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# The incomplete natural history of mitochondria

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**Abstract**

**Mitochondrial DNA (mtDNA) has been used to study molecular ecology and phylogeography for 25 years. Much important information has been gained in this way, but it is time to reflect on the biology of the mitochondrion itself and consider opportunities for evolutionary studies of the organelle itself and its ecology, biochemistry and physiology. This review has four sections. First, we review aspects of the natural history of mitochondria and their DNA to show that it is a unique molecule with specific characteristics that differ from nuclear DNA. We do not attempt to cover the plethora of differences between mitochondrial and nuclear DNA; rather we spotlight differences that can cause significant bias when inferring demographic properties of populations and/or the evolutionary history of species. We focus on recombination, effective population size and mutation rate. Second, we explore some of the difficulties in interpreting phylogeographical data from mtDNA data alone and suggest a broader use of multiple nuclear markers. We argue that mtDNA is not a sufficient marker for phylogeographical studies if the focus of the investigation is the species and not the organelle. We focus on the potential bias caused by introgression. Third, we show that it is not safe to assume *a priori* that mtDNA evolves as a strictly neutral marker because both direct and indirect selection influence mitochondria. We outline some of the statistical tests of neutrality that can, and should, be applied to mtDNA sequence data prior to making any global statements concerning the history of the organism. We conclude with a critical examination of the neglected biology of mitochondria and point out several surprising gaps in the state of our knowledge about this important organelle. Here we limelight mitochondrial ecology, sexually antagonistic selection, life-history evolution including ageing and disease, and the evolution of mitochondrial inheritance.**

*Keywords*: introgression, mitochondrial DNA, molecular population genetics, phylogeography, selection

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## Introduction

Mitochondrial DNA (mtDNA) has been used extensively in the last three decades as a tool for inferring the evolutionary and demographic past of both populations and species. This tool has proven invaluable for the new fields of molecular ecology and phylogeography. In recent years, however, the field has had an increasing awareness that inference based on this single molecule alone will not always be sufficient to answer the many interesting questions asked of it. With others, we feel that it is time to re-evaluate the strong reliance on mtDNA in phylogeography. We argue that more genetic markers are needed to answer most phylogeographical questions. Moreover, we suggest that there are many interesting questions about mitochondrial molecular evolution that have been overlooked by viewing it mainly as a tool.

This review considers the natural history of animal mitochondria and mtDNA and points out potential areas for future research. Many of the issues we raise might equally apply to the study of mtDNA in other taxa and to chloroplast DNA, but we limit our scope to animals. We focus on parameters that influence the evolution of mtDNA within and among closely related species and raise some issues with the phylogeographical interpretation of mtDNA data for species or populations, which are, or have recently begun to, separate. Mitochondrial DNA is used to address

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questions over a wider timescale, ranging from population structure issues where the timescales are set by recurrent dispersal between populations to generic level phylogenies where the timescale may be much larger than the time required for the evolution of complete reproductive isolation. At the population structure timescale, introgression and incomplete lineage sorting are not barriers to inference but are the objects of inference themselves. For long evolutionary timescales, the rate of hybridization may be reduced to the point where introgression is nearly impossible and lineage sorting will have occurred.

### *Biology and history*

Mitochondria occupy a central position in the biology of cells and are crucial to life, as we know it. Most eukaryotic cells contain many mitochondria, which contain many copies of mtDNA chromosomes. Mitochondria can move, fuse, and divide within a cell (Bereiter-Hann & Voth 1994). Collectively, they can occupy as much as 25% of the volume of the cytoplasm. The mammalian mitochondrial genome consists of a circular, double-stranded DNA molecule that is 15 000–17 000 base pairs in length. This is about 1/10 000 of the smallest animal nuclear genome.

Thirty-seven genes are encoded by mtDNA. Twenty-four encode the translational machinery of the mtDNA itself (22 tRNAs and two rRNAs). The additional 13 genes encode for subunits of the electron transport chain where carbohydrates and fats are oxidized to generate carbon dioxide, water and ATP. Indeed, mitochondria are responsible for the majority of ATP production. The energy released by oxidative phosphorylation in the electron transport chain of mitochondria is harnessed so efficiently that about 30 molecules of ATP are produced for each molecule of glucose oxidized, whereas only two molecules of ATP are produced by nuclear-controlled glycolysis alone. As a by-product of oxidative phosphorylation, mitochondria produce toxic reactive oxygen species such as superoxide, hydrogen peroxide and organic hydroperoxides. These reactive oxygen species can damage DNA, lipids and proteins, and their production increases with age. Figure 1 gives a summary of the structure and function of mitochondria.

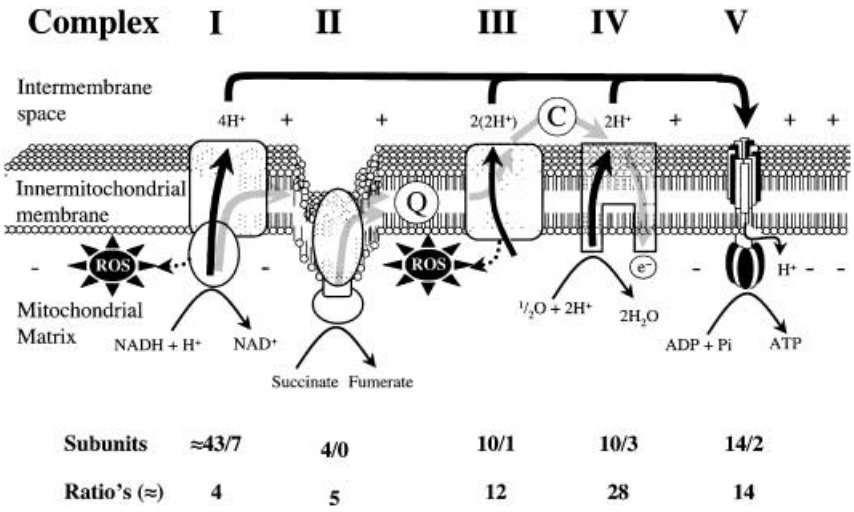


**Fig. 1** Mitochondria are the powerhouse of the cell but produce reactive oxygen species (ROS). Mitochondria oxidize metabolic substrates including carbohydrates and fats to generate water and ATP, with $O_2$ acting as the terminal electron acceptor for the electron transport chains that generate the proton gradient across the inner mitochondrial membrane. Reducing equivalents in the form of electron donors are recovered from carbohydrates in the tricarboxylic acid cycle, while those recovered from fats are obtained through β-oxidation. The resulting electrons are transferred to the mitochondrial electron transport chains (ETCs) via NADH dehydrogenase (complex I) or succinate dehydrogenase (complex II) and then flow to ubiquinone (coenzyme Q or CoQ) in the Q cycle (complex III) to give ubisemiquinone and ubiquinol. Ubiquinol transfers electrons to cytochrome *c* and eventually to cytochrome oxidase (in complex IV). Finally in complex IV, four one-electron transfers to oxygen occur resulting 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 *trans*-membrane proton gradient ($\Delta\mu H^+$). The potential energy stored in the gradient is used to condense ADP and Pi to make ATP via complex V, driven by the movement of protons back through a complex V proton channel. Matrix ATP is then exchanged for cytosolic ADP by the adenine nucleotide translocator. Generation of ROS (such as superoxide, hydrogen peroxide, and organic hydroperoxides) at complexes I and III is believed to be important in ageing, with up to 1–2% of the oxygen consumed being converted to ROS. These ROS can damage DNA, lipids and proteins, causing mitochondrial ROS production to increase with age. It is possible that mtDNA is a primary target of oxidative damage and as more copies of the mtDNA sustain mutations, complexes in the electron transport chain become less efficient as a result of stoichiometric mismatches between the mitochondrially vs. nuclear-encoded components of the electron transport chains. This gradual accumulation of damage to mtDNA would be expected to lead to a decline in mitochondrial function and an increase in residence time of the electrons.

The first studies considering mtDNA variation employed restriction fragment length polymorphisms (RFLP) (Avise *et al*. 1979a; Avise *et al*. 1979b; Brown & Wright 1979). These studies set the stage for much work to follow (Avise 1994) and were instrumental in developing mtDNA as a molecular tool. One of the more influential RFLP-based papers (cited over 1100 times) demonstrated that the rate of mtDNA sequence evolution in higher primates was high — about 2% per million years (Brown *et al*. 1979), a statistic that has been repeatedly employed in the literature. The focus started to shift from RFLP to sequence analysis when Kocher *et al*. (1989) published highly conserved primers that could be used to amplify by polymerase chain reaction the DNA from a wide range of taxa. This landmark paper opened the door to studies of molecular variation that hitherto had been impossible in most taxa. Intrigued and excited by the lack of recombination, maternal mode of inheritance, and ease of enzymatic amplification, researchers began to utilize mtDNA to study a variety of interesting ecological and evolutionary questions. As in many areas of science, the more we know about a particular system the easier it is to make the next advance. In terms of mtDNA, this is reflected by the sharp increase in mtDNA sequences uploaded to GenBank in the 1990s.

## Mitochondrial DNA is a small molecule with unique biology

There are many ways in which the biology of the mitochondria differs substantially from the nuclear genome, and these substantively affect the pattern and process of its evolution. For example, there are 16 569 base pairs in the human mitochondrial genome but around 3 billion in our nuclear haploid genome. Thus the mitochondrial genome is only about 0.00055% of the total human genome. The mitochondrial and nuclear genomes differ in many other ways, such as the ploidy, mode of inheritance, degree of recombination, number of introns, effective population size, mutation rate, repair mechanisms, etc. (Scheffler 1999). These differences are important if we are trying to make inferences about the biology of the whole organism and we would suggest that it is risky to infer general patterns from an idiosyncratic small fraction of the genome.

### *Recombination*

One unusual property of most animal mtDNA is that it does not usually undergo recombination (Birky 2001). This is not true of fungal and plant mtDNA (Birky 2001). When true, this can be extremely useful for phylogenetic studies because most methods of phylogenetic reconstruction assume no recombination. However, another consequence of the lack of recombination is that anything that affects one part of the molecule directly influences all other parts of the molecule, meaning that we cannot get independent replicated data about the history of a population from its mtDNA. This lack of recombination and the physical linkage of all sites also make traditional genetics nearly impossible.

Much has been written recently about the possibility of recombination and rearrangements in mitochondrial genomes (Awadalla *et al*. 1999; Eyre-Walker & Awadalla 2001; Innan & Nordborg 2002; Dowton *et al*. 2003). Some animal species indisputably show mitochondrial recombination, such as the mussel *Mytilus galloprovincialis* (Ladoukakis & Zouros 2001). For other species such as our own, even low rates of mitochondrial recombination seem unlikely (Innan & Nordborg 2002), although the biochemical machinery for recombination seems to be present in the mitochondria (Thyagarajan *et al*. 1996; Kajander *et al*. 2000).

For recombination to be important in terms of changing the patterns of descent of mitochondria, it is necessary that some individuals be heteroplasmic (that is, carry more than one mtDNA haplotype) as a result of biparental inheritance. Partial biparental inheritance (usually from paternal leakage) has been shown to occur at low frequency in a number of taxa including *Drosophila* (Kondo *et al*. 1990), mice (Gyllensten *et al*. 1991), birds (Kvist *et al*. 2003), and humans (Schwartz & Vissing 2002). Paternal leakage seems to occur at higher rates in hybrid individuals (Kaneda *et al*. 1995; Kvist *et al*. 2003). Perhaps the best-studied example occurs in *Drosophila*. There are three distinct types of mtDNAs in *D. simulans* (*si*I, *si*II and *si*III) and two in *D. mauritiana* (*ma*I and *ma*II) with the *D. mauritiana ma*I type resulting from the introgression of *D. simulans si*III (Ballard 2000a,c). Kondo *et al*. (1990) examined the possibility of incomplete maternal transmission of mtDNA in *Drosophila* by examining intra- and interspecific backcrosses of *D. simulans* and *D. mauritiana*. Among 331 lines that had been backcrossed for 10 generations, four lines from the interspecific cross *D. simulans si*II (female) × *D. mauritiana ma*I (male) showed clear evidence for paternal leakage of mtDNA. In three of these, the maternal type was completely replaced, while the fourth was heteroplasmic. Here the total number of fertilizations was 331 × 10 = 3310, so the proportion of successful paternal mtDNA per fertilization was about 0.1%. In *D. simulans* heteroplasmy has been observed between the *si*II and *si*III haplogroups in Reunion (Matsuura *et al*. 1991) and in east Africa (Dean *et al*. 2004) and within the *si*I haplogroup in New Caledonia (James *et al*. 2002).

### *Effective population size* ($N_e$)

From a population genetic perspective, two major differences between mitochondrial DNA and nuclear DNA are that mtDNA is a haploid genome and that it is usually only maternally inherited. This in turn means that for every

copy of mtDNA passed from one generation to the next, there are about four copies of autosomal nuclear DNA passed on. Because $N_e$ is usually expressed in terms of individuals, the $N_e$ for mtDNA ($N_{e,mit}$) is approximately half that for autosomal DNA ($N_{e,nuc}$), although because of ploidy differences the effective number of alleles is about one-quarter for mtDNA relative to nuclear DNA (nDNA). All else being equal, this would mean that mtDNA would fix new alleles faster than nDNA. (This calculation, however, assumes a bottleneck in the passage of mtDNA during oogenesis and low heteroplasmy, which may not be true in all animal species.) In contrast, effective population size does not affect substitution rates under neutral evolution, but substitution rates will be slower in genomes with smaller effective sizes if some substitutions result from positive selection. Neutral substitution rates depend only on the mutation rate per individual, but substitution rates of positively selected alleles can depend on the total number of new mutations in the population per generation.

To predict this fourfold difference in drift, however, requires a number of assumptions about the biology of the species. The effective population size is affected not only by the rote number of genomes in a population, but also by the distribution of reproductive success of these genomes. For example, if the sexes differ in their variance of reproductive success, as would be the case in polygamous systems where some males have much higher reproductive success than others, then the diploid autosomal effective size is much smaller than the census size: $N_e = 4MF/(M + F)$, where $M$ and $F$ are the effective numbers of males and females in the population. If the effective number of males in a population is less than one-seventh of the number of females, then the rate of drift in nuclear genes is actually higher than for the mitochondria. This ratio is quite plausible, particularly in species with strong sexual selection.

The difference between the effective population size of nuclear and mitochondrial DNA is further complicated by the fact that mtDNA is a single, linked molecule with low or no recombination. This means that selective sweeps (the fixation of one haplotype as a result of the fitness advantage of one or more of its component nucleotides) or background selection (the reduction in the effective population size associated with the purging of a population of low-fitness variants) can be important in determining the apparent rate of genetic drift. It should be noted, however, that some regions of the nuclear genome also have low recombination rates, for example, the regions near the centromere in *Drosophila* and many other species (Begun & Aquadro 1992; Nachman & Churchill 1996; Hamblin & Aquadro 1999).

The bottom line is that while mtDNA, on average, has a lower $N_e$ for many species this is not always true. For some species (like those with strong sexual selection) the expectation is reversed, and for all species there are periods of time (during selective sweeps on the mtDNA) when the relative effective size of mtDNA is very low indeed.

### *Mutation rates*

Mutation rates of mtDNA are generally higher than those of nDNA. As previously mentioned, Brown *et al*. (1979) demonstrated that the rate of mtDNA sequence evolution in higher primates was high — about 2% per million years. They also demonstrated that the rate of evolution of the mitochondrial genome exceeded that of the single-copy fraction of the nuclear genome by a factor of about 10. However, it is not safe to assume that all mtDNA evolves at 2% per million years or that mtDNA always evolves at a rate 10 times faster than nuclear DNA. As an example, Moriyama & Powell (1997) compared synonymous substitution rates, defined as the number of mutants reaching fixation per generation, in mitochondrial and nuclear genes of *Drosophila*. They found that mitochondrial genes have 1.7–3.4 times higher synonymous substitution rates than the fastest nuclear genes or 4.5–9.0 times higher rates than the average nuclear genes.

Phylogenetic estimates of the mutation rate can be influenced by the effective population size, though this bias is not confined to mtDNA. For neutral alleles, the substitution rate is expected to equal the mutation rate, independent of $N_e$, but this independence can fail if alleles are subject to any, even slight, selection. Ohta (1972, 1973) predicted that very slightly deleterious mutants are governed by random drift in small populations because they behave as if selectively neutral, but in large populations these same mutants are effectively selected against. Small populations may accumulate deleterious mitochondrial mutations at an increased rate (Bergstrom & Pritchard 1998).

Several workers have made direct estimates of the mtDNA mutation rates in birds (Lambert *et al*. 2002), nematodes (Denver *et al*. 2000), and humans (Howell *et al*. 1996; Parsons *et al*. 1997; Howell *et al*. 2003). Denver *et al*. (2000) measured the spontaneous mutation rate in *Caenorhabditis elegans* by sequencing 10.4 kilobases of the mitochondrial genome from 74 originally isogenic lines that had accumulated mutations over 214 generations of single individual propagation. They estimated the mutation rate to be $1.6 \times 10^{-7}$ per site per generation, which is two orders of magnitude higher than estimates based on phylogenetic comparisons. The three human studies (Howell *et al*. 1996, 2003; Parsons *et al*. 1997) reached similar conclusions: the pedigree rate of control region divergence was an order of magnitude higher than phylogenetically derived rates. When combined, these data suggest a dominant role for purifying natural selection in the evolution of the mtDNA in natural populations, even at so-called silent sites.

Mutational biases may influence the evolution of mtDNA. There is strong heterogeneneity of mutation rates in the

hypervariable region (e.g. Penny *et al*. 1995; Malyarchuk *et al*. 2002) and also in other parts of the molecule (Ballard 2000b), that have the potential to cause problems in phylogenetic and phylogeographical inferences. A strand-specific substitution bias has been shown to occur (Anderson *et al*. 1981; Clarey & Wolstenholm 1985; Garesse 1988; Rand & Kann 1998; Ballard 2000a). This can have a significant effect when pooling coding sequences from different strands (in a phylogenetic context this has been referred to as a total evidence approach) and when conducting sliding window statistical analyses using maximum likelihood. Biases toward A/T-ending codons have also been observed in the mtDNA of many species. This may be caused by factors that impinge on rates of DNA damage (Martin 1995) and the relative availability of each nucleotide in the cellular medium of the mitochondrion (Xia *et al*. 1996). Alternatively, it is likely that selection may also be involved in the high A/T content of mtDNA (Denver *et al*. 2000). A/T-rich genomes may replicate more quickly than G/C-rich genomes and, if all else is equal, have a selective advantage in a heteroplasmic population (Ballard 2000b).

When studying mtDNA care must be taken not to include somatic mutations or nuclear pseudogenes of mitochondrial origin (numts). Tissue-specific somatic mutations can accumulate within the lifespan of an individual (Kajander *et al*. 2000) and can confound estimates of genetic diversity. Numts have been found in the nuclear genome of over 64 eukaryote species, including humans (reviewed in Bensasson *et al*. 2001). Sequencing a numt can give incorrect results if it is interpreted as a true mitochondrial gene. Indeed, an early study implicating mtDNA heteroplasmy as a cause of Alzheimer's in humans was later rejected because the original authors apparently sequenced a numt (Wallace *et al*. 1997).

## The vocal minority: mtDNA and phylogeography

### *Similarities and differences between gene trees and demographic history*

In molecular ecology, most studies employing mtDNA have been directed towards estimating the demographic and phylogenetic history of the species. This approach has been very powerful, giving access to data on questions that previously were approached with difficulty. There are at least three sources of substantial error in this inference: error in inferring the gene genealogy from the available data, error in inferring a typical genealogy from that of a single molecule, and error in interpreting demographic history from gene genealogies. Several people have addressed the problem of distinguishing between gene and species trees. For excellent recent reviews, see Edwards & Beerli (2000), Nichols (2001), and Hudson & Turelli (2003). We briefly discuss the first two sources of error in this section and the third in the following two sections.

Gene genealogies are not inferred without error, but with enough sequence the error in reconstructing the true mtDNA genealogy can become small. 'Enough sequence' can be a daunting prospect, though. Even for much deeper phylogenies than we are concerned with here it can take much more than a single gene to infer the gene tree with accuracy (Cummings *et al*. 1995). This is reflected in the low bootstrap values seen in most phylogeographical trees. Note that Cummings *et al*. found that bootstrap support below about 90% did not reflect the probability that a clade was true, but strongly overestimated the probability that the clade was true. In some cases, especially when the timescale is small, we would not be able to accurately reconstruct the gene tree of mtDNA even with complete sequence data, because the number of mutational differences in the population would be too small.

Even if the gene genealogy were inferred without error, an mtDNA genealogy (like that derived from any single molecule) does not necessarily match the true history of the species — the genealogy of any particular molecule is just a random draw from an extremely variable distribution of genealogies, with many possible given the actual history of the population or species (Pamilo & Nei 1988; Edwards & Beerli 2000; Nichols 2001). This is compounded for mtDNA, because the lack of recombination means that the entire molecule has the same history. Mitochondrial DNA is just a single molecule with a singular molecular history, which may be unusual because of either sampling over possible coalescent processes, by greater sensitivity to certain processes like introgression, or by molecule-specific vicariant events such as selective sweeps or cytoplasmic infections. The only solution to this problem is to replicate across molecules, which in animals is only possible in the nuclear genome.

One of the most powerful statistical advances in population genetics over the last few decades has been the introduction of the coalescent (see review by Rosenberg & Nordborg 2002). With coalescent theory, the ancestry of genes is traced backwards in time until different lineages 'coalesce' — that is, until they share a common ancestor. The most recent common ancestor (MRCA) is the last individual copy of an allele that can be said to ultimately give rise to all subsequent copies in the population. If the MRCA of a species lived since the split between this species and its sister taxon, then the species can be said to be monophyletic and the gene tree will at least reflect the true topology of this part of the species tree. If the MRCA lived before the species split, then the two taxa are paraphyletic. We also say that the species displays 'incomplete lineage sorting' — that is, the genealogical lineages have not yet sorted themselves perfectly into species, but some lineages occur in more than one taxon (see Figs 2.13 and 2.14 in

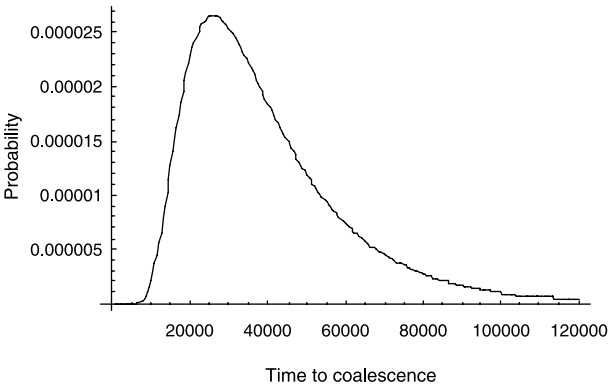


**Fig. 2** The distribution of time to Most Recent Common Ancestor (MRCA) of a diploid population of effective size 10 000. The mean time to MRCA is $4N_e$, or 40 000, but the distribution is very broad, with variance $4.64 \times 10^8$. A population of this size that had separated from another population 50 000 generations ago would still have a 25% chance of not being monophyletic. This distribution scales with $N_e$, so the time to coalescence would be one-tenth shorter for a population with a value of $N_e$ one-tenth as large.

Avise 2000). To completely avoid paraphyly independent MRCAs are required for each species, which can take several times $N_e$ of generations to happen with high probability (Rosenberg 2003). In a randomly mating diploid population, according to coalescent theory the MRCA existed on average $4N_{e,nuc}$ generations ago for nuclear genes, and $2N_{e,mit}$ for mtDNA with a constant population size. The higher the effective size, the longer it takes on average for all alleles to share a common ancestor; for some populations this can be an extremely long time. Here is one advantage of mtDNA over nDNA: mtDNA generally has a smaller effective size (but see above), and therefore its phylogenies resolve faster.

This value of $2N_e$ or $4N_e$ generations to the MRCA is only an average, however. The variance around this time to MRCA is very large, ~4. $\sim 4.64N_e^2$ for nuclear genes (or $\sim 1.16N_e^2$ for mtDNA (Tavaré 1984)! (See Fig. 2.) This means that for any given genealogy, the time to MRCA can be extremely different from the expectation, even without selection. This in turn means that relying on a single gene genealogy to reconstruct the population history is very risky. The gene genealogy can be very different from the true history of the species. Mitochondrial DNA (like any individual marker) is a single molecule with a single specific history that can differ substantially from the true history of the species. This problem is particularly important when the divergence time of the two lineages is fairly recent, either relative to the present or relative to the next branching event in the species tree. Branches shorter than several $N_e$ generations have a high probability of incomplete lineage sorting. Note that this variability occurs over different runs of the evolutionary process, not among different samples from the same population. Increasing sample size from a single population does not account for the variance among possible evolutionary gene genealogies that results from the uncertainty of the evolutionary process itself.

The only way to avoid these problems is to have independent gene trees that can represent different samples from the evolutionary process and therefore independent estimates of the species tree. With such replicated data, the variance in the estimate of the divergence time of two clades is reduced by the inverse of the number of replicate measures, which means that even a few extra loci can substantially reduce the error variance of the estimate (Edwards & Beerli 2000). Because the mtDNA in an individual is completely linked without recombination, information about different genes on the mtDNA does not give us this independence. Moreover, because the mitochondrial genome is small, it is often not possible to get a well-resolved gene tree even with complete data. Therefore it is essential to get information from other sources (Degnan 1993; Slade *et al*. 1994).

Nuclear DNA provides an obvious alternative, with statistical near-independence of unlinked sites. The value of getting additional and independent data is shown by the many conflicts between mitochondrial and nuclear data (Ferris *et al*. 1983; Powell 1983; Bernatchez *et al*. 1995; Taylor & McPhail 2000; Lu *et al*. 2001; Shaw 2002; Sota 2002; Rognon & Guyomard 2003; Seehausen *et al*. 2003). There are many potential reasons for these disagreements, including not only sampling error and coalescent variance but also real differences in the evolutionary processes of the different genomes. For example, a successful new mutation at a locus will cause that allele and alleles at all loci closely linked to it to coalesce for most or all individuals in a population. As we will see in the next section, this selection combined with a low level of hybridization can substantially change the phylogenetic pattern of multiple species as well.

### *Introgression*

Gene genealogies can differ from the typical pattern of the species for a variety of reasons other than just sampling. Almost every gene has a unique history determined by mutation and selection, and this selection can cause strong differences between the genealogy of a gene and other genes in the same population. One particularly important problem, for the intermediate timescale we focus on in this review (within and among closely related species), is the possibility of introgression of mtDNA from one species into another. With introgression, the phylogenies we see now can show us the recent dispersal history of the taxa in question, but the signal from previous events can have been erased. Moreover, introgression can result in a significantly different gene genealogy for mtDNA than for most genes in the species.

Mitochondrial DNA may be more likely to introgress than nDNA, and this can result in more discrepancies for this molecule than others. It is difficult to gather an unbiased data set about the relative impact introgression has on phylogenies from mtDNA in contrast to nDNA. In particular, it is difficult to distinguish the patterns caused by recent introgression from those generated by incomplete lineage sorting from gene genealogies alone (Nielsen & Wakeley 2001). There are many examples, however, of mitochondrial introgression unaccompanied by apparent nuclear introgression (e.g. Ferris *et al*. 1983; Powell 1983; Bernatchez *et al*. 1995; Ballard 2000c; Sota 2002; see below). 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).

The gene genealogy that results from introgressive hybridization is very similar to that expected by ancestral polymorphism and incomplete lineage sorting. In the cases reviewed in the last paragraph, introgression was inferred by either geographical or intergenome comparison. In most of these cases, the level of presumed introgression is higher in areas of sympatry or parapatry between the taxa than in areas of allopatry, and this was seen as necessary evidence that introgression had occurred. In other cases, the introgression of the mtDNA was inferred because of its difference from the patterns shown by nDNA. Note, however, that these criteria are very restrictive — any introgression that had been completely successful would not leave a geographical pattern but instead would result in an apparent lack of prior divergence between the groups. In the same way, if introgression were also successful in the nuclear genome then the signal would have been erased, making it impossible to find evidence of any previous differences between the groups. In either case, the gene genealogy would not reflect the previous history of the divergence between the taxa.

Why are mitochondrial introgressions common? There are two main contributing factors: selection and drift. Selection can drive introgression because the selective fixation of an allele in one population may also lead to the fixation of that same allele in a second population. This is particularly true if the two populations occupy a new habitat different from their common ancestor. For example, if two new species of fish such as sticklebacks invade a freshwater habitat from a marine ancestor, then both of the new populations would be experiencing a changed thermal and saline environment. Any local adaptation by mtDNA in one population would facilitate introgression by direct selection. If the two populations are not widely diverged (so that the cytonuclear interactions have not greatly diverged), then there might be little direct selection opposing the introgression.

Introgression as a result of fitness differences among distinct mtDNA haplogroups has been demonstrated in *Drosophila*. The most direct evidence for fitness differences comes from microinjection studies. Niki *et al*. (1989) injected the mtDNA from the g20 *D. mauritiana* line into *D. melanogaster*. This line has the *D. mauritiana ma*I mtDNA haplotype that is the 'naturally' introgressed mtDNA from *D. simulans si*III (Ballard 2000c). When the mtDNA from the g20 line was injected into *D. melanogaster* embryos, and a heteroplasmic line was produced, the foreign mtDNA most often completely replaced the resident *D. melanogaster* mtDNA.

The natural history of selection on mitochondria has been insufficiently studied, but one strong candidate for a type of selection that may cause repeatable patterns in mitochondrial introgression by selection is thermal adaptation in poikilotherms. In poikilotherms, the external temperature is experienced by the mitochondria, and the relative fitness of different genotypes is likely to change as a result (Somero 2002). Given the potential for temperature variations across species' ranges in nature and the sympatric distributions of closely related taxa, temperature may play a strong role in selecting for introgression of alien mtDNA from locally better-adapted species. Temperature may be an important agent of selection in human and *Drosophila* mtDNA (Doi *et al*. 1999; Mishmar *et al*. 2003). This introgression would be opposed by indirect selection on nuclear genes where the hybrid is unfit and unlikely to survive long enough to reproduce (hybrid breakdown), but the low linkage between mtDNA and nDNA would minimize the effects of hybrid breakdown. In contrast, nuclear genes are linked to more other genes, so the net effect of the indirect selection could be stronger for nuclear genes. As a result, mtDNA may introgress faster than nDNA.

Drift may also indirectly drive introgression in some circumstances. Genetic drift can allow the fixation of slightly deleterious alleles in small populations. If enough deleterious alleles fix in the mtDNA of one population, as might happen in a very small population, then it could have a mean fitness that was lower than a related species in the same area. Selection could then drive introgression of mtDNA from the more fit population into the less fit population. There is strong evidence for lowered fitness in mitochondria resulting from genetic drift. Lynch (1997) has shown that mitochondrial transfer RNAs are less fit than their nuclear counterparts, with lower binding stabilities and higher variability among species. Furthermore, there is evidence for more fixations of deleterious alleles in not only transfer RNAs, but also ribosomal RNAs and proteins (Lynch & Blanchard 1998). These data, however, pertain to long-term evolution in these taxa, and there is little available information about the rates of mitochondrial drift load on closely related taxa.

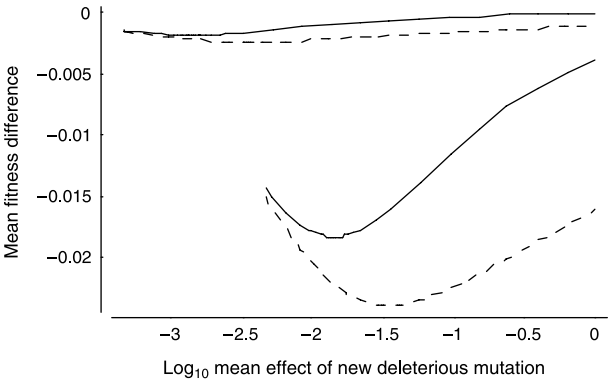


**Fig. 3** The expected difference in fitness of mitochondrial haplotypes from two distinct populations after 10 000 generations of separation. The top two curves correspond to the case where both populations have effective sizes of 1000 females, while the bottom pair has $N_{e,mito} = 100$. The solid lines plot the results for an exponential distribution of the effects of new mutations, while the dashed lines show the results from a gamma distribution with $CV^2 = 5$, each with mean mutational effect of 0.02. The genomic mtDNA mutation rate was estimated from mutation accumulation experiments in *Caenorhabditis elegans* (Denver *et al*. 2000) as approximately 0.0027. These differences are expected to increase with the square root of time. Smaller populations can have a substantial difference in mitochondrial fitness as a result of asymmetric fixation of deleterious alleles by genetic drift.

How different are the mitochondrial fitnesses of related taxa? Figure 3 shows mean fitness differences of populations of size 100 and 1000 following 10 000 generations of isolation. The rate of fixation of new mutations was calculated by assuming a gamma distribution of new mutational effects and a probability of fixation based on Kimura (1962). The actual number of new fixations per species was assumed to follow a Poisson distribution, and the difference in fitness between two species was calculated by summing over the distributions of fixed effects in each species. Each species was assumed to be equal in mutation rate and population size. With smaller population sizes, there are reasonably large expected differences in fitness between taxa, and this is increased at both population sizes under the more leptokurtic gamma model than with the exponential distribution. For small populations, fixation of deleterious alleles by drift may be an important source of fitness differences between populations that could result in selective introgression but in larger populations this is unlikely to be an important process.

In short, in taxa with some level of hybridization or migration, there is a non-negligible probability of introgression of mtDNA from one taxon into another. This can happen just by chance (because of the low effective size of mtDNA), by selective pressure (because of local adaptation of the mitochondria), or by selective introgression following mutational meltdown in small populations. In each case, the mtDNA is equally or more sensitive to introgression than nDNA, and this introgression can occur despite the levels of gene flow or hybridization between the populations being very low. Moreover, the introgression of mtDNA can reflect chance events, either in the form of genetic drift or the vicariance of selection. As a result, the rest of the genome may tell a very different story, and the results from mtDNA may not reflect the typical history of the taxa involved. The possibility of introgression deepens the importance of including multiple molecules with distinct evolutionary histories when making inference about the history of a taxon. The next section shows an important set of examples of the errors in inference that are possible as a result of introgression.

### *Example: mtDNA and apparent sympatric speciation*

Introgression is particularly important for closely related sympatric taxa — successful hybridization is more likely with closely related taxa and is only possible with some sympatry. Unfortunately, this overlaps exactly with an important evolutionary question: how common is sympatric speciation? Several studies have found a phylogeographical pattern consistent with a history of sympatric speciation; that is, several currently sympatric species have mitochondrial gene genealogies that show that sympatric species are also sister taxa, e.g. rift lake cichlids (Meyer *et al*. 1990); freshwater stickleback species (Taylor & McPhail 2000) and Hawaiian swordtail crickets (Shaw 1996). This is also the phylogenetic pattern expected by the less exciting process of allopatric differentiation followed by secondary contact and introgressive hybridization.

Many cases of putative sympatric speciation have been subsequently re-examined with nuclear DNA markers, with strikingly different results. In the case of the three examples given in the previous paragraph, subsequent work with either microsatellite or amplified fragment length polymorphism markers has shown that the sympatric species are not the closest relatives of each other [cichlids (Seehausen *et al*. 2003), stickleback (Taylor & McPhail 2000), crickets (Shaw 2002)]. This could reflect incomplete lineage sorting on the part of the nuclear markers, but in each case there is auxiliary evidence that supports separate origins of the taxa. For example, with the sticklebacks (*Gasterosteus* spp.) geological information (McPhail 1993), allozyme variability (McPhail 1994), and salinity tolerance differences (Kassen *et al*. 1995) argue for a temporally separate origin of the benthic and limnetic forms resulting from a 'double invasion' of the freshwater habitat from the marine population. Hybridization has been observed in nature (McPhail 1994), so clearly the parsimonious explanation is for separate origins of the sympatric species. Mitochondrial DNA gives a misleading pattern because of introgression. The rise and recent fall of phylogeographical inference of sympatric speciation offers an object lesson against the use of a single molecule to infer demographic history.

## Selection on the mitochondria

> 'If it's important enough to learn about in first-year biology, it's probably under strong selection.'
>
> The Freshman Law of Selective Neutrality

Empirically, contrasting patterns of phylogeography that point to mtDNA being out of line with other markers may provide the first hint of potential selective affects. For example, Ballard (2004) collected 1442 *Drosophila simulans* from 33 countries and 64 sampling localities and found that the global distribution of the three mitochondrial types was non-random. In contrast multiple nuclear genes do not exhibit the same distinct population substructure. Strong direct selection and the indirect effects of selection on many other parts of the genome influence mitochondria. Therefore, it is not safe to assume *a priori* that mtDNA evolves as a strictly neutral marker. Because of the importance of mitochondrial function, changes in the mtDNA sequence can have substantial impacts on the fitness of the organelle (within individuals) and on fitness of the individual host organism. In this section we review some of the evidence that demonstrates the effects of selection on mitochondrial evolution.

### *Direct selection*

The direct impact of mitochondrial haplotypes on fitness has been measured in copepods (Schizas *et al*. 2001), mice (Takeda *et al*. 2000) and *Drosophila* (Nigro 1994; Hutter & Rand 1995; Kilpatrick & Rand 1995; de Stordeur 1997; Rand *et al*. 2001; James & Ballard 2003). In an elegant paper, de Stordeur (1997) conducted microinjection studies between eggs carrying the three *D. simulans* mtDNA types and assayed the frequencies of the foreign injected mtDNA. He demonstrated that the haplotypes have different individual fitness (*si*II > *si*III > *si*I) following microinjection into a fly line harbouring a different mtDNA type. James & Ballard (2003) tested three life history traits on *D. simulans* lines with known sequence differences in the mtDNA genome after controlling for nuclear genome by backcrossing. They found that flies with the *si*I haplotype were fastest developing but had the highest mortality rate. Wild-type males with *si*III mtDNA were more active, while disruption of specific coadapted cytonuclear complexes by backcrossing caused a significant decrease in activity.

Mitochondrial fitness effects can be context-dependent: they can depend on the environment that the organism finds itself in, and they can depend on the nuclear genotype of the host. Mishmar *et al*. (2003) examined 104 complete human mtDNA genomes and observed that the *ATPase6* gene had the highest amino acid sequence variation of any mtDNA gene even though this gene is one of the more conserved proteins evolutionarily. They note that the gene was most variable in human lineages in arctic regions. The authors suggest that mtDNA variants that reduce the coupling efficiency of oxidative phosphorylation (Fig. 1) would reduce ATP production but increase heat production. Fitness of mtDNA types also appears to be temperature dependent in *Drosophila*. In a series of papers, Matsuura's group has been systematically looking at the transmission rates of mtDNA haplotypes in flies made heteroplasmic by microinjection (Nagata & Matsuura 1991; Matsuura *et al*. 1993). The most recent of these papers showed that the nuclear genome is involved in determining the temperature-dependency of mtDNA transmission (Doi *et al*. 1999).

The inherited variability of mtDNA has been extensively studied in humans where it has been used to understand human population history. However, the study of mitochondrial diseases, where point mutations of mtDNA triggered complex clinical phenotypes, questioned the view of neutrality of human mtDNA mutations. In 1988, three diseases were shown to be caused by mtDNA mutation: Leber's hereditary optic neuropathy (Wallace *et al*. 1988), chronic progressive external ophthalmoplegia, and Kearns–Sayre syndrome (Holt *et al*. 1988). Since that time about 50 pathogenic mutations have been identified (for a review see www.mitomap.html). More recently, mtDNA has also been shown to influence life-history trait characters in humans. For example, Ruiz-Pesini *et al*. (2000) reported that two common human haplogroups, H and T, displayed a significant difference in sperm motility.

### *Indirect selection*

Selection can change allele frequency even at a locus not responsible for fitness differences. Because there is little or no recombination in mitochondrial DNA, selection at one nucleotide affects the frequencies of all other variable nucleotides for the whole molecule. Selection on the nuclear genome, particularly nuclear-encoded proteins that are imported into the mitochondrion and X-linked markers that can have a high effective linkage to mtDNA, can also cause changes in the frequencies of mtDNA haplotypes. Equally importantly, selection on any other cytoplasmically inherited traits will directly affect the frequencies of mtDNA.

Selection on nuclear genes may influence the observed rate of mitochondrial evolution and potentially mtDNA haplotype frequencies in populations because mitochondrial function depends on the coordinated expression of genes encoded in the nucleus and mitochondrion (Fig. 1). Schmidt *et al*. (2001) studied the cytonuclear interactions in cytochrome *c* oxidase (Complex IV) in six species of mammals and noted that mtDNA-encoded residues in physical proximity to nDNA-encoded residues evolved

more rapidly than the other mitochondrial-encoded residues, indicating positive selection. The need for the coordinated expression of mtDNA- and nDNA-encoded genes is demonstrated by studies showing that interspecific and intersubspecific introgression causes a reduction in physical performance in mice (Nagao *et al*. 1998) and a decrease in cytochrome *c* oxidase activity in the copepod *Tigriopus californicus* (Burton *et al*. 1999). Willett & Burton (2001) also found evidence to suggest that significant variation in cytonuclear coadaptation exists between *T. californicus* populations and that the relative viability of specific allelic combinations is affected by sex.

Meiotic drive may influence the evolution of mtDNA. Meiotic drive is the differential production of gametes in heterozygotes that results in nonMendelian inheritance of chromosomes. Meiotic drive of sex chromosomes is relatively common and can result in large changes in the frequency of sex chromosomes in populations (Jaenike 2001). When it is the feminizing chromosome that is driven into a population, this can result in a large increase in the frequencies of the mtDNA haplotype associated with the driving chromosome (Hoekstra 2003). This will affect mammalian populations only under rare circumstances (because normally the heterogametic sex is male in mammals), but for female heterogametic species such as birds, butterflies, and many other taxa, mitochondrial hitchhiking with Z-chromosome meiotic drive could be an important factor in the evolutionary history of mtDNA.

Any maternally inherited factor can influence the evolution of mtDNA. For example, *Wolbachia* are a maternally inherited symbiotic α-proteobacteria that induces a variety of reproductive abnormalities in insects, nematodes and crustaceans that subvert the hosts' reproductive system to enhance the spread of the bacterium (Martin *et al*. 1973; Yen & Barr 1973; Wade & Stevens 1985; Hoffmann *et al*. 1986; Breeuwer *et al*. 1992; Hurst *et al*. 1993; Jiggins *et al*. 2000). One of these phenotypes is called cytoplasmic incompatibility. Incompatibility is expressed in many species when a male harbouring a strain of *Wolbachia* mates with a female that does not carry that same strain. In contrast, infected females produce normal numbers of offspring when they mate with uninfected males or with males infected with the same *Wolbachia* strain(s). For a wide range of experimentally observed incompatibility levels, fitness costs and maternal transmission frequencies, the frequency of infected individuals tends to increase rapidly in polytypic populations once a threshold has been reached. Because the *Wolbachia* spreads by maternal cytoplasmic transmission, mtDNA hitchhike along as well. As a result, *Wolbachia* invasions are characterized by great increases in the frequency of a single mtDNA haplotype (Turelli & Hoffmann 1991; Turelli & Hoffmann 1995).

### *Tests of selection*

Evidence for mildly deleterious evolution of mitochondrial DNA has been provided by population genetic analyses of mtDNA sequence data (Ballard & Kreitman 1994; Nachman 1998; Rand & Kann 1998; Rand 2001). These data typically show an excess of amino substitutions within species, suggesting the accumulation of slightly deleterious intraspecific changes. 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. Care must be taken, however, as selection may be operating in some intraspecific lineages but not others (Blier *et al*. 2001; Mishmar *et al*. 2003).

Deviations from a strictly neutral model of evolution have been found in a variety of organisms (Nachman 1998; Rand & Kann 1998; Rand 2001), and reviews have collated the battery of statistical tests that can be applied to mtDNA (Ballard & Kreitman 1994; Gerber *et al*. 2001). At least three types of test can be employed to examine the neutral equilibrium model for mitochondria. Most of these 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 and Ricardo Rozas (Rozas & Rozas 1997). Rejection of the null hypothesis probably 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 over-interpret the results, particularly when inferring dates of divergence or when making phylogeographical interpretations. In particular, a negative result cannot be taken as evidence of a lack of positive selection when the sample size is small. On the other hand, rejection of the null hypothesis often opens up new and exciting areas of study. Indeed, the rejection of neutrality of mtDNA in *D. simulans* has led to a decade of research on the problem by one of us (J.W.O.B.). In this section we briefly review some of these tests.

First, it is informative to compare intraspecific polymorphism with a closely related species. These tests assume that each sample is taken from a single randomly mating population and is influenced by the geographical and population history. Tajima's *D* (Tajima 1989), Fu and Li's *D** (Fu & Li 1993), and Fu's *Fs* (Fu 1997) can be used to test whether the observed mutation patterns in mtDNA data are consistent with a neutral model of molecular evolution. Most often these statistics include synonymous and silent sites only. Each of these tests has specific advantages. However, distinguishing between the contributions of demographic history and natural selection to a given departure from the null model can be difficult. For example, patterns of neutral DNA sequence variation closely linked to a site that has undergone a recent adaptive substitution or 'selective sweep' are similar to those in an expanding population.

A second class of approaches compares patterns of DNA variation between two or more genetic regions. Such comparisons attempt to distinguish between the effects of demographic history, which should have a roughly equal impact throughout the genome under neutrality, and natural selection, whose effect may be more localized. One such test that can be employed here is the Hudson, Kreitman and Aguade (HKA) test (Hudson *et al*. 1987). The test is based on the prediction that the rate of evolution of a region should be a function of polymorphism within species under the neutral theory. The test requires data from at least two regions of the genome (e.g. a mitochondrial and nuclear gene) both for an interspecific comparison and also intraspecific polymorphism data from at least one species. This is a relative test, and it is important to consider what evolutionary processes may be acting on all loci under study. This test should be applied to mitochondrial data with the caveat that the demographic history of the females and males can be quite different. It should be possible to get a significant difference between nuclear and mitochondrial genes as a function of the different dispersal histories of males and females, for example.

Third, the McDonald–Kreitman test (McDonald & Kreitman 1991) can be used to compare the synonymous and nonsynonymous variation within and between species within a region of interest. Under neutrality, the ratio of nonsynonymous to synonymous fixed substitutions between species should be the same as the ratio of nonsynonymous to synonymous polymorphism within species. It should be noted that this test is most useful among closely related species where multiple substitutions at a single site have not occurred. This test can easily be expanded to look at distinct lineages (Ballard 2000a), and in these cases the ratio of nonsynonymous to synonymous differences should be equal in all branches of the phylogenetic tree. However, Eyre-Walker (Eyre-Walker 2002) has shown that artefactual evidence of adaptive amino acid substitution can be generated within a McDonald–Kreitman test if some amino acid mutations are slightly deleterious and there has been an increase in effective population size. Smith & Eyre-Walker (2003) extended this basic approach with an analysis of variance test to examine the relative contributions of gene, lineage, and gene-by-lineage effects.

## What the future holds: the neglected biology of mitochondria

Mitochondria play a unique role in animal biology, but they have largely been studied — in the evolutionary biology and ecology communities at least — as a means to demographic and historical inference. We have tried to show how the unique biology of mitochondria makes this inference fraught, and we have argued that mitochondrial analysis at the least has to be reinforced with other sources of information. But this is only half of the story; the ecology and evolution of mitochondria is interesting in itself. In this final section we argue that there are many great opportunities for further study of mitochondria in a natural history context.

### *Ecology*

A very large gap in our understanding of mitochondria is that we do not know the ecological basis of selection. Given the extremely important role that the mitochondrion plays in metabolism, it is easy to imagine that there are important ecological differences mediated by mitochondrial changes. For example, evolutionary changes in metabolic rates under stress could be effected by changes in mtDNA sequence. Other predictions can be made. For example, in poikilotherms adaptation to a novel thermal environment should cause selection for mitochondrial gene products with different thermal stability. In poikilotherms, the external temperature is experienced by the mitochondria, and the relative fitness of different genotypes is likely to change as a result (Somero 2002). Temperature adaptation has been shown to be important in arctic fishes and other species (Somero 2002; Sommer & Portner 2002), but the role of mitochondria in this adaptation seems to have been little investigated to date. Another environmental axis for which mitochondria may be very important is sulphide exposure (Grieshaber & Völkel 1998). In many species, sulphide oxidation takes place in the mitochondria, and if sulphide is not detoxified, it can inhibit cytochrome *c* oxidase. Sulphides are common in many natural and human-polluted environments, allowing a great opportunity for evolutionary study. However, it should be noted that these studies require careful experimental design as mtDNA effects may be difficult to differentiate from maternal effects.

### *Sexually antagonistic selection*

Maternal inheritance of mtDNA was not firmly established for humans until 1980 (Giles *et al*. 1980); however, the mechanism for the exclusion of paternal mitochondria has proved to be elusive (Sutovsky *et al*. 1999; Sutovsky *et al*. 2002). Uniparental inheritance in a biparental species is the exception, not the rule. Why then, does uniparental inheritance of mitochondria evolve, and why is it evolutionarily stable? We lack full answers to these questions, but one obvious hypothesis is that uniparental inheritance prevents intermitochondrial conflict that may reduce individual fitness (Hurst 1995). Much work remains to be done in exploring the mechanism of uniparental inheritance, predicting the evolutionary forces that create and maintain such a system, and explaining the exceptions such as the double uniparental inheritance in *Mytilus*.

The differences between the inheritance of nuclear and cytoplasmic genomes can allow sexually antagonistic selection to create significant fitness differences between the sexes. Several mitochondrial disorders have more severe phenotypic effects in males than females (Frank & Hurst 1996; Finkel & Holbrook 2000). Recently, Matessi & Saino (2003) conducted a mathematical analysis of the evolutionary dynamics of sex-allocation mutants and demonstrated that natural selection will promote production of daughters by mothers of high mitochondrial quality while mothers with defective mitochondria may produce male-biased progeny, because this will liberate their descendants from low-quality mitochondria within a single generation.

In diploid sexual species with sex chromosomes and uniparental inheritance of mtDNA, the patterns of joint cytonuclear chromosomal transmission are different for the X chromosome compared with the autosomes. For example, in species where the female is the homogametic sex (such as most flies and mammals), a set of male and female parents carries four copies of each autosome but only three copies of the X chromosome. For any autosome, half of the copies are cotransmitted through the female with the mtDNA. For the X chromosomes, however, two-thirds of the copies within a mating pair are cotransmitted through the female with the mtDNA. This difference in the patterns of cotransmission for X chromosomes and autosomes motivated Rand *et al*. (2001) to re-examine models of cytonuclear fitness interactions that were based on autosomal loci. They developed a model of joint transmission of X chromosomes and cytoplasms and showed that cytonuclear polymorphisms can be maintained by selection on X–cytoplasm interactions. The study further demonstrated significant sex-by-genotype interactions for mtDNA type, cytoplasms and X chromosomes. These interactions were sexually antagonistic — i.e. the 'good' cytoplasms in females are 'bad' in males.

There are few examples in the literature like these, which we think reflects how little study has been made rather than the importance of such conflict between sexes. Intersexual conflict mediated by either mitochondria or other cytoplasmic factors is a ripe area for evolutionary investigation.

### *Life-history evolution, ageing and disease*

Mitochondria are not static within the cell, yet the physiology and biochemistry of mitochondria and mtDNA are understudied. It is possible to link biochemical and sequencing studies with tertiary structure analyses to investigate the importance of specific amino acid substitutions. Given the involvement of mtDNA in human disease and ageing, the lack of understanding of the physiology of mitochondria is an area in need of study. Studies examining the role of mtDNA in organismal fitness have been limited to a few species. This is surprising as mtDNA is responsible for about 90% of our energetic metabolism.

One area of life-history study in which mitochondria have been shown to be extremely important is longevity. Mitochondrial DNA has been strongly implicated in ageing (Tanaka *et al*. 1998; de Benedictis *et al*. 2000; Rose *et al*. 2001). The free radical theory first proposed by Harman (1956) hypothesized that free radicals produced during aerobic respiration cause cumulative damage to proteins, lipids and DNA, resulting in death. The identification of mitochondria as the major source of reactive oxygen species led to the mitochondrial theory of ageing (Harman 1972, 1992; Miquel *et al*. 1980; Miquel *et al*. 1983). Harman (1992) proposed that mtDNA is a probable target for attack by reactive oxygen species. Subsequently, Miquel *et al*. (1980) hypothesized that the ageing process is caused by oxyradical attack on the mitochondrial genome of fixed postmitotic cells with a decrease in the number of functional mitochondria resulting in decreased energy production and cell death. The theory has been supported by the observation that mitochondrial function declines and mtDNA mutation increases in tissue cells in an age-dependent manner (Kajander *et al*. 2000). Age-related impairment in the respiratory enzymes not only decreases ATP synthesis but also enhances production of reactive oxygen species through increased electron leakage in the respiratory chain (Fig. 1). While this theory is appealing, it has not been rigorously tested. One reason for this is that none of the current model organisms have sufficient mtDNA variability to test the hypothesis. *Drosophila simulans* is one species that has high mtDNA variability and that could be gainfully employed to test the mitochondrial theory of ageing.

## Conclusion

Many studies using mtDNA as an evolutionary marker are now considered modern literature classics and demonstrate that deep insight can be obtained from studying mtDNA. However, mtDNA provides a single perspective, and we now believe it is time to reflect on the current status of our understanding of the natural history of mitochondria and mtDNA. Mitochondria are often under strong selection and evolve under unusual evolutionary rules compared to other genomes. The vast majority of studies employing mtDNA as an evolutionary marker have not attempted 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. 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 selection operating on mitochondria. We suggest that the

focus of mitochondrial study should shift away from using mtDNA as a tool for inference of population history towards studies of the ecology and biochemistry of the mitochondrion itself. Mitochondria should be studied in their own right, rather than as a synecdoche of the whole organism.

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