

THE POPULATION BIOLOGY OF MITOCHONDRIAL DNA AND ITS PHYLOGENETIC IMPLICATIONS

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■ **Abstract** The reconstruction of evolutionary trees from mitochondrial DNA (mtDNA) data is a common tool with which to infer the relationships of living organisms. The wide use of mtDNA stems from the ease of getting new sequence data for a set of orthologous genes and from the availability of many existing mtDNA sequences for a wide array of species. In this review we argue that developing a fuller understanding of the biology of mitochondria is essential for the rigorous application of mtDNA to inferences about the evolutionary history of species or populations. Though much progress has been made in understanding the parameters that shape the evolution of mitochondria and mtDNA, many questions still remain, and a better understanding of the role this organelle plays in regulating organismal fitness is becoming increasingly critical for accurate phylogeny reconstruction. In population biology, the limited information content of one nonrecombining genetic marker can compromise evolutionary inference, and the effects of nuclear genetic variation—and environmental factors—in mtDNA fitness differences can compound these problems. In systematics, the limited gene set, biased amino acid composition, and problems of compensatory substitutions can cloud phylogenetic signal. Dissecting the functional bases of these biases offers both challenges and opportunities in comparative biology.

INTRODUCTION

Mitochondria are thought to have originated from a free-living, aerobic, and motile α -proteobacteria containing 3000–5000 genes (Boussau et al. 2004). In animals, mitochondria are controlled by a dual genome system, with cooperation between endogenous mitochondrial genes and nuclear-encoded genes with two origins, 1) mitochondrial genes translocated to the nucleus over the course of evolution, and 2) nuclear genes that have acquired targeting signals and been recruited to derived functions in the mitochondrion (Rand et al. 2004). Mitochondrial genomes show varying degrees of reduction, ranging from 97 protein coding genes of the

protozoan *Reclinomas americana* (Berg & Kurland 2000) to a mere 3 protein coding genes in the malarial parasite *Plasmodium falciparum*. Despite the enormous variations in size, the coding function of the mitochondrial genome remains relatively stable in animals. In general, mitochondrial DNAs (mtDNAs) code only for genes involved in the mitochondrial translation apparatus, electron transport, and oxidative phosphorylation.

Mitochondria oxidize metabolic substrates, including carbohydrates and fats, to generate water and ATP, with O₂ acting as the terminal electron acceptor for the electron transport chains that generate the proton gradient across the inner mitochondrial membrane. Reducing equivalents in the form of electron donors are recovered from carbohydrates in the tricarboxylic acid cycle, whereas those recovered from fats are obtained through β -oxidation. The resulting electrons are transferred to the mitochondrial electron transport chains via complex I (about 43 nuclear-encoded and 7 mtDNA-encoded loci) or complex II (4 nuclear-encoded loci) and then flow to ubiquinone. Ubiquinone transfers electrons to cytochrome c and eventually to complex IV (9 to 10 nuclear- and 3 mitochondrial-encoded loci), where 4 single-electron transfers to oxygen result in the formation of water. The energy released by the electron transport chain is used to pump protons out of the inner mitochondrial membrane, creating the transmembrane electron gradient. The potential energy stored in the gradient is used to condense ADP and Pi to make ATP via complex V (14 nuclear- and 2 mitochondrial-encoded loci). Generation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and organic hydroperoxides, at complexes I and III is believed to be important in aging with up to 1% to 2% of the oxygen consumed being converted to ROS. These ROS can damage DNA, lipids, and proteins, causing mitochondrial ROS production to increase with age.

In 1987, John Avise et al. illustrated how mtDNA can be used to bridge the gap between population genetics and systematics (Avise et al. 1987). This classic review considered many issues, including the impact of selection versus neutrality, heteroplasmy (the situation in which, within a single cell, there is a mixture of different mtDNA haplotypes), homoplasmy (resemblance due to parallelism or convergent evolution rather than to common ancestry), scale, and lineage sampling bias on phylogenetic hypotheses generated from mtDNA. Over the past two decades, a number of new issues have arisen while some debates have continued. One of the most important issues to have arisen is the influence of nuclear mitochondrial pseudogenes on inferences of population history (Bensasson et al. 2001, Thalmann et al. 2004). One dispute that continues is the selection versus neutrality debate. The selection versus neutrality debate is a focus of this review because it concerns the basic biology of mitochondria and mtDNA, and it has phylogenetic implications. We agree with Avise et al. that “the phylogenetic value of mtDNA does not . . . completely hinge on the outcome [of this debate].” We do, however, propose that it can no longer be assumed that mtDNA is evolving in a manner consistent with a strictly neutral equilibrium model. In this review, we present examples showing that mtDNA variation influences organismal fitness. We then

consider factors that may bias phylogenetic inference. Finally, we propose some statistical tests that should be routinely included in phylogenetic studies, which would help researchers determine the robustness of the genealogical hypotheses they have generated from mtDNA.

mtDNA AS A NEUTRAL MARKER

For practical reasons, mtDNA is widely used because it provides easy access to an orthologous set of genes with little or no recombination and rapid evolution. From a theoretical perspective, mtDNA is widely used in population genetic, phylogeographical, and phylogenetic studies owing to the belief that haplotype frequencies are governed primarily by migration and genetic drift and that most of the variation within a species is selectively neutral. Indeed, the majority of studies have assumed that mtDNA is evolving as a strictly neutral marker, and researchers have employed it to estimate a range of evolutionary and ecological parameters of interest such as divergence times and phylogeographic patterns. Ballard & Kreitman (1995) reviewed the literature and concluded that the widespread acceptance of the selective neutrality of mtDNA follows from a series of plausibility arguments connecting features of mtDNA evolution with (mis)conceptions of neutral theory. Deviations from a strictly neutral model of evolution have been found in a variety of organisms (Nachman 1998, Rand 2001, Rand & Kann 1998).

A priori, we suggest it is reasonable to predict that mtDNA variation may be under strong selection for three main reasons. First, the mitochondrion is the powerhouse of the cell and, in most organisms, a reduction in ATP production is expected to reduce fecundity. In humans, a reduction in the efficiency of ATP production is known to be highly deleterious and lethal in the extreme case. Second, proteins from mtDNA interact with those imported from the nuclear genome to form four of the five complexes of the electron transport chain. Third, the lack of normal recombination in mtDNA means that each genome has a single genealogical history and all genes will share that history. Any evolutionary force acting at any one site will equally affect the history of the whole molecule. Thus, the fixation of an advantageous mutation by selection, for example, will cause the fixation of all other polymorphisms by a process known as genetic hitchhiking (Maynard Smith & Haigh 1974). Even the quickly evolving noncoding origin-of-replication region cannot be assumed to have neutral allele frequencies: It is linked to the rest of the genome where selection has been documented, and conserved motifs within this region exhibit variation that affects mitochondrial transcription and replication in significant ways (Coskun et al. 2004). Alternatively, polymorphism within a mitochondrial genome may be depressed through selection against linked deleterious mutations, a process known as background selection (Charlesworth 1994, Charlesworth et al. 1993, 1995). We suggest that the hypothesis that mtDNA is under strong selection is rarely explored, and selective neutrality is assumed because there is a perceived lack of evidence suggesting that different mtDNA types

within a species have unequal fitness (Ballard & Whitlock 2004). In the following paragraphs, we review the evidence and conclude that different mtDNA haplotypes (sometimes called mitotypes) can have a significant influence on organismal fitness in a wide variety of species including humans.

The direct impact of mtDNA variation on fitness has been measured in humans (Ruiz-Pesini et al. 2000), mice (Roubertoux et al. 2003, Takeda et al. 2000), *Drosophila* (Ballard 2004; de Stordeur 1997; Fos et al. 1990; Hutter & Rand 1995; James & Ballard 2003; Kilpatrick & Rand 1995; Nigro 1991, 1994; Rand et al. 2001), and copepods (Schizas et al. 2001). In humans, there is increasing evidence showing that human sperm motility is strongly dependent on the ATP supplied by oxidative phosphorylation (Ruiz-Pesini et al. 1998). The frequency of the known pathological mtDNA mutations within humans is insufficient to explain a significant proportion of the asthenozoospermic (reduced sperm motility) patients. As a consequence, it was proposed that mtDNA mutations affecting mitochondrial ATP production could cause reduced sperm motility. Ruiz-Pesini et al. (2000) analyzed the distribution of mtDNA in Caucasian men having fertility problems and found that the sperm-motility phenotype was indeed conditioned by the mtDNA type. mtDNA is maternally inherited and is not expected to suffer strong selective pressure in males as it is an evolutionary dead end (Frank & Hurst 1996). We suggest that this striking result illustrates an underappreciated feature of mtDNA evolution. If sperm dysfunction (or any male specific effect) is the main, or the only, phenotypic consequence of a mtDNA mutation, specific mutation(s) could accumulate within a population and reach high frequency.

In a somewhat controversial and highly publicized study Roubertoux et al. (2003) showed that mtDNA influenced learning, exploration, sensory development, and the anatomy of the brain in mice. The effects of the mtDNA type persisted with age, increasing in magnitude as the mice got older. To complete this study, the authors were very careful to develop a well-controlled set of strains to effectively determine true mtDNA contributions as well as interactions with nuclear DNA. The experimental design excludes both the effects of genomic imprinting and the influences of the cytoplasmic and maternal environment of a parental strain on its mtDNA congenic strain.

A body of research shows that the three distinct and geographically subdivided mtDNA types of the fly *Drosophila simulans* (*siI*, *-II*, and *-III*) have significantly different effects on organismal fitness (Ballard 2004, Solignac et al. 1986). de Stordeur (1997) conducted microinjection transfection studies between eggs carrying the mtDNA types and assayed the frequencies of the foreign injected mtDNA in heteroplasmic strains. He observed that the mtDNA types have unequal fitness within cells during transmission between generations. James & Ballard (2003) controlled the nuclear genome of flies by backcrossing and observed that the mtDNA haplotype influenced physical activity, development time, and longevity. Ballard & James (2004) observed that the relative fitness of the three mtDNA types in perturbation cages was positively correlated with the observed worldwide distribution of the mitotypes (*siII* > *-III* > *-I*). However, it was also clear that mitochondrial-nuclear (hereafter mitonuclear) interactions also influenced the

fitness of flies in population cages (Ballard & James 2004). Mitonuclear interactions have also been shown to influence the frequencies of flies in cages and in biochemical assays of mitochondrial metabolism. Nigro (1991) employed wild-caught and microinjected lines to show that both the mtDNA and nuclear background influenced the competitive ability of flies in population cages. Mitonuclear interactions have been shown to influence cytochrome c oxidase (complex IV) activity in wild-type and introgressed *D. simulans* (Sackton et al. 2003).

Mitonuclear Interactions

Mitochondrial fitness effects may be conferred directly by the mitochondrial genotype, nuclear-encoded loci-producing proteins imported into the mitochondrion, and/or coadapted mitonuclear gene complexes. It has been suggested that this is a potential explanation for the basic conundrum, "Why are mitochondria maternally inherited?" Ross (2004) proposes that the specific degradation of the paternal mitochondrial genome could have been selected to prevent competition between mtDNA and nuclear DNA gene products. Minor mutations in either mtDNA or nuclear DNA coding for proteins essential for oxidative phosphorylation are known to lead to major and catastrophic diseases of humans, suggesting that very tight and precise interactions are required. Most often, paternal mtDNA within a species is quickly degraded following recognition of a ubiquitin tag (Sutovsky et al. 1999), but recognition of paternal mtDNA apparently depends on phylogenetic relatedness. It seems that when the paternal mtDNA is more than about 2.5% divergent from the maternal mtDNA the paternal mtDNA is not recognized and excluded. Under such conditions high rates of heteroplasmy are observed (Kaneda et al. 1995, Kondo et al. 1990, Satta et al. 1988). It remains to be determined whether diverged mitochondria simply escape tagging or the hybrid nuclear genomes from crosses between divergent species are compromised in the tagging process.

The evolutionary forces responsible for the movement of genes from the mitochondrion to the nucleus is better understood in plants than in animals (Adams et al. 2002, Bergthorsson et al. 2003). However, the physical proximity of mtDNA to sites where ROS are produced may be a major reason for the selective pressure reinforcing transfer of genes to the nuclear genome (ROS quickly damages mtDNA and this may cause a rapid decline in metabolic efficiency and fecundity). Certainly, the movement of genes from the mitochondrial to the nuclear genome places the gene in a very different chromosomal context, including differences in the genetic code and codon bias. As a consequence of this export, it may be expected that genes may show a burst of evolution or acceleration in the rate of evolution following transfer. Unfortunately, this may be very difficult to detect because there is likely to be overlap in the functioning of the mitochondrial- and the nuclear-encoded genes. From a phylogenetic perspective, researchers must be careful in the assigning of homology in such cases.

The movement of genes from the mitochondrial to the nuclear genome is documented both between and within species. Different species of *Rickettsia* show

varying degrees of “mutational meltdown,” suggesting that movement of genes from the mitochondrion to the nucleus is an ongoing process (Andersson & Andersson 1999, Andersson et al. 1998). A result of the export of DNA from the mitochondrion is that nuclear-encoded proteins must be reimported into the mitochondrion to ensure successful mitochondrial metabolism. Zeviani et al. (1999) grouped nuclear-encoded proteins, which influence the structure and function of mitochondria, into three categories: structural components of the electron transport chain, factors influencing the structural integrity or copy number of mtDNA, and proteins that control the formation, assembly, and turnover of respiratory complexes.

Mitochondrial interactions influence complexes I, III, IV, and V of the electron transport chain. Schmidt et al. (2001) studied the functional interactions between mitochondrial and nuclear-encoded proteins in the multisubunit respiratory complex cytochrome c oxidase (complex IV) in six species of mammals, using chickens as an outgroup. In this complex, mtDNA-encoded residues in physical proximity to nuclear DNA-encoded residues evolved more rapidly than the other mtDNA-encoded residues, indicating that mitochondrial interactions can alter rates of amino acid substitutions. The complexity of these mitochondrial gene interactions is compounded by the fact that genes from both genomes show significant sequence polymorphism. On average, two unrelated humans differ at more than 50 nucleotide polymorphisms in their mtDNAs, about 20–30 of which lead to amino acid changes (Ingman et al. 2000, Rand & Kann 1996). Two humans also differ at ~1 million polymorphic nucleotide sites across the nuclear genome, several thousand of which will alter a protein sequence (Sachidanandam et al. 2001).

Mitochondrial interactions influence a variety of biochemical and physiological processes and these may influence the pattern of evolution shown by specific genes. Kern & Kondrashov (2004) compared 86 pathogenic mutations in human tRNAs, encoded by mitochondrial genes, to the sequences of their mammalian orthologs and noted that 52 pathogenic mutations were present in normal tRNAs of one or several nonhuman mammals. The authors proposed that a pathogenic mutation and its compensating substitution are fixed in a lineage in rapid succession. At least 10%, and perhaps as many as 50%, of all nucleotide substitutions in evolving mammalian tRNAs participate in such interactions, indicating that the evolution of tRNAs proceeds along highly epistatic fitness ridges. Because mitochondrial translation requires many proteins encoded in the nucleus, a greater understanding of these processes will enhance our ability to interpret selection on tRNAs. This, in turn, will facilitate accurate determination of the models that are most appropriate for phylogenetic analyses.

Mitochondrial interactions have also been shown to influence organismal fitness, and this influences our interpretation of the selective neutrality of mtDNA. The first experiments considering the coevolution of nuclear and mitochondrial genomes were reported in 1971 (Clayton et al. 1971). In 1997, elongated cells from hominoid apes (chimpanzee, pigmy chimpanzee, gorilla, and orangutan) were fused with mtDNA-less human cells (Kenyon & Moraes 1997), thereby creating cells with ape

mtDNA and human nuclear DNA. Only the combinations of human nuclear DNA with mitochondria from the most closely related species (chimpanzee, gorilla) yielded cells even capable of oxidative phosphorylation.

More recently, it was shown that mice with introgressed interspecific and intersubspecific mtDNA exhibit reduced physical performance (Nagao et al. 1998). Nagao and colleagues backcrossed *Mus spretus* and *M. musculus* mtDNA into *M. domesticus*. These represent the conditions of interspecific and intersubspecific mitonuclear mismatch, respectively. Using these backcross mice, they examined physical performance by measuring running time on a treadmill until exhaustion. The result clearly showed that mtDNA backcross lines manifested a significant decrease in their physical performance compared to their progenitor lines. In the marine copepod, *Tigriopus californicus*, Rawson & Burton (2002) found that the cytochrome c variants isolated from two different populations each had significantly higher activity with the cytochrome c oxidase derived from their respective source population. Three amino acid substitutions in the cytochrome c protein appear to be sufficient to confer population specificity. These results suggest that electron transport chain proteins form coadapted sets of alleles within populations and that disruption of the coadapted gene complex leads to functional incompatibilities that may lower hybrid fitness. Mitonuclear coadaptation not only is an interesting feature of many species in nature, but can also be observed to evolve within only 2000 generations under replicated laboratory conditions. Experimental evolution in yeast populations has shown that the competitive ability of evolved strains relative to ancestral strains is governed by mitonuclear epistatic interactions (Zeyl et al. 2005).

In humans, the same mtDNA mutations may induce longevity or diseases, depending on their interactions with nuclear loci. De Benedictis et al. (2000) observed that Italian male centenarians had a significantly higher frequency of the European mitotype J than sex-matched younger subjects having the same ethnic and geographic origin. This result suggests mtDNA-specific effects on the rate and quality of aging. Somewhat surprisingly, however, complete mtDNA sequencing demonstrated that the J haplogroup is characterized by a particular suite of six mutations often associated with disease (Rose et al. 2001). From these data it has been argued that key mtDNA mutations may induce death or extend life span depending on other mtDNA mutations and on stoichiometric mismatches with nuclear-encoded proteins in each oxidative phosphorylation subunit.

SYSTEMATIC BIASES IN EMPLOYING mtDNA AS AN EVOLUTIONARY MARKER

In this section, we consider how phylogenetic hypotheses based on mtDNA may be systematically biased. Most obviously, this could occur when limited sampling occurs in a species harboring multiple mtDNA types (Ballard 2000c). This may be more widespread and difficult to detect than previously believed. In the copepod

T. californicus there are at least nine haplogroups with up to 23% nucleotide divergences and up to 3% amino acid divergences (Edmands 2001). It has recently been shown that slightly deleterious mutations segregating within haplogroups may be removed by selection prior to their fixation among haplogroups (Dean & Ballard 2005). This results in distinct intraspecific mtDNA lineages behaving more like lineages between species.

One strong candidate for a type of selection that may cause population subdivision is thermal adaptation (Ballard & Whitlock 2004). This may result in the clustering of species that exist, or existed, in similar climates. Given the potential for temperature variation across species' ranges in nature and the sympatric distributions of closely related taxa, temperature may also play a strong role in selecting for introgression of alien mtDNA from locally better-adapted species (Ballard & Whitlock 2004). A second candidate that may cause population subdivision is infection with a maternally inherited symbiont. In this review, we focus on *Wolbachia*, as it has been shown to influence mitochondrial evolution in a variety of arthropods (Karr & Ballard 2005).

Thermal Adaptation

The potential for temperature to influence the evolution of mtDNA was the focus of much attention a decade ago (Martin & Palumbi 1993, Martin et al. 1992, Rand 1993, 1994). Martin et al. (1992) reported that the nucleotide substitution rates in the cytochrome b and cytochrome oxidase I genes in sharks are seven- to eightfold slower than in primates or ungulates. In the following year, Martin & Palumbi (1993) show that, in general, exothermic vertebrates have slower mtDNA substitution rates overall than do endotherms of similar size. Such differences in mtDNA substitution rates suggest that the thermal environment may influence rates of evolution and indicate that it is inappropriate to use a calibration for one group to estimate divergence times or demographic parameters for another group.

Temperature adaptation has been shown to be important in arctic fishes and other species (Somero 2002, Sommer & Portner 2002), but the role of mitochondrial variation in this adaptation is little investigated. In *Drosophila*, the fitness of the mtDNA haplogroups appears to be temperature dependent. In a series of papers Matsuura and colleagues (Matsuura et al. 1993, Nagata & Matsuura 1991) have systematically examined the transmission rates of *Drosophila* mtDNA haplogroups in flies made heteroplasmic by microinjection. The most recent paper showed that the nuclear genome is involved in determining the temperature dependency of mtDNA transmission (Doi et al. 1999).

It has also been argued that thermal adaptation may occur within humans. Ruiz-Pesini et al. (2004) conducted a phylogenetic study including 1125 globally distributed human mtDNA sequences and observed that the relative frequency and amino acid conservation of internal branch amino acid mutations increased from tropical Africa to temperate Europe to arctic northeastern Siberia. Highly conserved amino acid changes were found at the roots of multiple mtDNA lineages

from higher latitudes prompting the authors to suggest that specific mtDNA non-synonymous mutations permitted our ancestors to adapt to more northern climates because their mitochondria produced more heat. However, Elson et al. (2004) analyzed complete mtDNA coding-region sequences for 560 maternally unrelated individuals of European, African, and Asian descent and were not able to replicate the results of Ruiz-Pesini et al. (2004). Elson et al. concluded that appropriate methodology with which to study climatic adaptation and the development of alternative methods is a goal for ongoing research.

A question for future research is, "How frequently has thermal adaptation within and among species affected mtDNA variation?" We suggest that selection is a viable alternative in two classic cases where mtDNA has been used as a phylogeographic marker. Populations of the American oyster from the Gulf of Mexico and the Atlantic coasts of the southern United States have a dramatic mtDNA discontinuity on the Florida panhandle. However, surveys of polymorphic allozymes reveal near uniformity of allele frequencies throughout the range (Reeb & Avise 1990). Subsequent surveys of four nuclear restriction fragment length polymorphisms (RFLPs) tended to support the Atlantic/Gulf mtDNA dichotomy (Karl & Avise 1992), although the pattern of variation of only one nuclear locus occurred in the same region of the coastline as the mtDNA discontinuity. Karl & Avise (1992) considered a variety of alternatives to reconcile these data and suggested that it is most likely that the allozyme loci are under balancing selection. One clear alternative is that the mtDNA is under strong thermal selection associated with the different temperature between Atlantic and Gulf waters.

A second example comes from the killifish. González-Villaseñor & Powers (1990) demonstrated that the distribution of mtDNA RFLP polymorphism among populations of the killifish showed a marked disjunction between 39.7°N to 40.7°N in northeastern North America. One mtDNA type was fixed in the north and another in the south. To investigate this result Ropson et al. (1990) examined the geographical variation in 15 nuclear loci. Four showed a pattern of variation similar to the mtDNA. However, in no case was the disjunction in the same geographic region as that observed for the mtDNA. Ropson et al. consider a variety of alternatives "in the absence of selection" but never fully explore the possibility that selection may operate on the mtDNA itself.

Wolbachia

In arthropods, infectious microorganisms like the bacterium *Wolbachia* are widespread (Werren et al. 1995). *Wolbachia*-induced incompatibility has been shown to cause the symbiont and the linked, maternally inherited mitochondrial genotype to rise in frequency in theory (Caspari & Watson 1959), in population cages (Kambhampati et al. 1992, Nigro & Prout 1990), and in nature (Turelli & Hoffmann 1991). For mtDNA, the key theoretical result is that, as the maternally inherited infection spreads, it carries along whatever mtDNA genotype was initially associated with it (Turelli et al. 1992). This horizontal spread of an infectious organism

throughout a group is a source of discrepancy between mtDNA-based and nuclear gene-based phylogenies. In *D. simulans*, the genealogy of mtDNA reflects the spread of *Wolbachia* rather than the relatedness of populations as assessed by nuclear DNA (Ballard et al. 2002, Dean & Ballard 2004).

Wolbachia symbionts may influence mtDNA diversity in at least three other ways. First, the resident strain of *Wolbachia* may have lost the ability to cause incompatibility. In this case, mutations may be accumulating in the mtDNA but still be depressed below the neutral equilibrium value [this may be the case in *D. melanogaster* (Ballard et al. 1996)]. Second, *Wolbachia* itself may directly affect the fitness of infected individuals. Males of *Sphyracephala beccarii* (a Stalk-Eyed Fly) infected with *Wolbachia* may have higher fertility than uninfected males (Hariri et al. 1998). *Wolbachia* can extend longevity and increase fecundity of some strains of *D. melanogaster* (Fry & Rand 2002, Fry et al. 2004). Conversely, a significant reduction in sperm production and fertility has been found in a *D. simulans* line, suggesting a negative fitness effect (Hariri et al. 1998, Snook et al. 2000). Third, *Wolbachia* may protect a less fit mtDNA type from going to extinction. One example of this protection may occur in the *D. simulans* *siI* type, which is an island endemic. In microinjection (de Stordeur 1997) and cage experiments (Ballard & James 2004), the *siI* mtDNA type is out-competed by flies harboring *siII* and *siIII* mtDNA. However, at least 99.9% of flies harboring *siI* mtDNA are infected with the *wHa* *Wolbachia* strain that induces high levels of incompatibility. In this case the microorganism-induced incompatibility may effectively limit the colonization potential of the fitter mtDNA types.

SAMPLING METHODS AND STATISTICAL TESTS THAT SHOULD BE ROUTINELY INCLUDED IN PHYLOGENETIC STUDIES

Reconstructing phylogenies involves inferring the branching sequence, but also describing the rate and the pattern of character-state change along each branch. In this way we can test alternative processes to explain the patterns of variation we observe. In this review, we argue that developing a more complete understanding of the biology of mitochondria is essential for interpreting evolutionary processes that influence mtDNA. However, mtDNA in an individual is completely linked with little recombination, and information from different mtDNA genes does not give us statistical independence. If the goal is to reconstruct the species-level phylogeny, we advocate the inclusion of nuclear DNA. In the following sections, we discuss some issues to resolving the mtDNA genealogy.

Phylogenetic Tests

From a sequence alignment, a phylogenetic tree can be reconstructed by several means. Each method, however, uses different assumptions (stated either explicitly

or implicitly), different optimality criteria, and different tree search algorithms. Evolutionary rate heterogeneity can have important phylogenetic implications. Here, we consider heterogeneity in rate along length of the sequence and then rate heterogeneity in different regions of the tree.

Heterogeneity in substitution rates can occur in regions of mtDNA (Ballard 2000a,b) and can influence phylogenetic hypotheses. Steinbach et al. (2001) compared the robustness of distinct genes and of different tree-building methods in resolving a well-corroborated *Drosophila* mitochondrial genealogy. Somewhat surprisingly, ND5, the longest gene (1713 bp), recovered the correct topology for fewer than 30% of the methods/models. One explanation for this result is that two regions of this gene have significant rate heterogeneity (Ballard 2000b), possibly causing its inconsistent performance. Rate heterogeneity can be investigated with the sliding-window maximum likelihood method, implemented in PLATO (partial likelihoods assessed through optimization) (Grassly & Holmes 1997). This method aims to detect regions that conflict with a single phylogenetic topology and nucleotide substitution process derived from the entire sequence. Such deviation along sequences, called spatial phylogenetic variation by Grassly & Holmes (1997), may reflect recombination or varying selective forces along the sequence. This approach calculates the likelihoods for each site independently. It then generates a measure of the average likelihood of a given window with respect to the rest of the sequence. Maximum values of this method are associated with regions showing low likelihoods given the maximum likelihood model derived from the complete sequence. If rate heterogeneity is detected, the phylogenetic and population inferences should be independently assessed (Dean & Ballard 2004).

Varying substitution patterns in different parts of a tree may affect the estimation of branch lengths and also possibly the branching pattern of a reconstructed tree. Cummings et al. (1995) analyzed complete mitochondrial genomes of 10 vertebrates and found that individual genes—or contiguous nucleotide sites—provided poor estimates of the tree taken from whole mtDNA sequences, reflecting biases in the information of individual mtDNA genes. Devauchelle et al. (2001) investigated proteins encoded by the mitochondrial genome of 26 multicellular animals, which include vertebrates, arthropods, echinoderms, mollusks, and nematodes, and showed that systematic deviations from a single-rate model are unmistakable and related to the evolutionary history of the species under consideration. Weiss & von Haeseler (2003) tested the assumption that there was homogeneity of the substitution process using simulation studies and analyzed two real data sets, one of which was complete hominid mtDNA data. Statistical analysis and the development of a new test showed that the substitution process was not homogeneous within the hominid mtDNA, suggesting a change in evolutionary pressures in different parts of the tree. If statistical tests suggest a rejection of the homogeneity assumption, results of a phylogenetic reconstruction should be interpreted with care. Varying substitution patterns affect the estimation of branch lengths and possibly also the branching pattern of the reconstructed tree. For example, it is well known

that compositional changes in nucleotide frequencies can produce misleading trees (Galtier & Gouy 1995, Lockhart et al. 1994). Two examples from earlier literature on mtDNA illustrate this point. In mammals and primates, the directional nucleotide substitution in favor of A + T nucleotides increases with weight-specific metabolic rate (Martin 1995). In addition, mitochondrially encoded proteins have highly hydrophobic amino acid compositions, limiting the information content in these sequences for phylogenetic analysis (Naylor & Brown 1997). One approach to minimize the potential for obtaining incorrect trees has been to develop more sophisticated models of nucleotide substitution with the hopes that the problems of analysis will be eliminated. Indeed, model-based approaches to phylogenetic reconstruction can facilitate determination of the correct tree if the correct model can be estimated (Sanderson & Shaffer 2002). Here we argue that testing the constraints on mtDNA sequence evolution, which stem from the biology of mitochondria, is in need of additional scrutiny. For example, empirically derived mtDNA substitution matrices of either nucleotides or amino acids could be used as a set of priors in a Bayesian analysis to improve accuracy. Branch length priors may also influence phylogenetic analyses and the effect of this could be considerable for species where mtDNA evolution is particularly rapid.

mtDNA and Deep Phylogenies

mtDNA has been used to infer deep phylogenies. A recent review of the current state of understanding of the evolution of the mitochondrial genome by Bullerwell & Gray (2004) comprehensively discusses the use of mtDNA to infer deep evolutionary relationships. Knoop (2004) summarizes the unique aspects of land plant mitochondrial evolution from a phylogenetic perspective. However, neither of these reviews presents a robust mechanism by which the phylogeny can be corroborated, and a concern exists over the level of homoplasmy in such data sets. One goal of Engstrom et al. (2004) was to examine strategies for analyzing highly homoplasious mtDNA data in deep phylogenetic problems where increased taxon sampling is not an option. The analyses of the combined data set from two mitochondrial protein-coding genes and an approximately 1-kb nuclear intron, and 59 morphological characters converged on a set of well-supported relationships. In this case, weeded and weighted parsimony, and model-based techniques, generally improved the phylogenetic performance of highly homoplasious mtDNA sequences, but no single strategy completely mitigated the problems associated with these highly homoplasious data. Indeed, many deep nodes in the softshell turtle phylogeny were confidently recovered only after the addition of largely nonhomoplasious data from the nuclear intron.

In an attempt to resolve deep, ordinal-level phylogenies with mtDNA a number of researchers have proposed using mtDNA gene order as a character set (Boore et al. 1995). If rearrangements in mtDNAs are relatively rare in evolutionary history, these events can be useful synapomorphies (derived character states that are shared by two or more taxa). Under such scenarios, convergent or parallel translocations and reversions in nonrelated lineages would be expected to be uncommon, though

convergent rearrangement of the mitochondrial genome has been observed in the reptile group Amphisbaenia (Macey et al. 2004). One potential source of concern is the lack of understanding of the mechanism of mtDNA gene order rearrangement (Dowton & Campbell 2001, Sun et al. 2005). This is particularly the case where large-scale rearrangements have occurred, such as in *Tigriopus japonicus* (Machida et al. 2002). The difficulty in identifying homologs in short tRNA genes is an area of particular concern. Rawlings et al. (2003) suggests that through a process of tRNA duplication and mutation in the anticodon triplet, remodeled leucine (L_{UR}) tRNA genes have repeatedly taken over the role of isoaccepting L_{CUN} leucine tRNAs within metazoan mtDNA. They further suggest that tRNA leucine duplication and remodeling events have occurred independently at least seven times within three major animal lineages. One clear area of future research is to examine the mechanism of mtDNA rearrangements in closely related groups. One such group is the insect order Hymenoptera (Dowton et al. 2003). Although different partitions or approaches to mtDNA sequence analysis are effective at different depths of evolutionary history, phylogeneticists need to accept the possibility that mtDNA may not be capable of resolving deep phylogenies. Phylogenetic analysis of whole nuclear genome sequences in yeasts revealed that at least 20 independent genes were needed to recover the whole-genome tree (Rokas et al. 2003).

mtDNA and Speciation

Many researchers have assumed explicitly or implicitly that differentiation within a character system is indicative of organismal differentiation or history. From the standpoint of mtDNA, attention has been focused on whether mtDNA differentiation is indicative of species trees or gene trees. A genealogical species is defined as a basal group of organisms whose members are all more closely related to each other than they are to any organisms outside the group and which contains no exclusive group within it (Baum & Shaw 1995, Cracraft 1983). In practice, a pair of species is so defined when phylogenies of alleles from a sample of loci show them to be reciprocally monophyletic at all, or monophyletic for some specified fraction of the loci (though not all genealogical species concepts require reciprocal monophyly). Hudson & Coyne (2002) investigated the length of time it takes to attain reciprocal monophyly when an ancestral population divides into two descendant populations of equal size with no gene exchange and when genetic drift and mutation are the only operating evolutionary forces. A clear lesson from their results is that one should be cautious about recognizing genealogical species using only mtDNA. Such DNA becomes monophyletic more rapidly than does a single nuclear gene, and it does so far more rapidly than does a sample of several nuclear genes. This may make inferences of species-level monophyly erroneous. Funk & Omland (2003) also argue that data from mtDNA may not provide an accurate measure of species status. To evaluate the importance of species-level polyphyly, Funk & Omland (2003) conducted an intense survey of studies that evaluate mtDNA variation in a phylogenetic context and observed that the mtDNA

monophyly of many biological species is not well supported. Funk & Omland (2003) detected species-level mtDNA paraphyly or polyphyly in 23% of 2319 assayed species, demonstrating that this problem is statistically supported, taxonomically widespread, and far more common than previously recognized. These patterns could be due to (a) sporadic hybridization among divergent lineages, (b) incomplete lineage sorting of mtDNA gene trees relative to organismal lineages, and/or (c) selection on mtDNA that might retain certain haplotypes within diverging lineages.

Moreover, mtDNA has great potential for becoming monophyletic by selective sweeps. This can decrease the time to monophyly of a clade and not be reflective of the geological processes in the nuclear genome. Advantageous mutations occurring on mtDNA will cause the entire organelle genome to become monophyletic because such genomes have little or no recombination. Although selective sweeps will also occur in nuclear DNA, causing monophyly for regions linked to the selected locus, recombination will whittle away the section of genome that becomes monophyletic through linkage.

Finally, when there is gene flow between diverging populations, one may encounter the opposite problem: mtDNA may be homogenized between the populations more readily than is nuclear DNA, so that mtDNA may appear paraphyletic when nuclear genes may be monophyletic. In fish, mice, and crickets, for example, mtDNA flows between taxa much more readily than does nuclear DNA (e.g., Bernatchez et al. 1995, Ferris et al. 1983, Shaw 2002, Taylor & McPhail 2000). In some cases, the mtDNA from one taxon completely replaces that in another, without any evidence of nuclear introgression or morphological signal. For example, the mtDNA in an allopatric population of brook trout in Lake Alain in Québec is identical to the Québec arctic char genotype, yet the brook trout are morphologically indistinguishable from normal brook trout and have diagnostic alleles at nuclear loci (Bernatchez et al. 1995). Ballard & Whitlock (2004) discussed the possible explanations of selection and drift on introgression.

Sampling and Neutrality Testing

When conducting phylogenetic analyses, we advocate including multiple individuals within all species possible. This will enable the researcher to test the basic assumptions and predictions of the neutral model: a constant mutation rate, a stationary allele frequency distribution, and a correlation between polymorphism levels and divergence. Specifically, we suggest researchers sample multiple individuals from a broadly distributed species or a species that occurs in a variety of niches. Consideration of the species to be sampled should also include contemplation of the outgroup of each species. In many statistical tests of neutrality, it is also important to include a closely related outgroup (Table 1).

Deviations from a strictly neutral model of evolution are found in a variety of organisms (Nachman 1998, Rand 2001, Rand & Kann 1998), and reviews have

TABLE 1 Approaches that can be employed to test the evolutionary dynamics of mitochondrial DNA (mtDNA) (modified from Ballard & Kreitman 1995)

Approach	Test	Prediction under neutrality	References
Direct	Competition between mitochondrial haplotypes	Haplotype frequency will not change in any predictable or repeatable way	Ballard & James 2004, Fos et al. 1990, Hutter & Rand 1995, MacRae & Anderson 1990, Nigro & Prout 1990, Singh & Hale 1990
Phylogenetic	Rates of evolution along different lineages	The variance should equal the mean after taking into account possible "lineage" effects, such as differences in mutation rate	Gillespie 1991, Tajima 1993
	Types of mutational changes along different branches of a tree	The type or pattern of substitution should be the same on all branches	Akashi 1995, Ballard 2000b, Ballard & Kreitman 1994
Statistical	Frequency spectrum of haplotypes	With no recombination and no selection, haplotype frequencies should conform to the neutral infinite alleles distribution	Ewens 1973, 1975; Fu 1997; Fu & Li 1993; Tajima 1989; Watterson 1977
	Distribution of polymorphism within species and divergence between species for two loci	The level of polymorphism and divergence, governed only by genetic drift and selective constraints, will be positively correlated	Hudson et al. 1987
	Distribution of synonymous and replacement changes within and between species. Easily extended to preferred or unpreferred synonymous sites, or conservative versus radical amino acids	If the synonymous and replacement variation is neutral, the divergence of the ratio of polymorphism will be the same when species compared are similar	Ballard & Kreitman 1994, McDonald & Kreitman 1991, Rand et al. 2000
	Distribution of substitutions on lineages	The substitution rate matrices do not differ for all lineages	Weiss & von Haeseler 2003
	Distribution of substitutions along the sequence	The substitution rate matrices do not vary along the length of the sequence	Grassly & Holmes 1997

collated the battery of statistical tests that can be applied to mtDNA (Ballard & Kreitman 1994, Gerber et al. 2001) (Table 1). These data typically show an excess of amino substitutions within species, suggesting the accumulation of slightly deleterious intraspecific changes. Most statistical tests are implemented in shareware computer programs, including Arlequin (<http://lgb.unige.ch/arlequin/>) written by Laurent Excoffier and DNASP (<http://www.ub.es/dnasp/>) written by Julio Rozas & Ricardo Rozas (1997). Rejection of the null hypothesis likely means that selection-and/or population-level processes (expansion, contraction, subdivision, etc.) are operating on the region of interest. In these cases it is unwise to overinterpret phylogeographic and phylogenetic hypotheses. Alternatively, rejection of the null hypothesis often opens up new and exciting areas of study comparing different properties of selection on mitochondrial genes and proteins (Rand & Kann 1996, Rand et al. 2000, Weinreich & Rand 2000). There are far more data available for population genetic analysis of mtDNA than have been used, and this represents a great opportunity for future study to gain a greater understanding of the biology of mtDNA.

CONCLUSIONS

The vast majority of studies employing mtDNA as an evolutionary marker have not attempted to expand our knowledge of the basic biology of mitochondria. We believe that this can and should be done in the forthcoming years so that hypotheses generated from mtDNA data are robust. We have identified a number of research areas in this review that would benefit from additional work. These include mechanisms of mtDNA rearrangement and research focusing on thermal adaptation as a mechanism for population subdivision. We have also identified specific tests that should be included with studies using mtDNA as an evolutionary marker. These include heterogeneity rate tests both along the length of the sequence, heterogeneity rate tests in different branches of the tree, and tests of the basic assumptions and predictions of the neutral model. The omission of these tests limits our ability to interpret the results of these analyses, but perhaps more importantly it misses an opportunity to understand the nature of the basic biology of mitochondria. We conclude that we should not throw the organelle baby out with the organismal bathwater. Rather, we should develop a greater understanding of the basic biology of the molecule so that evolutionary hypotheses are robust.

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