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The Units of Selection on Mitochondrial DNA

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■ **Abstract** Mitochondrial DNA (mtDNA) exists in a nested hierarchy of populations. There are multiple mtDNAs within each mitochondrion, a population of mitochondria in each cell, multiple oocytes within each reproductive female, multiple females in each population, and so on up through species and higher clades. The metabolic properties of mitochondria make them highly mutagenic environments for the naked, circular mtDNAs that lie within them. This mutational pressure introduces mtDNA variation (i.e., heteroplasmy) into the cytoplasmic population of cell lineages that are particularly prone to mutational decay and Muller's ratchet owing to the asexual, maternal inheritance of mtDNA. Neutrality tests show that deleterious mutations are common in mtDNA evolution. Population cage experiments further show that mtDNA fitnesses are influenced by nuclear-mitochondrial interactions. These selective processes are pervasive despite the long-standing use of mtDNA as a neutral marker in population and evolutionary biology. These evolutionary dynamics are also unique in the nested hierarchy of mtDNA populations because mutation, selection, and drift can act—and interact—at multiple levels. Multi-level selection can facilitate the escape from Muller's ratchet and help resolve intragenomic conflicts. This review addresses recent advances in the transmission genetics, population genetics, and evolution of mtDNA. A primary goal of the review is to motivate additional empirical studies that might clarify the many units of selection acting on mtDNA.

INTRODUCTION

Mitochondrial DNA (mtDNA) has been the most widely studied region of eukaryotic genomes and has played a critical role in the development of population and evolutionary genetics (Brown 1985, Avise et al. 1987, Moritz et al. 1987, Harrison 1989, Avise 1991, Rand 1994). During its first decade of use in evolutionary biology, it was an implicit—if not explicit—assumption that mtDNA was a neutral genetic marker. More recently, there has been growing interest in how selection might act on mtDNA, and two different approaches have been taken to address this question. Direct experiments with laboratory populations have attempted to identify

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fitness effects of distinct mtDNA haplotypes (e.g., MacRae & Anderson 1988, Clark & Lyckegaard 1988, Nigro & Prout 1990, Fos et al. 1990, Kambhampati et al. 1992, Hutter & Rand 1995, Kilpatrick & Rand 1995, Garcia-Martinez et al. 1998, Rand et al. 2001). Alternatively, evidence for selection on mtDNA has been sought using statistical tests of neutral models of molecular evolution based on static samples of DNA sequences from natural populations (Whittam et al. 1986; Excoffier 1990; Ballard & Kreitman 1994; Ballard 2000a,b; Nachman et al. 1994, 1996; Nachman 1998; Quesada et al. 1999; Rand et al. 1994, 2000; Rand & Kann 1996, 1998; Templeton 1996; Wise et al. 1998; Weinreich & Rand 2000; Blouin 2000). Both of these approaches have identified nonneutral evolution of mtDNA that has implicated selection at the level of the individual organism.

However, a large and rapidly growing literature in cell biology and molecular genetics has demonstrated the importance of selection and genetic drift within and among cytoplasms as primary mechanisms affecting the phenotypic expression of mtDNA mutations. It is clear from comparative and medical studies that deleterious mutations define much of the selection on mtDNA. The answer to how organelle genomes purge these deleterious mutations and escape Muller's ratchet in the absence of recombination and sex may lie in the dynamics afforded by multi-level selection (Birky 1995, Bergstrom & Pritchard 1998, Jacobs et al. 2000). The classic examples of multi-level selection are the *petite* mutants in yeast. These mtDNA mutants lack genes for oxidative phosphorylation but retain functional origins of replication. They can out-compete wild type mtDNAs by "selfish" replication in mixed (i.e., heteroplasmic) cytoplasms but are ultimately selected against at the level of the cell owing to reduced growth rates from deficiencies in oxidative phosphorylation (e.g., Reid 1980, Birky 1983). Analogous systems of multi-level selection involving mtDNA deletion mutants may well have operated among some of the retinal and brain cells that are processing the words that you are now reading (Wallace 1999).

At a macroevolutionary scale, a similar multi-level selection has operated. The origin and evolution of mtDNA—and the eukaryotic cell itself—has involved extensive gene transfer from a proto-mitochondrion to the nuclear genome, as well as outright gene loss (Lang et al. 1999, Gray et al. 1999). Deleted mtDNAs have undoubtedly won many cytoplasmic "replication races" during the history of life, but selection at the level of the integrated nuclear-mitochondrial system has undoubtedly produced the lineages that carry streamlined mtDNAs today. Because there are clear parallels between both micro- and macro-evolutionary (as well as clinical) models of the units of selection on mtDNA, this view provides an opportunity for a synthesis across a broad spectrum of the biological sciences.

The connection between organelle evolution and the units of selection has been discussed in other reviews (Lewontin 1970, Eberhard 1980, Cosmides & Tooby 1981, Buss 1987, Hastings 1992, Birky 1995, Blackstone 1995, Maynard Smith & Szathmáry 1995). Whereas these reviews have defined many fundamental questions, the derived state of current mitochondria and their free-living sister taxa (Lang et al. 1999) present problems for historical inference, and certainly

for direct experimental studies of phenotypes. The primary aim of this review is to survey recent studies of selection and drift acting on mtDNA in the hierarchy of populations from organelles, germ cells, and tissues through the individual and population levels. The review is biased towards the population genetics of animal mtDNA. However, certain plant and fungal systems offer clear advantages for some aspects of multi-level selection analysis and are mentioned (e.g., Albert et al. 1996). A second aim of the review is to motivate additional manipulative experiments that might reveal the importance of evolutionary forces acting across the hierarchy of organization inherent in organelle genomes.

THE CYTOPLASM AS A POPULATION

As Lewontin (1970) pointed out, selection will act among units at any level of organization that exhibits three basic principles: l. phenotypic variation, 2. differential fitness of phenotypes, and 3. transmission of fitness phenotypes. The hierarchical nesting of mtDNAs within organelles, cells, tissues, and individuals allows for each of these principles to apply at more than one level. For example, the phenotypic variation at the level of cells can be an emergent property of the heteroplasmic composition of individual cells; the phenotypic variation at the level of individual organisms can be an emergent property of the heteroplasmic composition of various tissues, each of which might have distinct effects on the life history of the organism. Moreover, the maternal inheritance and general lack of recombination in (animal) mtDNA provides a clear context in which to apply the basic population genetics of mutation, selection, and drift across this hierarchy of populations (see Figure 1).

Consider a cell that is fixed (homoplasmic) for one mtDNA type (see Figure 2). Mutation will introduce variation into this population, providing phenotypic variation (principle 1). Mutant mtDNAs will replicate at different rates, either by kinetic advantages in replication, or by differential rates of organelle or cell proliferation (principle 2). The frequencies of the two (or more) mtDNA variants within a cell will be subject to random genetic drift during the sampling event imposed by mitosis and cytokinesis. Organelles or cells that enjoy a fitness benefit from the novel frequencies of mtDNA will transmit these fitness states to their offspring (principle 3). However, random drift at each of these levels can undermine principle 3 and provides a clear population genetic distinction between the units of selection and the units of evolution (cf. Vrba & Gould 1986).

Mutation

MUTATION VERSUS SUBSTITUTION A hallmark of mtDNA is rapid sequence evolution, at least in mammals (Upholt & Dawid 1977, Brown et al. 1979). Given the functional importance of oxidative phosphorylation (OXPHOS) in eukaryotic metabolism, this was surprising. The rapid rate of evolution was explained

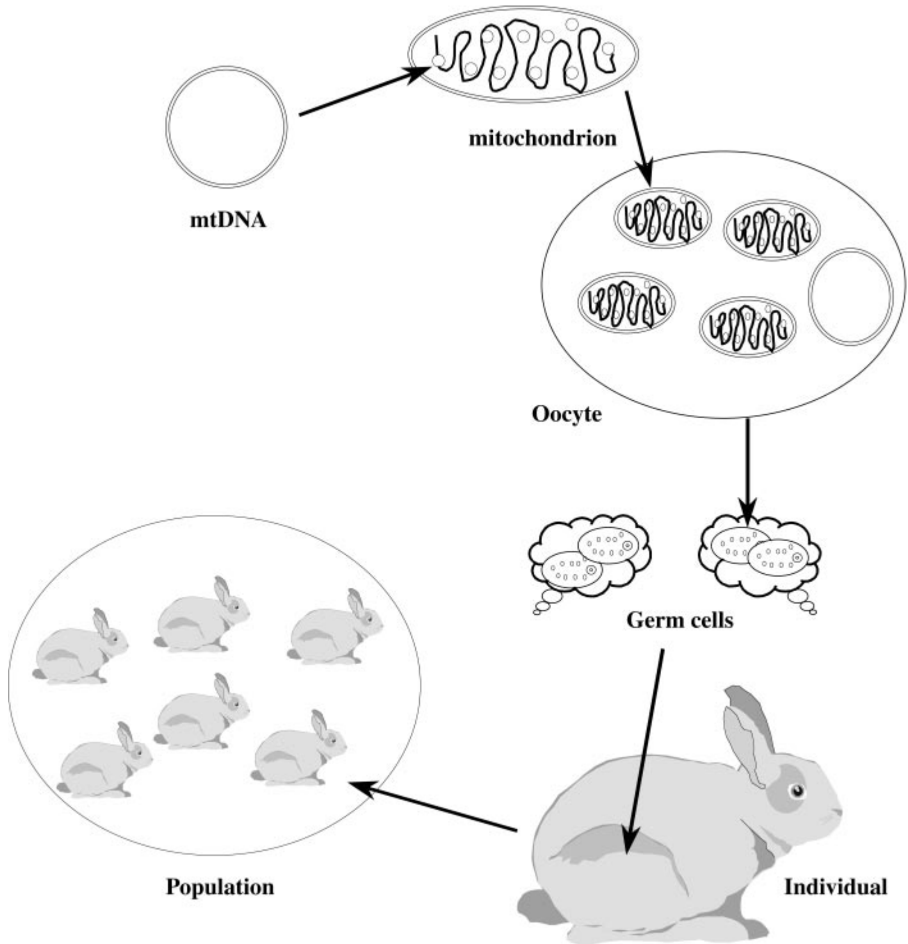

Figure 1 mtDNA exists in a nested hierarchy of populations spanning the mtDNA molecule, the organelle, the cell, the individual organism, and on up through natural populations and species.

by a high mutation rate, relaxed selective constraints, or limited mtDNA repair mechanisms (Brown 1985, Avise 1991). Mutation and substitution (or fixation) need to be distinguished. The rate of DNA sequence evolution (i.e., substitution) between species is a function of the mutation rate (u) and the probability of fixation (p_{fix}) (Kimura 1983). Many more mutations arise than ultimately become fixed, because most mutations are deleterious. The substitution rate inferred from comparative sequence data fails to record mutations that never enter populations as polymorphisms and polymorphisms that never reach fixation. This distinction is particularly important in the context of a units-of-evolution view of mtDNA

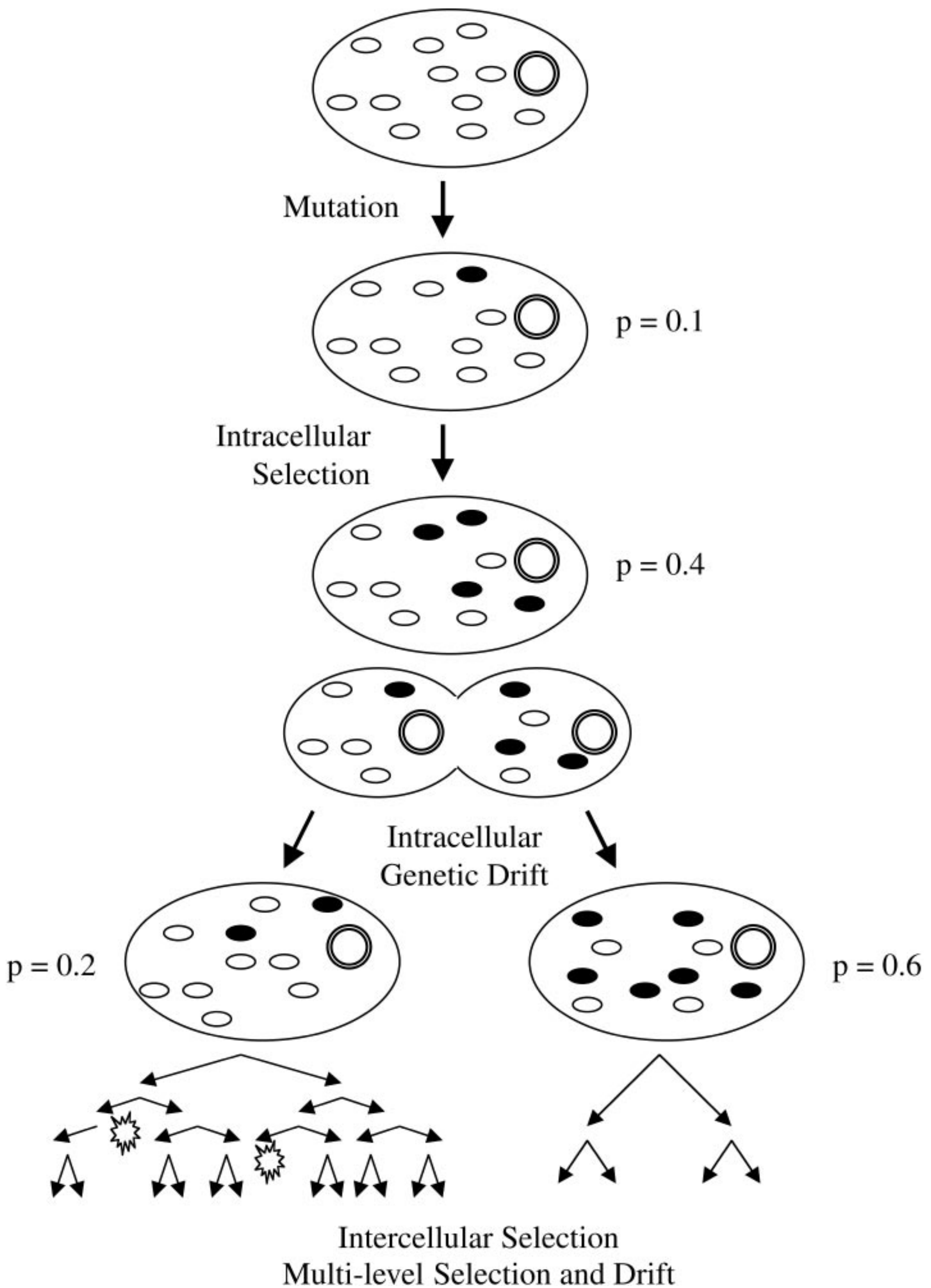

Figure 2 Population genetics of the cytoplasm. Mutation introduces variation at the lowest level of the hierarchy. Natural selection and random genetic drift act to change mtDNA haplotype frequencies within and among cytoplasms. Selection and drift can act at multiple levels in the hierarchy of populations: within organelles, within cells, or among maternal lineages.

because the cytoplasmic population is an additional level of the biological hierarchy that can affect the distinction between mutation and substitution. Hence, heteroplasmy is an obligatory, if transient, phase in the evolution of mtDNA. The hierarchical population genetics of mutation, drift, and selection thus defines the spectrum of phenotypic variation upon which selection can act at each level from the mtDNA molecule to the organismal population.

DIRECT MEASUREMENTS OF MUTATION RATES Mitochondrial mutation rates have been measured by sequencing partial or complete mtDNAs in defined pedigrees. Parsons et al. (1997) sequenced the control region (CR or D-loop) from grandparent- or parent-offspring pairs in 134 human lineages spanning 327 generational events. They detected 10 mutations, resulting in a rate estimate of 1 mutation per 33 generations, or 2.5 mutations per site per million years. This is 20 times higher than the mutation rates estimated using phylogenetic methods of sequence comparisons between humans and chimps (Parsons et al. 1997). In the nematode *Caenorhabditis elegans*, sequences from 75% of the mtDNA genome were obtained from 74 mutation accumulation lines maintained by single-worm transmission for an average of 214 generations (Denver et al. 2000). This protocol reduces natural selection to a minimum. Of the 26 mutations observed, 16 were single base changes and 10 were insertions or deletions (indels). The majority of the point mutations were nonsynonymous, and some of the deletions disrupted reading frames. The estimated mutation rate (14.3 mutations per site per million years) is two orders of magnitude higher than the phylogenetic approach for humans discussed above, and about five times higher than the mutation rate estimate in human pedigrees. This discrepancy between mutation and substitution rates illustrates the clear deleterious nature of the spontaneous mutations and underscores the important qualitative distinction between mutational processes operating at these different time scales.

HOMOPLASMY AND HETEROPLASMY Initial studies of mtDNA variation in nature showed that all mtDNA molecules within an individual were identical (homoplasmy). The preponderance of homoplasmy, coupled with high levels of variation within and between species, dictates that the necessary heteroplasmic phase is transient during mtDNA evolution. The first studies that uncovered high levels of heteroplasmy involved insertion and deletion of repeated sequences with high mutation rates (Solignac et al. 1983, Hauswirth et al. 1984, Harrison et al. 1985; reviewed in Rand 1993). In the pedigree analyses discussed above, both Parsons et al. (1997) and Denver et al. (2000) detected individuals heteroplasmic for point mutations or indels. Because complete shifts in mtDNA genotypes were also observed between lineages in the pedigrees, germ line mutations had clearly arisen and gone through the heteroplasmic phase to fixation. These studies illustrate the timescales that are relevant for the transition between distinct homoplasmic states.

The biochemical processes of the mitochondrion itself are believed to be a major source of mutations. Most of the cell's energy production and oxygen consumption occur during OXPHOS, which is performed by five enzyme complexes embedded

in the inner mitochondrial membrane. Complex I (NADH dehydrogenase) and particularly complex III (cytochrome bc_1) are major sources of reactive oxygen species, which are potent mutagens (Finkel & Holbrook 2000). The proximity of mtDNA to the sites of OHPHOS and reactive oxygen species production, and the fact that mtDNA is not associated with histones, are two primary explanations for high mutation pressure on mtDNA (Shigenaga et al. 1994, de Gray 1999).

Coupled with the lack of recombination (Ballard 2000a, Ingman et al. 2000), the high rate of deleterious mutation in mtDNA should result in an accumulation of mutations leading to a "mutational meltdown" and extinction of lineages (Lynch 1997, Lynch & Blanchard 1998). A solution to this problem is likely provided by both drift and selection within and among cytoplasms. Replicative purging of the cytoplasmic population can occur if mtDNAs with the most lesions are replicated less efficiently (Birky 1983, Lightowlers et al. 1997, Marchington et al. 1997). Indeed, age-dependent accumulation of heteroplasmies is greater in nondividing tissue than in dividing tissue (Cortopassi et al. 1992). Random drift of mtDNA mutants during germ line divisions leads to the loss of variation within cells but increases the variance among oocytes, somatic tissues, or maternal lineages. This will increase the efficacy of selection at the level of the cell or individual, and possibly counter the "ratchet" effects of reactive oxygen species–induced mutations (Bergstrom & Pritchard 1998). Nevertheless, mtDNA evolution may be characterized by a mutation-drift-selection balance in which most cells carry transient levels of heteroplasmy for point mutations, deletions, and rearrangements.

We need additional studies of the accumulation of mtDNA mutations in a diverse array of organisms (e.g., Parsons et al. 1997, Denver et al. 2000). Such studies done in organisms with very different patterns of germ line differentiation will provide important comparative data on the fate of mtDNA mutations through the mitochondrial population booms and bottlenecks of extended germ lines. Plants offer excellent material for these studies as well. Because the germ line is dispersed, the opportunity to study somatic mosaicism and potential intra-individual selection on chloroplast or mtDNA is great (Albert et al. 1996, Klekowski 1998).

Genetic Drift Within and Among Cytoplasms

BEAN-BAG POPULATION GENETICS AND BOTTLENECKS IN THE CYTOPLASM Some of the earliest analyses of the transmission of a population of organelles through the cytoplasm came from studies of mutant and wild-type chloroplasts in variegated plants (reviewed in Gillham 1978, Beale & Knowles 1979). Studies of the segregation of mtDNAs in heteroplasmic yeast cultures lead to the application of population genetic theory to mitochondrial genetics (Thrailkill & Birky 1980, Birky 1983). The ability to construct experimental heteroplasmies by mating yeast strains with different mutant forms of mtDNA has obvious advantages over relying on naturally occurring heteroplasmies. However, these single-cell studies are limited in their ability to characterize the genetics of mitochondria in organisms with clearly defined germ lines. Transmission of mitochondria from one organismal

generation to the next involves repeated sampling events during the mitotic divisions that occur during the formation of the germ line and subsequent production of competent oocytes.

The evolution of this cytoplasmic population can be modeled using a drift equation for the evolution of the variance among demes (Wright 1968, p. 346; Solignac et al. 1984, Rand & Harrison 1986):

$$V_n = p(1-p)[1-(1-1/N_{e\text{-}mt})^{gn}], \qquad 1.$$

where V_n is the variance among cells (i.e., demes) after n organismal generations, p and $(1-p)$ are the initial frequencies of the two mtDNA types in the founding mother (or oocyte), $N_{e\text{-}mt}$ is the effective population size of mitochondria during cell division, and g is the number of germ line cell divisions per organismal generation. V_n and p are relatively straightforward to estimate using molecular methods. Recent advances in competitive PCR have made the "field work" of this population genetic problem accurate and repeatable (Chen et al. 1995; Barritt et al. 2000). The main limitation to this approach is the number of oocytes or offspring that can be sampled from any one mother, because estimates of variance are sensitive to sample size. This is not a problem in insects but can be in mammals, and especially in human pedigrees.

The number of germ cell generations per organismal generation has been estimated to be 7 in *Drosophila* (Solignac et al. 1987) and about 15 in mice (Jenuth et al. 1996) and humans (Howell et al. 1992, Poulton et al. 1998; although $g = 24$ was used by Jenuth et al. 1996), and possibly as high as 50 in cows (Upholt & Dawid 1977, Hauswirth & Laipis 1982). Empirical studies of the evolution of the variance across organismal generations can be used to estimate the effective population size for mtDNA through the germ line. This sampling-effective population size may involve mtDNAs, organelles, or aggregations of organelles (see below). As illustrated in Figure 3, the demography and population structure of the germ line, as defined by the drift equation, can have important consequences for the evolution of mtDNA.

STUDIES OF MITOCHONDRIAL TRANSMISSION Transmission studies of mtDNA in yeast, insects, and mammals indicate that this "bean bag" population genetics provides a relatively good fit with the number of mitochondria sampled from a cytoplasmic population. Ultrastructural observations suggest that there are about 500–1000 mitochondria per cell (Gillham 1978). Assuming that this population is sampled during cell division, one would expect $N_{e\text{-}mt}$ to be lower than the census size, or on the order of a few hundred mitochondria.

Using the drift equation above, $N_{e\text{-}mt}$ is approximately 50 in the budding yeast *Saccharomyces* and somewhat higher in the fission yeast *Schizzosaccharomyces* (Birky 1983, Thrailkill & Birky 1980). In *Drosophila* $N_{e\text{-}mt}$ ranges from 545 to 700 (Solignac et al. 1984, 1987; Kann et al. 1998). Crickets have a somewhat lower estimate of $N_{e\text{-}mt}$ [87–395, (Rand & Harrison 1986)]. A transmission study

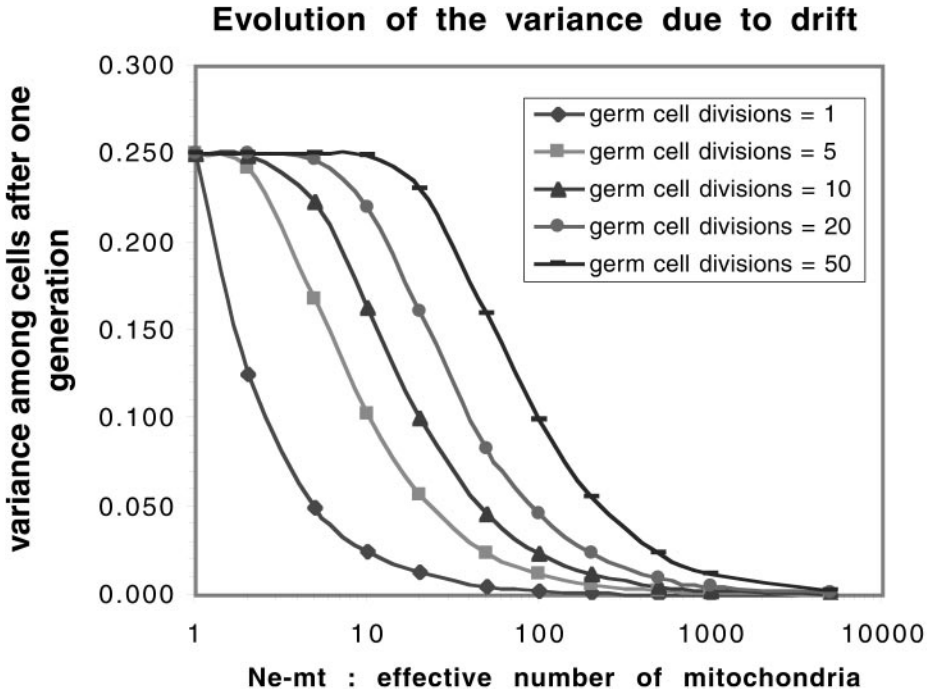

Figure 3 The evolution of the variance among populations resulting from random drift. The amount of variation among germ cells (or F1 offspring) from a single heteroplasmic female is shown on the y-axis, as a function of the effective number of segregating mitochondria (or other units such as mtDNAs or clusters of organelles) on the x-axis. Separate curves are generated from Equation 1 based on the number of germ cell generations that span a single organismal generation. The heteroplasmic mother is assumed to have two mtDNAs at equal frequency ($p = q = 0.5$); hence, from Equation 1, fixation occurs at a variance of $V = 0.25$. Note that a single-celled organism with two mitochondria has comparable transmission dynamics to that of a multicellular organism with 50 germ cell generations per organismal generation, but with $N_{e\text{-}mt} \sim 100$ (variances of about 0.125).

in an experimental strain of heteroplasmic mice carrying wild-type mtDNAs reported estimates of $N_{e\text{-}mt}$ that ranged from 76 to 867. Importantly, analyses of primary oocytes, mature oocytes, and F_1 offspring from these experimental females produced statistically indistinguishable estimates of the effective number of mitochondria, leading to a general estimate of $N_{e\text{-}mt} = \sim 200$ (Jenuth et al. 1996). Similar results have been reported for a distinct strain of heteroplasmic mice carrying wild-type and deleted mtDNAs (Inoue et al. 2000). This is surprising given the pathological nature of the deleted mtDNA, but the close fit to neutral drift suggests there is no major effect of OXPHOS deficiency on transmission through germ cells in this strain.

Very different, and conflicting, results have been obtained from cows and humans. In cows, complete shifts from one mtDNA haplotype to another have been observed in one or two generations (Ashley et al. 1989, Koehler et al. 1991). These observations suggest a strong bottleneck in the effective population size of mtDNA through the germ line, possibly as small as a single mtDNA template. Rapid shifts from one homoplasmic state to another have also been observed in human pedigrees (Parsons et al. 1997, Blok et al. 1997), but this is not a general rule; Howell et al. (1992) obtained estimates of $N_{e\text{-}mt}$ ranging from 36 to 2400 (see also Poulton et al. 1998, Chinnery et al. 2000 for results similar to mice). Moreover, very different estimates of $N_{e\text{-}mt}$ have been reported when transmission was studied using blood samples ($N_{e\text{-}mt}$ from 800 to 4000) versus oocyte ($N_{e\text{-}mt}$ from 15 to 70; Blok et al. 1997). This is surprising given the many more cell generations that intervene between blood samples of two children and two oocytes. During the differentiation of mature oocytes from primary oocytes in mammals, the mitochondrial population is amplified at least 50-fold and the number of mtDNAs per mitochondrion is reduced from ~10 to an average of 2 (Hauswirth & Laipis 1982, Blok et al. 1997). However, little mtDNA replication occurs during the early divisions following fertilization (Piko & Matsumoto 1976, Meirelles & Smith 1998). If alternative mtDNA haplotypes or mitochondria are sampled at random to serve as a "master copy" during the amplification phase, a population bottleneck would result.

WHAT ARE THE SEGREGATING UNITS? The spatial distribution of mitochondria is very different in different tissues and changes dramatically during development, suggesting considerable control by nuclear genes (reviewed in Yaffe 1999). Moreover, mitochondria are associated with a number of cytoskeletal proteins involved in cytoplasmic movement (kinesin, cytoplasmic dynein, and dynamin homologs of GTP binding proteins) (Yaffe 1999). Whereas mtDNAs appear to be sampled randomly for replication during the cell cycle (Bogenhagen & Clayton 1977), this does not mean that subsequent transmission is a passive process. The dynamic associations between cytoskeletal proteins, nuclear signaling, and mitochondria suggest that a regulated mitochondrial "mitosis" may be involved in the partitioning of mitochondria prior to cytokinesis (Yaffe 1999, Jacobs et al. 2000). Rapid shifts in mtDNA haplotypes between generations could easily be achieved with pulses of replication, targeted mitochondrial trafficking, and a random distribution of mutant mtDNAs in the cell.

It is not clear whether clusters of distinct mitochondria (Jacobs et al. 2000) or individual heteroplasmic mitochondria (Howell et al. 1992) are the units sampled for transmission. Nor is it clear how this differs between somatic and germ cells. We need additional transmission studies in organisms with different patterns of germ line development and somatic partitioning.

Drift at More than One Level

GERMLINE VS. MATRILINE It is intuitive that genetic drift within germ cells should occur faster than drift among maternal lineages. Jenuth et al. (1996) have quantified

this difference. Using a mean value of $N_{e\text{-}mt} = 187$, taken from several studies of human mtDNA inheritance, the drift model indicates that the mean number of cell generations to reach fixation in homoplasmic cells is 370. At 24 germ cell generations per human generation, a mutant mtDNA will drift to fixation in a human matriline in 15 generations. Under neutrality the sojourn time to fixation for a novel mtDNA mutation in an organismal population is $2N_e$ generations. Based on an accepted estimate of the human population size ($N_e = 10{,}000$ individuals, or ~5,000 females) (Takahata 1993), a neutral mtDNA mutation will drift to fixation in ~10,000 generations, vastly longer than the fixation time of any given heteroplasmy (Jenuth et al. 1996).

This difference in the sorting time for drift within germ lines and drift among maternal lineages justifies the assumption that heteroplasmy can be ignored in some theoretical treatments of the evolutionary dynamics of mtDNA (Avise et al. 1984, Takahata & Palumbi 1985). From a units-of-evolution perspective, the fact that drift is operating at two (or more) levels of a hierarchy offers a unique opportunity to dissect the interaction among levels. Standing amounts of polymorphism for neutral mutations are determined by an equilibrium between mutational input and loss by genetic drift. Drift operating within germ cells leads to the loss of variability at the level of the individual organism. Thus, the action of an evolutionary force at one level affects the uniqueness of the higher-level individuals. In the terminology of hierarchy theory, there is "upward causation" (Vrba & Gould 1986). Because drift and selection can operate in different directions at the level of tissues (see below), the individual organism can acquire a uniqueness from the infinity of ways an organism can exhibit somatic mosaicism. However, drift at the level of individual organisms has a downward effect on variation at the cytoplasmic level and will tend to reduce it. The development of mature oocytes from a heteroplasmic stem cell in the germ line sorts variation from within to among populations. If sampling at the level of the individual is added on top of this, the approach to homoplasmy will be accelerated. In the terminology of hierarchy theory, drift at the individual level has "downward causation" effects on drift at the cytoplasmic level (Vrba & Gould 1986).

APPORTIONMENT OF DIVERSITY AMONG LEVELS Mutation will introduce variation only at the lowest level, i.e., among mtDNAs within organelles. However, the relative contributions of drift at the multiple levels in the hierarchy will lead to some equilibrium apportionment of gene diversity within and among these levels. At very high mutation rates, most of the gene diversity will lie within cells because drift will not be "fast" enough to eliminate it. At lower mutation rates, the levels of the hierarchy carrying the greatest diversity will characterize the net effect of drift at multiple levels. The apportionment of diversity across this hierarchy can be quantified in a manner analogous to population structure analyses using F-statistics (with the caveat that Mendelian expectations do not apply to cytoplasmic systems).

Gene diversity is defined as $K = 1 - \Sigma x_i^2$, where x_i is the frequency of the ith variant at the focal level of the hierarchy (Birky et al. 1989, Rand & Harrison 1989). Following the notation of Birky et al. (1989), K_a is the diversity within a cell, K_b

is the diversity within an individual organism, K_c is the diversity within a deme (or collecting locality), and K_t is the diversity within the total sample. Following Lewontin (1972), these diversity measures can be apportioned into different hierarchical components: the within-individual component of the total diversity, the among-individual-within-deme component of the total diversity, and the among-deme component of the total diversity. With the appropriate data, apportionment of diversity across additional levels of a hierarchy are easily incorporated into these statistics (Birky et al. 1989, Rand & Harrison 1989).

Apportionment of mtDNA variability across this hierarchy has been done for mitochondrial VNTR (variable number of tandem repeat) systems in which the mutation rate is sufficiently high that heteroplasmy is easily observed (Rand & Harrison 1989, Arnason & Rand 1992, Wilkinson & Chapman 1991, Lunt et al. 1998). These studies showed that differences in the mutation-drift balance lead to distinct apportionment of diversity for different organisms (e.g., codfish have a higher within-individual component than evening bats, owing to a highly mutable VNTR). This approach has not been applied to the many heteroplasmies reported in mice and humans, but such studies would provide important contributions to the units of evolution view of mtDNA. With the advent of high-resolution methods for detecting heteroplasmy (Michikawa et al. 1999) and for quantifying the mtDNA in individual mitochondria (Cavelier et al. 2000), the apportionment of variation could be quantified for multiple levels in a variety of organisms.

Selection Within and Among Cytoplasms

MATERNAL INHERITANCE With biparental inheritance of organelles, the evolutionary interests of the organelle may be in conflict with the evolutionary interests of the host cell or organism. It has been argued that uniparental inheritance is one means by which this conflict can be resolved (e.g., Cosmides & Tooby 1981, Hastings 1992). Some insight into the nature of these events can be provided by comparative analysis of the mechanisms that ensure uniparental inheritance of organelle DNA (Birky 1995). Studies indicating that "male" mitochondria are actively destroyed by the egg cytoplasm or early embryo provide compelling proximate evidence for interference competition in the cytoplasm. That these mechanisms are under control of multiple nuclear genes further suggests an ultimate mechanism for the continued avoidance of the presumed intracellular genomic conflict.

In *Drosophila*, sperm tail structures composed of proteins and mitochondrial derivatives persist throughout embryogenesis but are eventually enveloped by the developing midgut and defecated after larval hatching (Pitnick & Karr 1998). In mice, mitochondria from sperm that are microinjected into eggs get eliminated by the time of birth, but mitochondria from liver do not (Shitara et al. 2000), indicating that mitochondria from different tissues may be differentially tagged and recognized by the host cell. A study of the domestic cow has shown that sperm mitochondria are tagged with ubiquitin and targeted for destruction in the early embryo (Sutovsky et al. 1999).

The doubly uniparental inheritance of mtDNA in mussels is a special case. In *Mytilus* two distinct types of mtDNAs are found in males and females. Females transmit their mtDNA (F type) to female and male offspring, whereas males transmit their mtDNA (M type) only to males (Zouros et al. 1994, Skibinski et al. 1994). At fertilization, both male and female embryos receive M mtDNA from the sperm, but within 24 hours the M mtDNA is eliminated in female embryos and maintained in male embryos (Sutherland et al. 1998). In male germline the M mtDNA is preferentially amplified. These M and F mtDNA types predate the divergence of *Mytilus* species (Rawson & Hilbish 1995), but independent M and F mtDNA types have evolved in the history of marine mussels (Hoeh et al. 1997). A similar system exists in freshwater mussels that diverged from *Mytilus* more than 450 mya (Liu et al. 1996). Thus, cells of different tissue type and sex can detect and eliminate alternative mitochondrial types. Although direct homology of these pathways has yet to be determined, they may provide a means to dissect the mechanisms by which the nuclear host genome avoids intragenomic conflict by ensuring the uniparental inheritance of mtDNA.

Additional insight into the mechanisms of maternal inheritance comes from cases in which paternal leakage has been detected. Paternal leakage tends to be detected in crosses between distinct subspecies or species of animals, rather than between strains of the same species. Mechanisms that eliminate "male" mtDNA may thus be species specific and break down in disrupted, hybrid nuclear genomes. Kondo et al. (1990) observed a paternal leakage rate of 0.1% in crosses between *Drosophila simulans* females and *D. mauritiana* males. Gyllensten et al. (1991) detected paternal mtDNA in all tissues in crosses between *Mus musculus* and *M. spretus*, albeit at low levels (0.1%). Their analyses further indicated increases in proportion of paternal mtDNA through additional backcross generations. Shitara et al. (1998) detected paternal leakage in mice, but paternal mtDNA was restricted to the F_1 generation animals and was not found in all tissues, unlike the results of Gyllensten et al. (1991). In contrast to domestic cattle, ubiquitin tagging was not evident on sperm mitochondria in crosses between domestic cow and the Asian wild gaur, and male mitochondria persist beyond the third embryonic division (Sutovsky et al. 1999, 2000).

In *Mytilus* a breakdown of the typical doubly uniparental pattern of inheritance is also observed in hybrid zones between *M. galloprovincialis* and *M. trossulus* but not between the former and *M. edulis* (see Rawson et al. 1996). Moreover, where hybridization has occurred, the F type of *M. edulis* has invaded the M route of transmission in *M. trossulus* (see Quesada et al. 1999), suggesting occasional breakdown of the transmission barrier. Breakdown of the elimination of male mitochondria in hybrids could provide a powerful means to dissect the underlying nuclear control of this presumed mechanism for the avoidance of intragenomic conflict.

A RACE FOR REPLICATION IN THE CYTOPLASM Organelle genomes with functional origins of replication should out-compete full-length wild-type molecules during replication (see Selosse et al. 2001). When these small genomes are not beneficial to

the cell, their frequency within a heteroplasmic cytoplasm is inversely proportional to fitness at the cell level, providing a clear example of conflicting levels of selection (Reid 1980, Birky 1983, Hurst et al. 1996). Similar systems exhibiting conflicting selection at two levels have been known in *Aspergillus* and *Nerurospora* for some time (Lewontin 1970, p. 4). However, a simple kinetic race for replication does not fully account for the selective advantage of deleted mtDNAs. A complex signaling network regulates the replication and transcription of mtDNA, with ATP balance and OXPHOS activity of individual mitochondria serving as potential signals (Allen 1993). The nucleus of a cell carrying a heteroplasmic mixture of wild type and defective mtDNAs may receive signals that some mitochondria are respiring at reduced levels and send signals to those mitochondria. Hence, active mechanisms under nuclear control may play important roles in the over-replication of defective mtDNA in heteroplasmic cells (de Gray 1999, p. 91).

In both *Drosophila* (Solignac et al. 1984) and crickets (Rand & Harrison 1986), in which mtDNA heteroplasmy is due to VNTR length variation in the origin of replication region, the smaller mtDNA showed significant increases in frequency across animal generations. These results were interpreted as evidence for a race for replication favoring smaller molecules. However, subsequent studies in both *D. mauritiana* (Solignac et al. 1987) and *D. melanogaster* (Kann et al. 1998) showed that as a heteroplasmic female ages, the longer mtDNA shows a transmission advantage from mother to offspring, indicating that the selection coefficient for the short and long mtDNAs changes sign as heteroplasmic females age. Because these heteroplasmies involve indels of ~400 bp repeats in the origin of replication region that has secondary structure potential (Lewis et al. 1994), the length of the mtDNAs may be less important than the ability of these repeat arrays to initiate and complete mtDNA replication.

The construction of heteroplasmic lines using microinjection of intact mitochondria provides a powerful means with which to study selection between competing mtDNAs (see King & Attardi 1988 for a study in cell culture). Mitochondria from different strains and species of *Drosophila* have been transplanted in various combinations to construct experimental heteroplasmic lines, revealing clear evidence for selection in the cytoplasm (Matsuura et al. 1989, 1991; de Stordeur et al. 1989, 1997). However, manipulation of temperature (Matsuura et al. 1993) and nuclear background (Doi et al. 1999) can have considerable effects on the selection coefficients of mtDNAs in heteroplasmic germlines.

All pairwise combinations of microinjections were performed among the divergent strains of *D. simulans* (*si*I, *si*II, and *si*III) and *D. mauritiana* (*ma*I and *ma*II) (de Stordeur et al. 1989, de Stordeur 1997). When the donor was *si*I or *ma*II, no successful heteroplasmic lines were established. In all remaining heteroplasmies, *si*II showed a consistent advantage over other mtDNAs in most nuclear backgrounds. Overall, a fitness hierarchy among heteroplasmic mtDNAs could be defined as *si*II > *si*III = *ma*I > *si*I = *ma*II (de Stordeur 1997). Manipulative experiments of multi-level selection can be addressed in this system by competing these same mtDNA haplotypes against one another in population cages of homoplasmic

flies. The available data suggest that the fitness hierarchy at the cytoplasmic level is maintained at the organismal level, but additional experiments are needed to determine the generality of this pattern in different nuclear backgrounds (see below).

TISSUE-SPECIFIC SELECTION The two mouse models for mtDNA heteroplasmy discussed above provide contrasting pictures of tissue-specific selection for mtDNA haplotypes. In one study the two mtDNAs were essentially wild-type genomes from different lab strains (BALB and NZB) and showed random transmission when individual oocytes or whole F_1 offspring were assayed (Jenuth et al. 1996). Analyses of heteroplasmies in blood, kidney, liver, and spleen showed distinct tissue-specific selection for the alternative mtDNAs as the mice aged from 2 to 17 months. Because the two mtDNAs had identical sequences in origin of replication, selection may have been acting among mitochondria based on the proportion of the two mtDNAs within each organelle (Jenuth et al. 1997).

Different results were obtained for the heteroplasmic strain of mice carrying wild type and deleted mtDNAs (Inoue et al. 2000). Transmission between generations was essentially random, but in contrast to the BALB/NZB study, the deletion strain showed very little tissue-specific variation in heteroplasmy (Inoue et al. 2000). Low variation among tissues is in contrast with human studies, which show marked tissue-specific variation in heteroplasmies when pathological deletions are present with wild-type mtDNAs (Shoubridge 2000a). A comparable system of heteroplasmy has been studied in *Drosophila subobscura*, with a wild strain carrying a large deletion (Volz-Lingenhohl et al. 1992, Petit et al. 1998). Germ tissue and oocytes show the lowest levels of the deletion mutant (60%), which increases to over 80% in the third larval stage and remains stable in the adult.

Barritt et al. (2000) determined that the frequency of the "common" mtDNA deletion was 33% within heteroplasmic human oocytes. However, in later stage embryos the frequency of the mtDNA mutant was 8%, suggesting that oocytes with higher frequencies of the mtDNA mutant were less likely to be implanted as embryos. A replication race is not believed to cause this pattern because little mtDNA replication takes place from the mature oocyte through the blastocyst stage in mammals (Meirelles & Smith 1998). Furthermore, because $\geq$1000 mtDNAs are present in each cell, pathological mutations must be present at frequencies between 60 and 95% to have an effect, depending on the severity of the mutation (Blok et al. 1997, Jacobs et al. 2000). Thus, there is some disagreement in the literature about the evidence for selection among oocytes owing to heteroplasmy (Shoubridge 2000b).

Experimental work is needed to determine the relative importance of (*a*) selection between two mtDNAs in replication, (*b*) different rates of organelle replication, and (*c*) selection at the level of the cell. Such studies will help clarify the role that multi-level selection could play in stopping the advance of Muller's ratchet by modulating the efficacy of among-lineage purging of deleterious mutations (see Bergstrom & Pritchard 1998).

SELECTION AT THE LEVEL OF THE INDIVIDUAL ORGANISM

Fitness Assays

The possibility of selection on mtDNA was mentioned in a seminal paper by Upholt & Dawid (1977) and raised by D. Ellis in the symposium questions following a paper presented by John Avise (see Avise 1986). The first studies that explicitly addressed selection on mtDNA were theoretical (Clark 1984, Gregorius & Ross 1984). These were followed by empirical studies seeking to detect fitness differences between mtDNA haplotypes collected from nature.

SELECTION ON HAPLOIDS Clark (1984) reasoned that the extensive mtDNA polymorphism in natural populations might have three explanations: (*a*) neutrality with a high mutation rate, (*b*) multiple-niche polymorphism or frequency dependence, or (*c*) nuclear-mitochondrial fitness interactions. For haploid genomes, the maintenance of polymorphism through selection in multiple niches, or by frequency-dependent selection, requires a very restrictive set of reciprocally balanced fitness values. Clark (1984) and Gregorius & Ross (1984) showed that the addition of various cytonuclear fitness interactions did little to promote the stable maintenance of haploid variation. By inference, most of the nonneutral mtDNA variation within a panmictic population would be eliminated by selection. A consequence of this, however, is that mtDNA fitness variation might accumulate between populations.

Empirical support for this theory has been obtained using chromosomal fitness assays in *Drosophila* (Clark 1985, Clark & Lyckegaard 1988). Pairwise combinations of wild second chromosomes and cytoplasms were constructed such that the fitness of each chromosome could be tested with each cytoplasm and vice versa. No significant nuclear-cytoplasmic fitness interactions were detected within geographic populations of *D. melanogaster*, but when chromosomes and cytoplasm derived from Old World and New World populations were combined, significant nuclear x cytoplasmic interactions were detected. Statistical analyses of the mtDNA haplotypes in these experiments revealed that the fitness of individual chromosomes was influenced by mtDNA haplotypes, but only when divergent populations were involved. These experimental designs identify the unit of selection as individual nuclear chromosomes in transient heterozygous combinations with distinct cytoplasms (and mtDNA).

These results have been extended to ask if cytonuclear fitness effects are distinct for sex chromosomes and autosomes (Rand et al. 2001). The patterns of chromosomal transmission for males and females, coupled with maternal inheritance of mtDNA, establishes distinct patterns of cotransmission for autosomal-mtDNA versus sex chromosome–mtDNA combinations. In mammals and insects, for example, females are diploid at all chromosomes, whereas males are effectively haploid for the X-chromosome. In a random mating population the probability of cotransmission of an autosome with the cytoplasm is 0.5, whereas for an X-chromosome the probability is 0.67. By modifying earlier simulation studies,

A. Clark was able to show that X-chromosome-cytoplasm fitness interactions can maintain joint nuclear and cytoplasmic polymorphisms, unlike previous autosomal models (Rand et al. 2001). Chromosome segregation studies further showed that X-chromosome-cytoplasm fitness interactions could be detected within geographic populations of *D. melanogaster*, and that these interactions were sexually antagonistic: The high-fitness mtDNAs, cytoplasms, and X-chromosomes in females tended to have low-fitness states in males (Rand et al. 2001). These studies show that selection on cytonuclear interactions are very distinct for X-chromosomes and autosomes.

POPULATION CAGE STUDIES Most population cage experiments have shown that one mtDNA variant increases in frequency over another across several generations (MacRae & Anderson 1988, Nigro & Prout 1990, Fos et al. 1990, Kambhampati et al. 1992, Nigro 1994, Hutter & Rand 1995, Kilpatrick & Rand 1995, Datta et al. 1996, Garcia-Martinez et al. 1998). In some cases, the apparent mtDNA fitness effect could be attributed to cytoplasmic incompatibility resulting from *Wolbachia* infections (e.g., Nigro & Prout 1990, Kambhampati et al. 1992). Cytoplasmic incompatibility occurs when an uninfected female mates with an infected male, resulting in reduced egg hatch rates. In the reciprocal cross normal egg hatch rates are observed. Thus, cytoplasms carrying *Wolbachia* have a fitness advantage over cytoplasms free of *Wolbachia*, resulting in the "hitchhiking" of other cytoplasmic factors, such as mtDNA. Nigro & Prout (1990) showed that this incompatibility was sufficient to account for the observed changes in mtDNA frequencies in their experimental populations, and that the nonneutral trajectories could not be attributed to any fitness differences between the mtDNAs themselves.

NUCLEAR-CYTOPLASMIC INTERACTIONS In *Drosophila* population cage studies in which *Wolbachia* was eliminated by tetracycline treatment, repeatable changes in mtDNA frequencies were detected, but the fate of competing mtDNA variants was influenced by nuclear genetic background. Using distinct strains of *D. melanogaster* from Argentina and Central Africa, Kilpatrick & Rand (1995) showed that mtDNA haplotypes behaved as neutral variants when competed on either the Argentinian or the Central African nuclear background, but the Argentinian mtDNA had a transient advantage on a mixed (hybrid) nuclear background. In reciprocally backcrossed lines of *D. pseudoobscura* and *D. persimilis* the *D. pseudoobscura* mtDNA showed a strong and repeatable home team advantage on its own nuclear background, owing almost entirely to greater egg production. On the *D. persimilis* nuclear background, however, the two mtDNAs were effectively neutral variants (Hutter & Rand 1995). Because mtDNA haplotypes behave as unlinked loci with respect to nuclear chromosomes, the repeatable association of a mtDNA haplotype with fecundity differences across multiple generations and a frequency perturbation suggests a causal role for mtDNA. Datta et al. (1996) developed a neutrality test of change in joint nuclear and mtDNA frequencies applicable to these sorts of population cage studies. The test derives expectations for the decay of the cytonuclear disequilibrium measures defined by Asmussen

et al. (1987). Applied to population cage data from *D. melanogaster*, the decay of cytonuclear disequilibria was significantly slower than expected, suggesting cytonuclear fitness interactions.

In *D. subobscura*, two distinct mtDNA haplotypes (I and II) are found throughout its range in Europe. In replicated population cages, haplotype II showed significant increases in frequency (Garcia-Martinez et al. 1998). Whereas the experimental results are clearly nonneutral, the data are difficult to reconcile with the observation that the haplotypes remain polymorphic in natural populations. If the natural polymorphism is due to selection, exactly reciprocal cytonuclear fitness interactions, habitat fitnesses, or frequency-dependent fitnesses are required (Clark 1984, Gregorius & Ross 1984). The difference between the experimental and natural populations is likely due to cytonuclear fitness interactions that were introduced in establishing the population cages.

These population cage experiments do suggest that selection at the level of the individual organism can be detected experimentally and that nuclear-cytoplasmic interactions are a fundamental context of this selection. Whereas there are limitations in applying the results of laboratory experiments to natural populations, it is clear that mtDNA markers can behave as nonneutral variants that could compromise evolutionary inference in studies of population structure, gene flow, and hybrid zones.

SELECTION AT THE CYTOPLASM AND ORGANISMAL LEVELS Two-level selection on distinct mtDNAs can be addressed by comparing the data from heteroplasmic transmission of microinjected strains of *Drosophila* (Matsuura et al. 1989, de Stordeur 1997) and population cage studies (Aubert & Solignac 1990, Nigro 1994). Aubert & Solignac (1990) established replicate bottles of a single *D. simulans si*III female and 32 *D. mauritiana ma*II females; the *si*III mtDNA increased markedly in three generations. Whereas this experiment involved mixing of nuclear genomes as well, the selection is in the same direction as the heteroplasmic competition between *si*III and *ma*II at the cytoplasmic level (de Stordeur 1997). Using microinjected strains of *D. simulans* in which the *si*II haplotype had gone to fixation on the *si*III background, the *si*II haplotype was selected against when competed at the individual level in population cages on the *si*III background (Nigro 1994). This selection is opposite in direction from that reported for heteroplasmy (de Stordeur 1997). When the *si*II and *si*III mtDNAs were competed on a mixed nuclear background (from reciprocal F_1 crosses), the *si*II mtDNA increased in frequency. Additional population cage experiments are needed in each of the nuclear backgrounds to complement the network of pairwise combinations of selection at the cytoplasm level.

Neutrality Tests of mtDNA Sequences

MILDLY DELETERIOUS MTDNA EVOLUTION The first suggestion that mtDNA polymorphisms depart from a neutral model were based on the frequency spectrum of polymorphic restriction sites in human mtDNA (Whittam et al. 1986). A test

of the Ewens-Watterson model of neutrality using mtDNA restriction map data showed that high-frequency alleles (restriction sites) were more common than expected, intermediate frequency alleles were observed less often than expected, and singletons were more common than expected under the neutral assumptions (Whittam et al. 1986). Whereas demographic issues and population structure may have played a role in these patterns (Excoffier 1990, Merriwether et al. 1991), the action of purifying selection was supported by the observation that amino-acid altering restriction sites were observed as singletons more frequently than expected (Whittam et al. 1986).

An excess of amino acid polymorphism is the most consistent pattern of non-neutral evolution that has emerged from efforts to detect selection on mtDNA (Kaneko et al. 1993; Ballard & Kreitman 1994; Nachman et al. 1994, 1996; Rand et al. 1994, 2000; Rand & Kann 1996, 1998; Hasegawa et al. 1998; Nachman 1998; Templeton 1996; Wise et al. 1998; Quesada et al. 1999, Weinreich & Rand 2000, Ballard 2000a). This "excess" is defined in the context of the McDonald-Kreitman, or MK, test (McDonald & Kreitman 1991): The ratio of polymorphism to divergence at nonsynonymous sites ($r_{pd\text{-}nonsynonymous}$) tends to be greater than the ratio of polymorphism to divergence at synonymous "silent" sites ($r_{pd\text{-}synonymous}$). These data are generally interpreted as evidence for mildly deleterious selection on amino acid polymorphisms (Hasegawa et al. 1998, Nachman 1998, Rand & Kann 1998). Mutations that change the amino acid sequence of a protein are presumed to experience weak negative selection, which should reduce their rate of fixation. Relative to a strictly neutral mutation, a mildly deleterious mutation will contribute more to polymorphism than to divergence (Figure 3.7 in Kimura 1983). Because selection on synonymous sites is presumed to be weaker than for amino acid–altering sites, ($r_{pd\text{-}nonsynonymous}$) > ($r_{pd\text{-}synonymous}$) (see Akashi 1995, Rand & Kann 1996, 1998, Nachman 1998).

The data applied to an MK test can be used to estimate the historical selection coefficient (e.g., Mcdonald & Kreitman 1991, Sawyer & Hartl 1992; Akashi 1995). An index that correlates well with this selection coefficient can be derived from the basic data of an MK test [referred to as a neutrality index (NI)] (Rand & Kann 1996):

$$NI = (r_{pd\text{-}nonsynonymous})/(r_{pd\text{-}synonymous}). \quad 2.$$

NI values >1 indicate negative selection and NI values <1 indicate positive selection. Note that a ratio of (dN/dS within species) to (dN/dS between species) is comparable to NI (where dN and dS are rates of nonsynonymous and synonymous substitutions, respectively) (see Hasegawa et al. 1998). With one degree of freedom, similar NI values can result from very different departures of neutrality. NI >1 can result from an excess of mildly deleterious nonsynonymous polymorphism, diversifying selection on nonsynonymous sites within species, or an excess of synonymous fixed differences. Several additional lines of evidence point to weak negative selection. At the ND3 gene in mice, polymorphic amino acid sites are not found at deep nodes in the genealogy of mtDNA haplotypes, which might

be expected under balancing selection (Nachman et al. 1994). At the ND5 gene in *Drosophila*, amino acid polymorphisms have significantly negative Tajima's D values, whereas synonymous polymorphisms do not (Rand & Kann 1996). Nielsen & Weinreich (1999) showed that the mean age of nonsynonymous polymorphisms was less than that for synonymous polymorphisms, consistent with the mildly deleterious hypothesis. Nematodes provide a striking example in which mtDNA shows $NI<1$ (Blouin 2000). Although indicative of positive selection, this result stems from saturation of synonymous changes between species owing to a strongly biased base composition. An underestimation of the number of fixed synonymous differences will lead to an underestimation of NI values.

DELETERIOUS MUTATIONS VS. POLYMORPHISMS Studies of mutation rates in mtDNAs provide further direct evidence for the deleterious effects of mtDNA mutations (Parsons et al. 1997, Denver et al. 2000). In the mutation accumulation lines in *C. elegans* (Denver et al. 2000), 6 synonymous and 8 nonsynonymous mutations were detected. Between 2 wild isolates of *C. elegans*, 26 synonymous and 3 nonsynonymous polymorphisms were detected (Denver et al. 2000). This is a significant ($P<0.001$) "excess" of amino acid mutations relative to amino acid polymorphisms. This comparison is essentially an MK test conducted at a lower level of organization and gives an NI value of 11.5. This is considerably higher than the average NI of 4.41 ± 4.5 for 31 mtDNA data sets tabulated from the literature (Weinreich & Rand 2000), indicating somewhat stronger negative selection than that inferred from polymorphism and divergence data.

Using the nonsynonymous/synonymous ratio for the mutation accumulation lines ($8/6 = 1.333$), Denver et al. (2000) estimated that 34.7 nonsynonymous polymorphisms would be detected between the two strains of *C. elegans* under strict neutrality (with 26 observed synonymous polymorphisms, $1.33 \times 26 = 34.7$). The efficiency of selection on amino acid changes is thus $1-$(3 observed polymorphisms/34.7 expected) $= 91.4\%$, implying the width of the "selective sieve" of 8.6%. Using 31 data sets on polymorphism and divergence reported for mtDNA in Weinreich & Rand (2000), a similar calculation provides a mean estimate of the efficiency of selection of $73.7\% \pm 26.8\%$, or a selective sieve of 26.3%. The mutation data from *C. elegans* are on the high end of the selective spectrum for these types of data. The selective filtering that prevents a spontaneous nonsynonymous mutation from entering the population as a polymorphism appears to be slightly stronger than the selective filtering that prevents existing nonsynonymous polymorphisms from reaching fixation.

MILDLY DELETERIOUS SYNONYMOUS EVOLUTION If the mildly deleterious story is general, then this pattern should hold for any two classes of nucleotide change that show different levels of functional constraint. Insects tend to have highly AT-rich mtDNAs [78.6% in *Drosophila* (Clary & Wolstenholme 1985), 84.9% in honeybee (Crozier & Crozier 1993)], and the third codon position shows even greater skew in base composition than the average for the entire genome. Thus, A- and T-ending

codons are used much more frequently than C- or G-ending codons. Mutations that change an A- or T-ending codon to a C- or G-ending codon should be deleterious or "unpreferred" relative to a change from C- or G-ending codon to A- or T-ending codons (Ballard & Kreitman 1994; see also Akashi 1995). Consistent with the standard MK tests, there is an excess of "unpreferred" synonymous changes from A- or T-codons to C- or G-codons within *Drosophila* mtDNA (Ballard & Kreitman 1994, Rand & Kann 1998). This result is also evident in a reciprocal pattern in the genes encoded on opposite strands in *Drosophila* mtDNA (Rand & Kann 1998, Ballard 2000a).

If synonymous sites show a pattern of excess "unpreferred" polymorphism in mtDNA, it would follow that functionally distinct classes of amino acid changes should exhibit a similar pattern, but this does not appear to be the case. When amino acids are classified as conservative or radical based on charge, polarity, or volume, there is no excess of radical polymorphisms (comparable to nonsynonymous polymorphism at the codon level) (Rand et al. 2000). It may be that the amino acid polymorphisms that are detectable in sequencing surveys have been filtered by purifying selection to a degree that they are effectively neutral by the criteria of an MK test. However, the partitioning of amino acid polymorphism into distinct classes reduces the sample size, and hence the power, of the MK tests. Additional studies of this sort are needed.

MILDLY DELETERIOUS EVOLUTION AND MITOCHONDRIAL GENETICS To address why mtDNA might be predisposed to a mildly deleterious evolution, Weinreich & Rand (2000) compared 36 nuclear data sets from *Drosophila* with 31 mtDNA data sets from diverse species. The mean of NI values for the nuclear loci was significantly smaller than for the mitochondrial data sets, and this difference between genomes was even greater when only those genes showing significant MK tests were considered (Figure 4).

Among the several ways that the genetics of nuclear and mitochondria genomes differ, recombination is a likely source of the difference in average NI values (Weinreich & Rand 2000). Linkage does not prevent genes or nucleotide sites with different levels of functional constraint from evolving at different rates (e.g., Birky & Walsh 1988). However, linkage has a considerable effect on the levels of polymorphism, as a consequence of hitchhiking (Maynard Smith & Haigh 1974) or background selection (Charlesworth et al. 1993). For mtDNA this discrepancy emerges as a nonneutral MK test, which is a consequence of the relevant units of selection. For divergence the relevant units of selection are the different classes of functionally constrained sites, but for polymorphism the unit of selection is the entire mtDNA haplotype. An alternative explanation for the nuclear-mtDNA difference in NI values is that the genes in modern mitochondria have cellular functions more likely to evolve under purifying selection. Many nuclear genes have functions in which diversity is key: pathogen recognition and avoidance, sex peptides, etc. No such genes are carried in mtDNA, and hence are less likely to evolve by positive selection (Weinreich & Rand 2000).

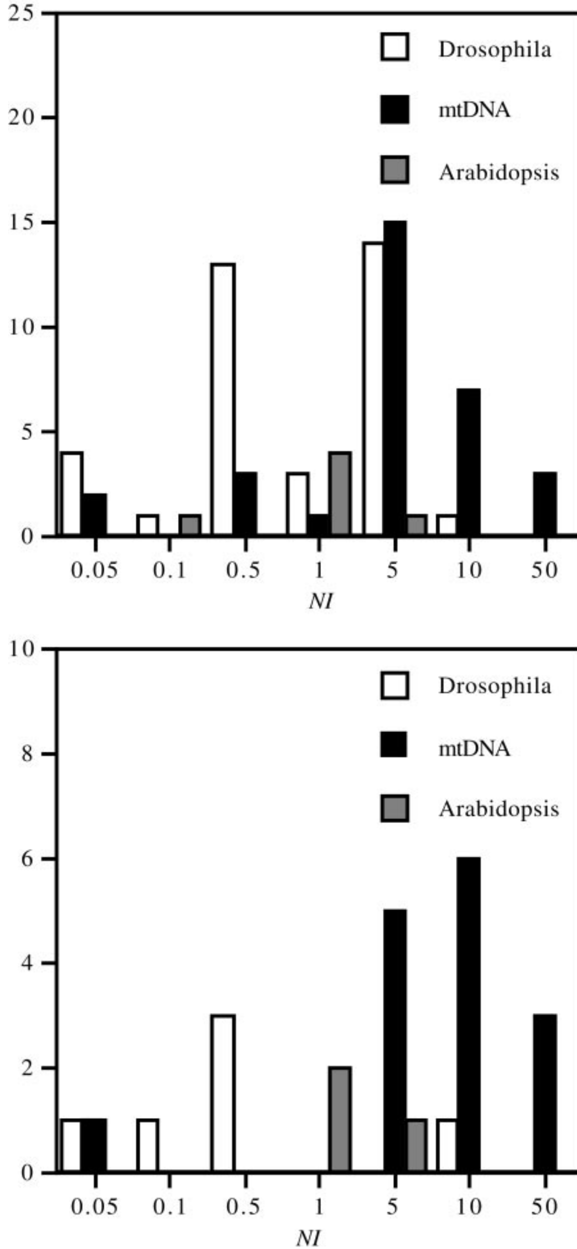

Figure 4 The distribution of neutrality index (NI) scores from McDonald-Kreitman (MK) tests for Drosophila nuclear, animal mtDNA, and Arabidopsis nuclear data sets. mtDNA and Arabidopsis tend to show NI values >1, indicative of negative selection. Top, all data sets; bottom, data sets in which the MK tests reject neutrality at the 5% level. Data from Weinreich & Rand (2000) and Rand et al. (2000).

SELECTIVE SWEEPS Evidence for selective sweeps in mtDNA was first revealed by "unusually low" levels of restriction site polymorphism in geographic samples of *D. simulans* (Baba-Aïssa et al. 1988, Nigro 1988). Subsequent Hudson, Kreitman, Aguadé (HKA) tests (Hudson et al. 1987) of mtDNA sequences and neutral contrast loci showed that this low polymorphism was below that predicted by a neutral model (Ballard & Kreitman 1994, Rand et al. 1994). A whole-mtDNA sequence analysis by Ballard (2000b) showed that three independent selective sweeps have occurred in each of the three distinct mtDNA haplotypes of *D. simulans* (*si*I, *si*II, and *si*III). Because each of these strains carries a distinct type of *Wolbachia*, it is currently believed that these selective sweeps are driven by the *Wolbachia*-mediated cytoplasmic incompatibility systems, not by adaptive mutations in mtDNA (Ballard 2000b, Rand 2001).

Recently, data sets sufficient to perform tests of selective sweeps in mtDNA have accumulated in a diversity of taxa. The virtual absence of studies documenting selective sweeps targeted to mtDNA is surprising given the general pattern of low recombination regions of nuclear genomes showing reduced variation. Either mtDNA provides a small target for adaptive mutations, or selective sweeps may occur with normal frequency in mtDNA and their footprint is rapidly erased by the replenishment of polymorphism owing to a high mutation rate.

EVOLUTION ABOVE THE SPECIES LEVEL

Rate Heterogeneity

Rate heterogeneity exists both among nucleotide sites within mtDNA and among lineages of organisms (reviewed in Mindell & Thacker 1996, Xia 1998, Gissi et al. 2000). Considerable attention has been focused on potential mutation-based explanations for evolutionary rate variation among lineages, such as the generation time and metabolic rate hypotheses (Martin & Palumbi 1993, Rand 1994, Nunn & Stanley 1998, Gissi et al. 2000, Weinreich 2001). From the perspective of selection on mtDNA, however, evolutionary rate analyses in species with very different population sizes provide a means of studying the modulation of effective selection.

RATE ACCELERATION IN SMALL POPULATIONS As predicted by the nearly neutral theory, the efficacy of selection should be reduced in small populations (Ohta 1992). One of the first studies to suggest this for mtDNA was a comparison of substitution rates in lineages of Hawaiian *Drosophila* based on restriction site maps (DeSalle & Templeton 1988). In the lineage that had experienced repeated founder events, mtDNA evolution was accelerated, consistent with a relaxation of purifying selection (see also Lynch 1997). Primates have a higher ratio of nonsynonymous to synonymous divergence (dN/dS ratio) for samples of nuclear genes than rodents, owing to presumed smaller effective population size in primates

(Ohta 1995). A parallel pattern has been documented for mtDNAs of primates and rodents, also consistent with the mild-deleterious model (Weinreich 2001). However, this study showed that changes in mutation rate—and hence dS values—in rodents were partly responsible for the reduced dN/dS ratio. Rates of divergence for nonsynonymous and synonymous sites in mitochondrial genes have been described for island-endemic lineages with small populations that should lead to greater lineage-specific accelerations for nonsynonymous sites than for synonymous sites. This prediction is upheld in island birds compared with their mainland sister taxa (Johnson & Seger 2001).

In *Drosophila* the situation has proved more complex (Ballard 2000a). *D. melanogaster* is believed to have a lower effective population size than its sister species, *D. simulans* (see Akashi 1995). Indeed, there is an excess of amino acid substitutions on the branch leading to *D. melanogaster* (Ballard 2000a). However, among synonymous sites, the more constrained "unpreferred" G- or C-ending codons do not show an excess of substitutions along the *D. melanogaster* branch. In island endemic lineages of the *D. simulans* clade, excess substitution for the functionally more-constrained classes of nucleotide sites is not consistently observed (Ballard 2000a). Hence, mtDNA evolution in this group of flies rejects both the neutral theory and simple predictions of the nearly neutral theory (Rand 2001). While lineage-specific changes in mutation rates are a possibility, fluctuating selection associated with changing population sizes seems more parsimonious.

Whole genome comparisons are providing new approaches for studying selection on mtDNA. Relaxed purifying selection in small effective population sizes should affect the entire mtDNA, but changes in the texture of selection could have different effects on specific mitochondrial genes. This can be detected statistically using a contingency table approach in which lineages are considered "rows" and genes are considered "columns." Lineage x locus interactions provide a powerful approach for discovering how selection acts on specific gene loci in nonrecombining mtDNA. Different genes show different patterns of rate acceleration in distinct lineages of the *D. melanogaster* subgroup (Ballard 2000a) and mammals (Gissi et al. 2000, Weinreich 2001).

A particularly promising variation on this theme of comparative genomics will be to explore the coevolution of nuclear and mitochondrial genomes. Lineage-specific covariation in evolutionary rates for nuclear- and mtDNA-encoded components of mitochondrial enzyme complexes is one means of taking a predictive approach to the lineage x locus tests of neutrality (Schmidt et al. 2001). If covariation in rate changes is detected for a nuclear and mitochondrial protein pair, the null hypothesis is that any random nuclear protein would show the same pattern of covariation. However, when the comparative sequence analysis can be done with nuclear and mitochondrial subunits that are known to interact, one has a set of candidate genes for the presumed coevolution (Weinreich 1998). As the biochemistry of OXPHOS proteins progresses, this analysis can be extended to individual amino acid sites. Moreover, comparisons of patterns of sequence divergence with functional assays of mitochondrial enzyme complexes is a promising approach

to the coevolution of nuclear and mitochondrial genes (Kenyon & Moraes 1997, Edmands & Burton 1999).

PURIFYING SELECTION IS PERVASIVE Despite clear modulation of dN/dS ratios, all mitochondrial proteins that have been examined to date have dN/dS ratios well below 1.0. Among hominoid mtDNAs, specific codons with dN/dS ratios >1 have been detected, but these represent <1% of the coding sites (Yang et al. 2000). This pattern is consistent with all of the data that have been presented in this review: mtDNA evolves under the scrutiny of purifying selection at the level of the individual organism.

Macroevolutionary Patterns of Genome Selection

If selection on mtDNA acts among genomes within cells, tissues, individuals, and populations, what is the important level of selection for mtDNA? The answer depends on the timescale of inquiry, but will always involve an interaction among levels. At the macroevolutionary scale the relevant units of evolution have been the dynamic interactions of the cyto-nuclear genome. These interactions have governed the two major evolutionary themes for organelle DNA: the reduction of genome size and the evolution of uniparental inheritance. Both of these transitions require the transfer of considerable control from the organelle "parasite" to the nuclear "host" genome.

GENOME SIZE The extensive reduction in genome size during the evolution of mtDNA from a protomitochondrial genome (Gray et al. 1999, Lang et al. 1999) and the extreme genetic economy of modern animal mtDNA (Attardi 1985) indicate a long history of selection. The reduction in size is a combination of the transfer of genes to the nuclear host genome and the outright loss of genes that may be redundant or not necessary (Sogin 1997, Selosse et al. 2001). These patterns support the notion of selection at the level of the integrated cytonuclear genotype. A pattern of sequential gene transfer and loss occurs, with genes of central metabolism being the first to go and rRNA genes, cytochrome b, and subunits of cytochrome oxidase generally being retained (Gray et al. 1999, Lang et al. 1999). In plastid genomes the same set of genes has been retained in different lineages, indicating parallel gene loss or transfer for a diversity of other proto-plastid genes. Typically, organelle genome size has been reduced beyond the "standard" set of genes only in cases in which the organism—the cytonuclear genomic unit–has become parasitic (e.g., nonphotosynthetic, saprophytic plants) (Wolfe et al. 1992, Palmer et al. 2000). Thus, selection for specific biochemical functions appear to have dictated the outcome of genomic evolution (Race et al. 1999). These patterns of parallel evolution are as suggestive of adaptive evolution as parallel dog or cat ecomorphs in placental and marsupial mammals.

The transitions to reduced organelle genomes must have involved a heteroplasmic state, however brief, in which the smaller genome out-competed the resident

wild-type genome. The establishment of the reduced organelle genome as a new wild-type requires selection at the level of the integrated nuclear-cytoplasmic unit because such a transition seems unlikely without some level of gene redundancy in the nuclear host genome (Selosse et al. 2001). These large-scale genome reorganizations are relatively rare in animal mtDNAs. Given the rate of organelle deletion and rearrangement in plants, species with major organelle genome rearrangements may be discovered in which wide crosses allow direct genetic experimentation of nuclear and cytoplasmic genomes. This could provide material for direct manipulation of the units of selection.

UNIPARENTAL INHERITANCE Cytonuclear coevolution has also been a driving force in the evolution of uniparental inheritance. The best explanation for this focuses on the units-of-selection problem associated with competition in the cytoplasm for variant genomes. The conflict inherent in the opportunity for selfish organelle genomes to spread under biparental transmission can be resolved by the evolution of asexual, uniparental inheritance (Eberhard 1980, Cosmides & Tooby 1981, Hastings 1992). Again, this is not achieved by the organelle or the nuclear genome, but by an intricate coevolution of the two. The sequestering of organelles in sex-specific germ cells dictates the presence of distinct surface proteins of the organelles from sperm versus egg and the recognition of these epitopes by different tissues (see above) Sutovsky et al. 1999, Shitara et al. 2000). These transitions have largely been completed among extant organisms, and polymorphisms for degree of uniparental inheritance are not common. For direct experimentation, the doubly uniparental inheritance in mussels provides the most accessible system. However, with increased knowledge of the proteins that tag—and recognize—male and female mitochondria in a diversity of species, we may be able to disentangle the complex selective events that have lead to the common pattern of uniparental inheritance (Birky 1995).

SUMMARY AND PROSPECTS FOR FUTURE STUDIES

The hierarchical organization of organelle genomes (nested populations of DNAs within organelles, cells, germlines, populations, species, and higher clades) dictates a units-of-evolution perspective for organelle evolution. The reduction of genome size, uniparental inheritance, and the purging of deleterious mutations represent important themes in the macroevolution of organelle genomes. These themes have played out under the pressures of mutation, selection and drift operating at multiple levels across this nested hierarchy. Understanding the relative contributions of these forces at each level remains the biggest challenge for a mechanistic view of organelle evolution. We need additional analyses of mtDNA mutation accumulation, transmission, tissue-level variation, and whole-organism phenotype. A promising area of research will be the cytoplasmic population genetics of stem cells and the formation of mature oocytes. Comparisons of the mtDNA

populations of oocytes with those that survive to emerge as F_1 offspring could help establish if this transition between levels is an especially important phase in the purging of deleterious mutations. Some of the most promising systems are those in which experimental heteroplasmies can be constructed and mtDNAs tested for fitness effects at more than one level. In these systems, the context of conflict and cooperation among mtDNAs can be manipulated, allowing true units-of-selection experiments to be done. Much of the statistical machinery for cytoplasmic population genetics is in place, as is much of the theoretical underpinning of what cooperation and conflict might look like (Buss 1987, Keller 1999, Michod 1999).

As the nuclear machinery for mitochondrial function becomes elucidated, comparative genomics will provide rich opportunities for empirical studies of evolutionary questions. Population samples of whole mitochondrial genomes are the new standard for neutrality tests (Ballard 2000a,b) and will provide crucial haplotype information for fitness studies in distinct nuclear backgrounds. Systems in which direct genetic manipulations are not tractable will still play an important role in the dissection of selection on mtDNA. Particular attention should be focused on comparative cytonuclear genomics of organisms in which the standard rules of mitochondrial genetics may not hold (e.g., maternal inheritance, lack of recombination, atypical gene sets or orders). When sister groups exhibit variation in these properties, strong inferences about the mechanisms leading to these transitions can be made.

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