the probability of obtaining a K statistic greater than the observed value by chance. In this case, $P = 0.14$.

The conclusion that mtDNA variation does not affect segregation only gains credibility after the power of the statistical test (the probability of concluding significance when their is an effect) is quantified. This too was addressed by computer simulation. Consider a hypothetical case with two mtDNA clones in the chain experiment, where there are 14 lines with clone A and 14 lines with clone B. If clone A had a segregation advantage of 20%, then the ranking of segregations would probably place all 14 lines with the A mtDNA at a higher segregation than all 14 of the B lines. The likelihood that this would not be so depends on the sampling effort in ascertaining segregation, and the magnitude of the segregation difference. To address this by simulation, for different magnitudes of segregation differences, random numbers were drawn representing the counts of flies in the segregation study, with the sample size and error distribution of the real experiment. These sampled segregations were then ranked and the Kruskal-Wallis test performed as for the real data. This entire procedure was then repeated 100 times in order to see what fraction of the time a significant K is obtained. When this was done with a segregation difference of 0.5% (*i.e.*, one clone yielded a segregation of 0.500 and the other 0.505), a value of K significant at the 5% level was obtained 57% of the time. A difference of 1% was detected as significant in 100 out of 100 trials.

Another analysis of the effects of mtDNA haplotypes on segregation made use of the phylogenies described above. Using several different minimum levels of divergence, the mtDNA types were classified into clusters based on restriction fragment variation, and the linear model was again fitted by GLM. In the case of the chain and the two Rothrock experiments, there were again no significant nuclear-mitochondrial interactions detected. In the case of the

diverse lines, the tree revealed that the Egà-1 and DV92S lines had the most divergent mtDNA haplotypes, with the Egà-1 being an outlier. When the analysis of variance was repeated, treating Egà-1 as one mtDNA type and all the others as another, the P value for the nuclear-cytoplasmic interaction fell from 0.017 in the first analysis (Table 5) to 0.003. Similarly, when DV92S was treated as separate from the others, the P value was 0.010. In contrast, when the AH198S, GB8S, R1 and St-5 lines were treated as outgroups and the ANOVA was repeated, the P values for the nuclear-cytoplasmic effect were 0.300, 0.282, 0.576 and 0.491, respectively. This analysis is consistent with the hypothesis that the mtDNA modulates the relative viability of different nuclear genotypes, but falls short of proving causality.

DISCUSSION

If an adaptive mutation were to occur within the mitochondrial genome, simple population genetic theory shows that the mutation should increase in frequency to fixation at a more rapid rate than a mutation in the nuclear genome having similar effect, especially if there is within-individual (within-generation) selection (TAKAHATA 1984). Similarly, deleterious mutations should rapidly be lost from the mitochondrial genome. Models allowing nuclear-cytoplasmic interactions do not change this picture much, except in the case of frequency dependent fitnesses, where stable polymorphisms can be maintained. By an all-else-being-equal argument, this implies that the variation found in the mtDNA within a panmictic population is less likely to confer differences in fitness than will variation in nuclear genes. The rapid fixation within local populations would also result in increased differentiation among subpopulations for adaptively significant mutations. These patterns are exactly what were found in our segregation analysis. The tests actually involved assays of second chromosome segregations, but the same arguments made above apply to the conditional cytoplasmic fitnesses that our tests measure. Only the experiment using flies of diverse geographic origin revealed significant nuclear-cytoplasmic interactions. Posterior tests that make use of the identifications of mtDNA clones failed to find any relation between mtDNA and the segregation patterns within populations, even though sample sizes were large enough to give considerable statistical confidence.

Even in the experiment using lines of diverse geographic origin, where nuclear-cytoplasmic interactions were detected, it is not possible to ascribe the effect with certainty to mtDNA. The Kruskal-Wallis test lacked adequate power in this case because of the small number of different lines used. Posterior comparisons showed that the Egà-1 and DV92S cytoplasms had an effect on second chromosome segregation that was significantly different from the others, and the mtDNA haplotypes of these lines were also the most deviant. It would be desirable to prove causality of the effect to the mtDNA by some type of transformation experiment, and several designs come to mind.

Another problem is that crossing among lines of diverse geographic origin might be inducing a phenomenon analogous to hybrid dysgenesis (BREGLIANO and KIDWELL 1982). Extractions were done in the P cytotype, but this does not guarantee that P element transposition is quiescent among these lines. The observation of a trend in the error variance (CLARK 1985) is particularly suggestive of some type of inflated mutagenesis. The critical point is that our design only guarantees that the cytoplasmic component be maternally transmitted, and any cytoplasmic effects that are observed cannot be ascribed with certainty to the mitochondria. Other investigators have also detected significant nuclear-cytoplasmic effects when using stocks of diverse geographic origin (HIRAIZUMI 1985; A. F. MACRAE and W. W. ANDERSON personal communication; HOFFMAN, TURELLI and SIMMONS 1986).

In a number of situations the application of mtDNA variation has implicitly assumed selective neutrality of that variation. The inference of phylogeny, so often touted as a big advantage of mtDNA because of its rapid rate of sequence divergence, assumes an underlying molecular clock (KIMURA 1983). The rate of the molecular clock is constant under a purely neutral model with constant mutation rate, but many forms of selection will not only perturb the clock, but make its rate depend on population size as well. The magnitude of the deviation from a purely neutral clock will depend on the frequency of non-neutral mutation, and hence the portion of extant variation that is non-neutral. Under a purely neutral molecular clock in an unstructured population, the phylogenies inferred from nuclear and mitochondrial genes would be identical, but discordant phylogenies inferred from these two genomes have been cited (DESALLE and GIDDINGS 1986). Selection need not be inferred in this case however, since the data are more suggestive of asymmetries in interspecific hybridizations.

Biogeographic inferences would also be best served by strictly neutral variation. Selective differentiation among local demes can inflate or decrease the degree of population structuring, and can alter the apparent rates of migration and times of isolation. Much of the analysis of hybrid zones would also be influenced by the knowledge of extensive variation in adaptive mtDNA polymorphisms. Our failure to detect selective differences among mtDNA clones within populations lends support to these applications of mtDNA variation.

The remarkable economy of animal mtDNA and the conservation of gene order suggests that the genome is under tight size constraints. It may therefore appear paradoxical that much of the polymorphism seen in the mtDNA of several organisms is due to length variation (BROWN and DESROSIERS 1983; CANN and WILSON 1983; HARRISON, RAND and WHEELER 1985; RAND and HARRISON 1986; REILLY and THOMAS 1980; SOLIGNAC *et al.* 1984; SOLIGNAC, MONNEROT and MOUNOLOU 1983). The observation of heteroplasmy also suggests that spontaneous rate of generation of length variants is fairly high (DENSMORE, WRIGHT and BROWN 1985; HALE and SINGH 1986; BERMINGHAM, LAMB and AVISE 1986; BOURSOT, YONEKAWA and BONHOMME 1987). There were length variants in our series of lines, and they did not confer detectable selective differences. Experimental tests of the transmission properties of heteroplasmic individuals have shown a bias in favor of the smaller molecule in both field crickets (RAND and HARRISON 1986) and *Drosophila mauritiana* (SOLIGNAC *et al.* 1984). In both of these cases, the sampling of mitochondria in germline cells is such that the expected time to fixation is on the order of 500 generations. The population frequencies of heteroplasmic individuals were on the order of 10–15% in this study and those above, and simple deterministic models suggest that bidirectional mutation among classes of length variants may explain the association between length variation and heteroplasmy (CLARK 1988).

This study was done in the spirit of classical selection components analysis: genetic variants were identified and manipulative experiments were done to assess differences in the performances of these variants. This approach is contrasted sharply by analytical methods that are now available for inference of population genetic forces directly from the patterns of molecular variation (*e.g.*, HUDSON, KREITMAN and AGUADE 1987). Observation of a significant excess of silent substitutions over the neutral expectation has lead to the conclusion that selective constraints operate on Drosophila mtDNA (WOLSTENHOLME and CLARY 1985). The power of the molecular approach is advocated by LEWONTIN (1985), who indicates that sequence analysis can reveal past selective events that could not be inferred by experimentation on extant variation. For example, meiotic drive might be inferred from fixation of alleles all along a chromosome, revealed by a high level of high order linkage disequilibrium. Despite these advantages, studies that exclusively analyze variation in sequences miss the details of the biological causes of variation in fitness. The importance of selection component analysis to population genetics is to underscore the multidimensionality of fitness, and to do so in a way that allows direct experimental tests of those effects. Many insights and counterintuitive results have been derived from this approach. Sequence analysis will not be able to tell if a particular locus were under strong selection due to effects on viability as opposed to fertility. Indeed, one cannot conclude from sequence analysis alone whether a particular allele is selectively favored. Molecular methods lend considerable power to more classical experimental approaches, and the general approach of establishing a relation between a molecular phylogeny and a fitness-associated phenotype will doubtless be widely exploited.

An example of the mechanistic complexity of selection can be found in considering the causes of reciprocal cross differences in Drosophila. Six causes for differences in segregation from reciprocal crosses can be identified, including, (1) a true cytoplasmic effect, (2) maternal effects caused by the maternal nuclear genome, (3) meiotic drive in oogenesis, (4) meiotic drive in spermatogenesis, (5) sex-specific rates of recombination and (6) hybrid dysgenesis. The joint observation of a cytoplasmic effect and no reciprocal cross effect in our segregation tests lends support for a true cytoplasmic effect. An understanding of the evolution of a gene requires such a detailed accounting of its potential pleiotropic effects. The limitation of classical methods of selection component analysis is that they require inordinate sample sizes in order to obtain adequate statistical power. In the present study we obtained a resolution of about 0.5% in measurements of selection coefficients, and this was only possible because we were able to obtain sample sizes in excess of one-half million flies by scoring marginal fitnesses using visible markers. In the case of microorganisms, competitive growth experiments in chemostats allow even finer resolution than this. DYKHUIZEN, FRAMOND and HARTL (1984) detected no difference in growth rates of strains of *Escherichia coli* transduced with different *zwf* alleles, even though the resolution in growth rates was less than 0.002 hr^{-1}. Results for other loci, reviewed in DYKHUIZEN and HARTL (1983), underscore the power of the technique of direct experimental measurement of fitness. Molecular techniques will continue to improve the data available to population geneticists for the inference of evolutionary forces, and by using a full complement of experimental techniques, including direct tests of selection components, the most complete picture of evolutionary past and potential will emerge.

We thank WILLIAM GELLMAN, DENISE SCAGLIONE, ROSS JOHNSON, JOHN FETCHKO and LISA KEITH for technical assistance, CHARLES AQUADRO, ROBERT CHAPMAN and CATHY LAURIE for sharing protocols, and THOMAS WHITTAM for insightful comments on the manuscript. This work was supported by grants HD-18379 and HD-00743 from the U.S. National Institutes of Health.

LITERATURE CITED

ANDERSON, S., A. T. BANKIER, B. G. BARRELL, M. H. L. DE BRUIJN, A. R. COULSON, J. DROUIN, I. C. EPERON, D. P. NIERLICH, B.

A. Roe, F. Sanger, P. H. Schrier, A. J. H. Smith, R. Staden and I. G. Young, 1981 Sequence and organization of the human mitochondrial genome. Nature **290:** 457–465.

Asmussen, M. A., J. Arnold and J. C. Avise, 1987 Definition and properties of disequilibrium statistics for associations between nuclear and cytoplasmic genotypes. Genetics **115:** 755–768.

Avise, J. C., 1986 Mitochondrial DNA and the evolutionary genetics of higher animals. Philos. Trans. R. Soc. Lond. B **312:** 325–342.

Avise, J. C., and N. C. Saunders, 1984 Hybridization and introgression among species of sunfish (*Lepomis*): analysis by mitochondrial DNA and allozyme markers. Genetics **108:** 237–255.

Beale, G., and J. Knowles, 1978 *Extranuclear Genetics*. Edward Arnold, London.

Bender, W., P. Spierer and D. S. Hogness, 1983 Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the bithorax complex in *Drosophila melanogaster*. J. Mol. Biol. **168:** 17–33.

Bermingham, E., T. Lamb and J. Avise, 1986 Size polymorphism and heteroplasmy in the mitochondrial DNA of lower vertebrates. J. Hered. **77:** 249–252.

Birky, C. W., T. Maruyama and P. Fuerst, 1983 An approach to population and evolutionary genetic theory for genes in mitochondria and chloroplasts, and some results. Genetics **103:** 513–527.

Boursot, P., H. Yonekawa and F. Bonhomme, 1987 Heteroplasmy in mice with deletion of a large coding region of mitochondrial DNA. Mol. Biol. Evol. **4:** 46–55.

Bregliano, J. C., and M. G. Kidwell, 1982 Hybrid dysgenesis determinants. pp. 363–404. In: *Mobile Genetic Elements*, Edited by J. A. Shapiro. Academic Press, New York.

Brown, G. G., and L. J. Desrosiers, 1983 Rat mitochondrial DNA polymorphism: sequence analysis of a hypervariable site for insertions/deletions. Nucleic Acids Res. **11:** 6699–6708.

Brown, W. M., M. George and A. C. Wilson, 1979 Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. USA **76:** 1967–1971.

Cann, R. L., and A. C. Wilson, 1983 Length mutations in human mitochondrial DNA. Genetics **104:** 699–711.

Cann, R. L., K. Stoneking and A. C. Wilson, 1987 Mitochondrial DNA and human evolution. Nature **325:** 31–36.

Carr, S. M., S. W. Ballinger, J. N. Derr, L. H. Blankenship and J. W. Bickham, 1986 Mitochondrial DNA analysis of hybridization between sympatric white-tailed deer and mule deer in west Texas. Proc. Natl. Acad. Sci. USA **83:** 9576–9580.

Chapman, R. W., J. C. Stephens, R. A. Lansman and J. C. Avise, 1982 Models of mitochondrial DNA transmission genetics and evolution in higher eucaryotes. Genet. Res. **40:** 41–57.

Clark, A. G., 1984 Natural selection with nuclear and cytoplasmic transmission. I. A deterministic model. Genetics **107:** 679–701.

Clark, A. G., 1985 Natural selection with nuclear and cytoplasmic transmission. II. Tests with Drosophila from diverse populations. Genetics **111:** 97–112.

Clark, A. G., 1987 Neutrality tests of highly polymorphic RFLP's. Am. J. Hum Genet. **41:** 948–956.

Clark, A. G., 1988 Deterministic theory of heteroplasmy. Evolution. In press.

Clayton, D. A., 1984 Transcription of the mammalian mitochondrial genome. Annu. Rev. Biochem. **53:** 573–594.

Densmore, L. D., J. W. Wright and W. M. Brown, 1985 Length variation and heteroplasmy are frequent in mitochondrial DNA from parthenogenetic and bisexual lizards (genus *Cnemidophorus*). Genetics **110:** 689–707.

DeSalle, R., and L. V. Giddings, 1986 Discordance of nuclear and mitochondrial DNA phylogenies in Hawaiian Drosophila. Proc. Natl. Acad. Sci. USA **83:** 6902–6906.

DeSalle, R., L. V. Giddings and K. Y. Kaneshiro, 1986 Mitochondrial DNA variability in natural populations of Hawaiian Drosophila. II. Genetics and phylogenetic relationships of natural populations of *D. silvestris* and *D. heteroneura*. Heredity **56:** 87–96.

DeSalle, R., A. Templeton, I. Mori, S. Pletscher and J. S. Johnston, 1987 Temporal and spatial heterogeneity of mtDNA polymorphisms in natural populations of *Drosophila mercatorum*. Genetics **116:** 215–223.

Dewey, R. E., C. S. Levings III and D. H. Timothy, 1986 Novel recombinations in the maize mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell **44:** 439–449.

Dykhuizen, D. E., and D. L. Hartl, 1983 Selection in chemostats. Microbiol. Rev. **47:** 150–168.

Dykhuizen, D. E., J. de Framond and D. L. Hartl, 1984 Selective neutrality of glucose-6-phosphate dehydrogenase allozymes in *Escherichia coli*. Mol. Biol. Evol. **1:** 162–170.

Ewens, W. J., 1979 *Mathematical Population Genetics*., Springer-Verlag, Berlin.

Feinberg, A. P., and B. Vogelstein, 1983 A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6–13.

Ferris, S. D., R. D. Sage, C. M. Huang, J. T. Nielsen, U. Ritte and A. C. Wilson, 1983 Flow of mitochondrial DNA across a species boundary. Proc. Natl. Acad. Sci. USA **80:** 2290–2294.

Gregorius, H. R., and M. D. Ross, 1984 Selection with gene-cytoplasm interactions. I. Maintenance of cytoplasmic polymorphisms. Genetics **107:** 165–178.

Haldane, J. B. S., 1956 The estimation of viabilities. J. Genet. **45:** 294–296.

Hale, L. R., and A. T. Beckenbach, 1985 Mitochondrial DNA variation in *Drosophila pseudoobscura* and related species in Pacific Northwest populations. Can. J. Genet. Cytol. **27:** 357–364.

Hale, L. R., and R. S. Singh, 1986 Extensive variation and heteroplasmy in size of mitochondrial DNA among geographic populations of *Drosophila melanogaster*. Proc. NAtl. Acad. Sci. USA **83:** 8813–8817.

Harrison, R. G., D. M. Rand and W. C. Wheeler, 1985 Mitochondrial DNA size variation within individual crickets. Science **228:** 1446–1447.

Harrison, R. G., D. M. Rand and W. C. Wheeler, 1987 Mitochondrial DNA variation in field crickets across a narrow hybrid zone. Mol. Biol. Evol. **4:** 144–158.

Hiraizumi, Y. 1985 Genetics of factors affecting the life history of *Drosophila melanogaster*. I. Female productivity. Genetics **110:** 453–464.

Hoffman, A. A., M. Turelli and G. M. Simmons, 1986 Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution **40:** 692–701.

Hudson, R. R., M. Kreitman and M. Aguade, 1987 A test of neutral molecular evolution based on nucleotide data. Genetics **116:** 153–159.

Kirk, J. T. O., and R. A. Tilney-Bassett, 1967 *The Plastids*. Freeman, San Francisco.

Kimura, M. 1983 *The Neutral Theory of Molecular Evolution*. Cambridge University Press, Cambridge.

Lansman, R. A., R. O. Shade, J. F. Shapira and J. C. Avise 1981 The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J. Mol. Evol. **17:** 214–226.

LATORRE, A., A. MOYA and F. J. AYALA, 1986 Evolution of mitochondrial DNA in *Drosophila subobscura*. Proc. Natl. Acad. Sci. USA **83:** 8649–8653.

LEHMANN, E. L., 1975 *Nonparametrics: Statistical Methods Based on Ranks*. Holden-Day, San Francisco.

LEVINGS, C. S., III, 1983 The plant mitochondrial genome and its mutants. Cell **32:** 659–661.

LEWONTIN, R. C., 1985 Population genetics. Ann. Rev. Gen. **19:** 81–102.

MERRIL, C. R., and M. G. HARRINGTON, 1985 The search for mitochondrial inheritance of human diseases. Trends Genet. **1:** 140–143.

MONNAT, R. J., and L. A. LOEB, 1985 Nucleotide sequence preservation of human mitochondrial DNA. Proc. Natl. Acad. Sci. USA **82:** 2895–2899.

MORITZ, C., and W. M. BROWN, 1986 Tandem duplication of D-loop and ribosomal RNA sequences in lizard mitochondrial DNA. Science **233:** 1425–1427.

POWELL, J. R., 1983 Interspecific cytoplasmic gene flow in the absence of nuclear gene flow: evidence from Drosophila. Proc. Natl. Acad. Sci. USA **80:** 492–495.

POWELL, J. R., A. CACCONE, G. D. AMATO and C. YOON, 1986 Rates of nucleotide substitution in Drosophila mitochondrial DNA and nuclear DNA are similar. Proc. Natl. Acad. Sci. USA **83:** 9090–9093.

RAND, D. M., and R. G. HARRISON, 1986 Mitochondrial DNA transmission genetics in crickets. Genetics **114:** 955–970.

REILLY, J. G., and C. A. THOMAS, 1980 Length polymorphisms, restriction site variation, and maternal inheritance of mitochondrial DNA in *Drosophila melanogaster*. Plasmid **3:** 109–115.

RIGBY, P. W. J., M. DIECKMANN, C. RHOADES and P. BERG, 1977 Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113:** 237–251.

SAS Institute, Inc., 1985 The GLM procedure. pp. 433–506. In: *SAS User's Guide: Statistics, Version 5 Edition*. SAS Institute, Inc., Cary, N.C.

SCHAFFER, H. E., and R. R. SEDEROFF, 1981 Improved estimation of DNA fragment lengths from agarose gels. Anal. Biochem. **115:** 113–122.

SCHAEFFER, R. E., M. G. KIDWELL and A. FAUSTO-STERLING, 1979 Hybrid dysgenesis in *Drosophila melanogaster:* morphological and cytological studies of ovarian dysgenesis. Genetics **92:** 1141–1152.

SEARLE, S. R., 1971 *Linear Models*. John Wiley & Sons, New York.

SLOTT, E. F., R. O. SHADE and R. A. LANSMAN, 1983 Sequence analysis of mitochondrial DNA in a mouse cell line resistant to chloramphenicol and oligomycin. Mol. Cell. Biol. **3:** 1694–1702.

SOLIGNAC, M., and M. MONNEROT, 1986 Race formation, speciation and introgression within *Drosophila simulans, D. mauritiana* and *D. sechellia* inferred from mitochondrial DNA analysis. Evolution **40:** 531–539.

SOLIGNAC, M., M. MONNEROT and J. C. MOUNOLOU, 1983 Mitochondrial DNA heteroplasmy in *Drosophila mauritiana*. Proc. Natl. Acad. Sci. USA **80:** 6942–6946.

SOLIGNAC, M., M. MONNEROT and J. C. MOUNOLOU, 1986 Mitochondrial DNA evolution in the *melanogaster* species subgroup of Drosophila. J. Mol. Evol. **23:** 31–40.

SOLIGNAC, M., J. GENERMONT, M. MONNEROT and J. C. MOUNOLOU, 1984 Genetics of mitochondria in Drosophila: mtDNA inheritance in heteroplasmic strains of *D. mauritiana*. Mol. Gen. Genet. **197:** 183–188.

SOUTHERN, E. M., 1975 Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:** 503–518.

TAKAHATA, N., 1983 Population genetics of extranuclear genomes under the neutral mutation hypothesis. Genet. Res. **42:** 235–256.

TAKAHATA, N., 1984 A model of extranuclear genomes and the substitution rate under within-generation selection. Genet. Res. **44:** 109–116.

TAKAHATA, N., 1985 Introgression of extranuclear genomes in finite populations: nucleo-cytoplasmic incompatibility. Genet. Res. **45:** 179–194.

TAKAHATA, N., and T. MARUYAMA, 1981 A mathematical model of extranuclear genes and the genetic variability maintained in a finite population. Genet. Res. **37:** 291–302.

TAKAHATA, N., and S. R. PALUMBI, 1985 Extranuclear differentiation and gene flow in the finite island model. Genetics **109:** 441–457.

TAKAHATA, N., and M. SLATKIN, 1983 Evolutionary dynamics of extranuclear genes. Genet. Res. **42:** 257–265.

TAKAHATA, N., and M. SLATKIN, 1984 Mitochondrial gene flow. Proc. Natl. Acad. Sci. USA **81:** 1764–1767.

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

WALLACE, D. C., 1981 Assignment of the chloramphenicol resistant gene to mitochondrial DNA and analysis of its expression in cultured human cells. Mol. Cell Biol. **1:** 697–710.

WHITTAM, T. S., A. G. CLARK, M. STONEKING, R. L. CANN and A. C. WILSON, 1986 Allelic variation in human mitochondrial genes based on patterns of restriction site polymorphism. Proc. Natl. Acad. Sci. USA **83:** 9611–9615.

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. Biol. J. Linn. Soc. **26:** 375–400.

WOLSTENHOLME, D. R., and D. O. CLARY, 1985 Sequence evolution of Drosophila mitochondrial DNA. Genetics **109:** 725–744.

Communicating editor: M. T. CLEGG