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INVITED REVIEW AND META-ANALYSES

A disproportionate role for mtDNA in Dobzhansky–Muller incompatibilities?

RONALD S. BURTON and FELIPE S. BARRETO

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA, 92093-0202, USA

Abstract

Evolution in allopatric populations can lead to incompatibilities that result in reduced hybrid fitness and ultimately reproductive isolation upon secondary contact. The Dobzhansky–Muller (DM) model nicely accounts for the evolution of such incompatibilities. Although DM incompatibilities were originally conceived as resulting of interactions between nuclear genes, recent studies have documented cases where incompatibilities have arisen between nuclear and mitochondrial genomes (mtDNA). Although mtDNA comprises only a tiny component (typically $\ll$0.01%) of an organism's genetic material, several features of mtDNA may lead to a disproportionate contribution to the evolution of hybrid incompatibilities: (i) essentially all functions of mtDNA require interaction with nuclear gene products. All mtDNA-encoded proteins are components of the oxidative phosphorylation (OXPHOS) system and all mtDNA-encoded RNAs are part of the mitochondrial protein synthetic machinery; both processes require interaction with nuclear-encoded proteins for function. (ii) Transcription and replication of mtDNA also involve mitonuclear interactions as nuclear-encoded proteins must bind to regulatory motifs in the mtDNA to initiate these processes. (iii) Although features of mtDNA vary amongst taxa, metazoan mtDNA is typically characterized by high nucleotide substitution rates, lack of recombination and reduced effective population sizes that collectively lead to increased chance fixation of mildly deleterious mutations. Combined, these features create an evolutionary dynamic where rapid mtDNA evolution favours compensatory nuclear gene evolution, ultimately leading to co-adaptation of mitochondrial and nuclear genomes. When previously isolated lineages hybridize in nature or in the lab, intergenomic co-adaptation is disrupted and hybrid breakdown is observed; the role of intergenomic co-adaptation in hybrid breakdown and speciation will generally be most pronounced when rates of mtDNA evolution are high or when restricted gene flow results in significant population differentiation.

Keywords: hybridization, hybrid breakdown, intergenomic coadaptation, molecular evolution, mtDNA, speciation

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Introduction

In the absence of high levels of gene flow, conspecific populations may diverge genetically and ultimately become reproductively incompatible, completing the process of speciation. But why would alleles leading to incompatibility evolve in isolated populations? What types of genes might be involved? A general model for the evolution of interpopulation genetic incompatibilities was independently derived by Dobzhansky (1936) and Muller (1942). The scenario involves the fixation of a mutant **a** allele at the **A** locus in one population, whereas a mutant **b** allele fixes at the **B** locus in a second population. Although **a** or **b** are not deleterious on

Correspondence: Ronald S. Burton, Fax: 858 534 7313;
E-mail: rburton@ucsd.edu

their respective genetic backgrounds, neither has been evolutionarily 'tested' on the background of the second population. F_1 hybrids have both the wild-type **A** and **B** alleles and the **a** and **b** mutant alleles; with all alleles present, harmonious interactions may continue and fitness is not negatively affected. However, some F_2 hybrids will be homozygous for both mutant alleles; as these alleles evolved in independent lineages, there is a certain chance that they will function poorly together and result in reduced fitness (hybrid breakdown). This model nicely explains a common feature of the early stages of population differentiation, where incompatibilities are more frequently manifested in the F_2 and later generations than in F_1 interpopulation hybrids. As we discuss below, the Dobzhansky–Muller (DM) model also gives important clues about the types of genes and gene interactions that may be required to achieve hybrid breakdown.

Within eukaryotic organisms, the function of mitochondria and chloroplasts as cellular organelles is wholly dependent on harmonious interactions between the nuclear genome and the genomes residing in the organelles themselves. Many lines of evidence demonstrate that within taxa, and sometimes within populations, natural selection has led to the co-evolution of organellar genomes with nuclear genomes to maintain efficient physiological function. Some of the most direct evidence for this co-evolution comes from analyses of organelle dysfunction in hybrids between differentiated populations. As the fate of hybrids is key to the origin of species, the role of intergenomic incompatibilities in hybrid breakdown points to a potential role in speciation that has recently received considerable interest (Levin 2003; Burton *et al.* 2006; Gershoni *et al.* 2009; Greiner *et al.* 2011; Lane 2011). Indeed, given that the structure and evolution of organellar genomes appears to differ substantially amongst plants, animals and fungi, it is remarkable that intergenomic interactions appear to play important roles in intra- and interspecific incompatibilities in all of these groups. Although diverse mechanisms can underlie nuclear-cytoplasmic incompatibilities, recent studies suggest that disruption of intergenomic regulatory systems can play a prominent role in hybrid breakdown across a broad range of eukaryotic taxa. Here we focus on mitochondrial/nuclear ('mitonuclear') interactions and offer some new perspectives on why intergenomic incompatibilities may frequently arise and ultimately play a key role in speciation in some (but not other) groups of organisms.

Looking across eukaryotic diversity, organellar genomes present some remarkable contrasts. Even when we restrict consideration to the mitochondrial genome (mtDNA), there are significant differences amongst eukaryotic groups. First, mtDNA of animals is small and highly conserved in size (typically between 14 and 18 kb) and gene content (13 protein coding genes, 2 rRNAs and 22 tRNAs). In contrast, plant and fungal mtDNAs are much larger (up to 2000 kb) and contain 120–140 genes (Schuster & Brennicke 1994). Plant mtDNA contains many repeated elements and introns (comprising ~90% of the total sequence, Galtier 2011), features that are absent from animal mtDNA. Another interesting contrast regards rates of evolution. At the level of point mutations, animal mtDNA generally evolves more quickly than the nuclear genome. Although the difference in evolutionary rates between genomes is relatively low in some groups like *Drosophila*, where mtDNA substitution rates average less than twice the nuclear rate, the difference is over 20-fold in many groups and 40-fold in primates (Osada & Akashi 2012). Rates of point mutations in plant mtDNA are typically lower than nuclear DNA; low substitution rates in plant mtDNA may be due to high rates of recombination in plant mtDNA and the evolution of efficient recombination-associated DNA repair activity (Davila *et al.* 2011). Such recombination-associated DNA repair is absent in most metazoans (octocorals being a notable exception, Bilewitch & Degnan 2011). Notably, despite their lower rate of substitutions, extensive repeat structures and recombination make plant mitochondrial genomes more dynamic than animal mtDNA in both gene content and genome size.

Before going further, we note that various aspects of intergenomic interactions have recently been reviewed and we attempt to avoid replication here. In particular, Greiner *et al.* (2011) have provided an excellent review of the plant literature on nuclear–plastome incompatibilities and their role in speciation. The presence of the chloroplast genome in plants (in addition to the mtDNA), adds further potential for intergenomic interactions. Like mitochondria, chloroplasts derive from an ancient endosymbiosis, in this case between a heterotrophic cell and a photosynthetic symbiont. As in the mitochondrial case, most of the proto-chloroplast genome has been transferred to the host nuclear genome; approximately 90% of the proteins required for chloroplast function are imported nuclear gene products (Leon *et al.* 1998). These nuclear-encoded gene products include key enzymatic and structural factors that control chloroplast gene expression (Gillham *et al.* 1994).

Here we focus on the interactions between mitochondrial and nuclear genomes. Such interactions have been implicated in numerous cases of hybrid breakdown, manifested as Dobzhansky–Muller incompatibilities (DMI) when differentiated taxa interbreed in field or laboratory situations. In understanding these incompatibilities, it is important to consider two aspects of mitochondrial

biology. First, the fundamental processes of mtDNA replication, transcription and translation are completely dependent on nuclear gene products that all interact with mtDNA-encoded RNAs or the mtDNA itself. Second, although mitochondria play multiple roles in cellular metabolic processes including carbohydrate and lipid metabolism, all 13 of the mtDNA-encoded proteins are components of the oxidative phosphorylation (OXPHOS) system responsible for the aerobic synthesis of ATP. At a minimum, then, mtDNA replication, transcription, and translation and the aerobic generation of ATP all require mitonuclear interaction (Fig. 1). We suggest that *all these processes* are particularly susceptible to disruption when populations harbouring divergent mitochondrial lineages hybridize, bringing together nuclear and mitochondrial genomes that have not had a common co-evolutionary history.

The scenario by which mitonuclear co-adaptation evolves and differentiates between populations is not dissimilar to the widely discussed DM model (Greiner *et al.* 2011). In fact, the extensive interactions necessary between mitochondrial and nuclear genes should, a priori, lead to the expectation that mitonuclear incompatibilities will arise between allopatric populations (Fig. 2). This is particularly true because the largely non-recombining mtDNA is susceptible to fixation of mildly deleterious mutations either by genetic drift or by hitch-hiking with a favourable mutation sweeping to fixation ('genetic draft,' e.g., Oliveira *et al.* 2008); such mutations set the stage for selection favouring compensatory nuclear alleles to regain optimal mitochondrial function in the population (Rand *et al.* 2004). When interpopulation gene flow is restricted, each population follows a unique co-adaptive mitonuclear trajectory and mtDNA ultimately functions best in the genetic background of its co-adapted nuclear genome. High rates of mtDNA evolution will be a potent intrinsic selective force likely to elicit a nuclear genome response. Whether the response comes from selection on standing variation or *de novo* mutations, the extent of co-adaptation will depend on time since the populations became isolated. Although there is no reason to expect that loss of fitness in hybrids will be linear through time (e.g. Matute *et al.* 2010), the probability of accumulating mitonuclear incompatibilities will certainly increase through time. Hybrid breakdown can then be explained by mitochondrial dysfunction resulting from incompatibilities between nuclear and mitochondrial genomes that independently evolved in allopatry (e.g. Ellison & Burton 2006).

How widespread is mitonuclear co-adaptation? Because mitochondrial function requires interactions between the genomes, mitonuclear co-adaptation is obviously ubiquitous. The more relevant question is 'how tight is the co-adaptation or how interchangeable are mtDNA genomes across populations or taxa?' To address this question, experimental methods rely on creating cell or organismal mitonuclear hybrids. The extent of mitochondrial dysfunction can then be determined by measuring phenotypes directly dependent on interactions between cytonuclear components (such as

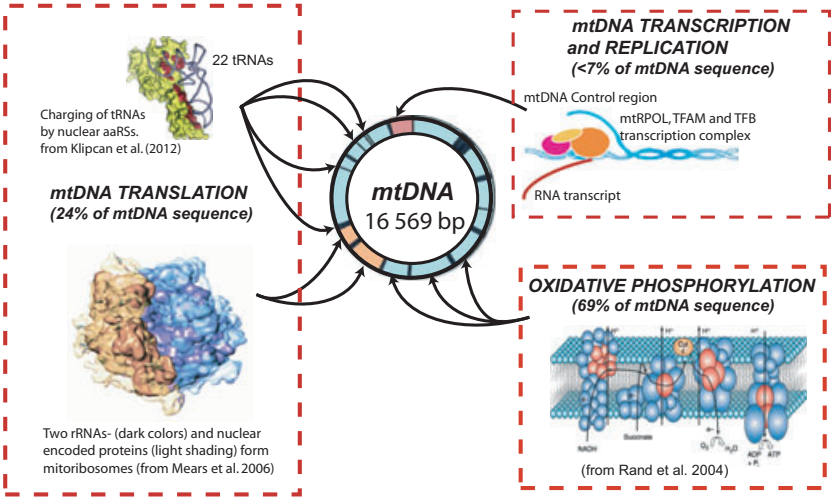

Fig. 1 Functions encoded in the mtDNA. The mtDNA molecule can be characterized in terms of its roles in its own replication, transcription, and translation and the role of the proteins it encodes. Using human mtDNA as a reference, most control functions (e.g., initiation of transcription, origin of replication) occur in the non-coding 'control region' that comprises <7% of the 16 569 bp sequence. Translation involves both the tRNAs and rRNAs that together account for 24% of the sequence, while the remaining 69% encodes subunits of the oxidative phosphorylation system. The figure depicts some of the many nuclear proteins that play essential roles in each of these functions through their interactions with elements or products of the mtDNA. Portions of the figure are adapted from Rand *et al.* (2004), Mears *et al.* (2006), and Klipcan *et al.* (2012).

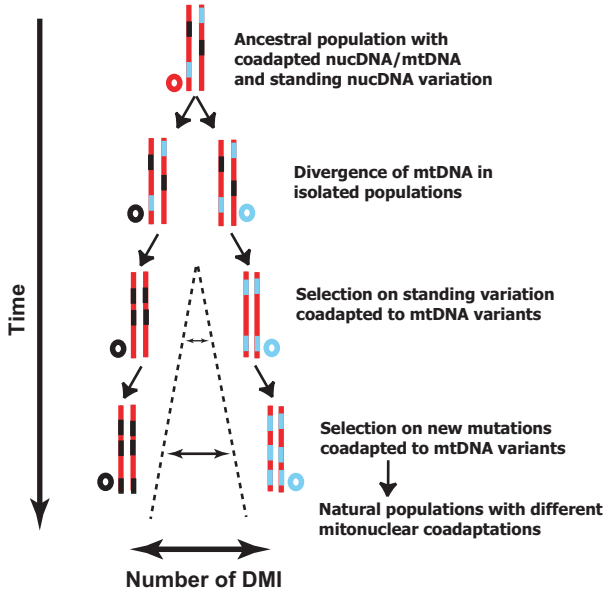

Fig. 2 Evolution of mitonuclear co-adaptation. Fixation of mtDNA mutants by drift and selection results in fixation of co-adapted nuclear alleles from standing variation and new mutations leading to different co-adapted gene sets in allopatric populations. DMI, Dobzhansky–Muller incompatibilities.

levels of OXPHOS enzyme activity), as well as quantifying components of organismal fitness (e.g., fecundity, viability, growth rates) that ultimately result from such interactions. Without exhaustively reviewing this literature, we present some representative examples of different approaches (see Ballard & Melvin 2010 for an excellent review of phenotypic consequences of mtDNA variation).

Experimental approaches

Given that mtDNA is maternally inherited, one approach to generating mitonuclear hybrids simply involves repeated backcrossing of a female lineage (mtDNA source) to males from alternate nuclear backgrounds (e.g., Breeuwer & Werren1995; Nagao *et al.* 1998; Edmands & Burton 1999). A significant limitation of this approach is that whereas it is easy to calculate the expected level of introgression of nuclear genes (first backcross generation has 75% of its nuclear genes from the backcross male lineage, the next 87.5% and so on), these expectations assume no selection. Selection, mitonuclear or otherwise, can significantly alter the genetic composition of the backcrossed progeny in unknown ways.

An alternate approach, especially in mammalian systems, has involved the construction of xenomitochondrial cybrids—cultured cells harbouring nuclear and mitochondrial genomes derived from different taxonomic lineages. Most commonly, these cybrids are produced by enucleation of mitochondrial donor cells followed by fusion of the 'cytoplasts' with mtDNA-less (ρ^0) cells. The resulting cells are screened for respiratory-competent transformants; phenotypes of these transformants (with mtDNA from one lineage and nucDNA from another) are then determined. For example, McKenzie *et al.* (2003) created a panel of xenomitochondrial cybrids, with mitochondria from six murid species with divergence from *Mus musculus domesticus* estimated at 2–12 million years before present. Cellular production of lactate, a sensitive indicator of decreased respiratory chain ATP production, correlated with divergence.

McKenzie *et al.* (2004) moved beyond cybrid analyses by successfully producing viable trans-mitochondrial mice (with homoplastic replacement of endogenous mtDNA with foreign mtDNA). They found that when *Mus spretus* or *Mus dunni* mitochondria replaced the native mitochondria in *Mus musculus* cells, there was no gross change in respiratory function. However, on finer inspection, it was found that the cells produced significantly more lactate, as might be expected when aerobic metabolism was deficient. To date, results from studies of trans-mitochondrial mice have been surprising in that effects on gross respiration have been weaker than expected based on *in vitro* cybrid-based studies. Cannon *et al.* (2011) found evidence for some changes in gene expression in a line of trans-mitochondrial mice that might play a role in mitigating the loss of co-adaptation, but understanding phenotypic differences between cybrid cells and transmitochondrial mice remains an active area of research.

An entirely different approach to assessing the role of nuclear/mitochondrial genomic interactions was employed by Ellison & Burton (2008a). If F_1 hybrids have wild-type (or higher) fitness, it appears that co-adaptation is in some sense a dominant trait—a single copy of co-adapted nuclear genes (as in a heterozygote) is sufficient for the expression of wild-type fitness (e.g., Liepins & Hennen 1977). Typically F_2 hybrids are homozygous for some non-co-adapted genes, and consequently, F_2 fitness is negatively impacted. This model predicts that a diploid individual with a full complement of nuclear genes co-adapted with its mtDNA will have wild-type fitness (Fig. 2). As shown in Fig. 3, the model is easily testable by backcrossing hybrids to the maternal and paternal lineages. In the copepod *Tigriopus californicus*, interpopulation crosses consistently result in F_2 (but not F_1) hybrid breakdown, which is manifested in several easily measured fitness components (viability, fecundity and development time). Ellison & Burton (2008a) directly tested the model using laboratory crosses of *T. californicus*. F_2 (and F_3)

hybrids were generated by crossing amongst three natural populations; low fitnesses were confirmed by measurements of fitness components. In addition, ATP production rates of isolated mitochondria were determined to be lower in hybrids than in parental controls. Virgin F_3 hybrid female subjects were then backcrossed to male subjects from either the original paternal parent or the original maternal parent (i.e., the source of the mitochondria in the hybrid). The results clearly supported the co-adaptation model: no improvement of fitness or mitochondrial function was observed in the paternal backcrosses whereas the maternal backcrosses showed wild type performance in most measures of fitness. Paternal backcrosses, although having an intact nuclear genome, do not have the genome copy that has co-evolved with the maternally inherited mtDNA; the results further indicate that the compensating nuclear genes act as dominant alleles.

Returning to the original question—how interchangeable is mtDNA across taxa?—We find that the answer is not completely clear. In some systems, there is good evidence for strong mitonuclear co-adaptation (e.g., *T. californicus*); there is clear breakdown in hybrid fitness that can be mapped to mitonuclear interactions. However, other systems show little or no evidence for co-adaptation playing a role in interspecific incompatibilities (e.g., Montooth *et al.* 2010). Indeed, there are numerous cases where mtDNA has introgressed across species boundaries (e.g., Doiron *et al.* 2002; Berthier *et al.* 2006;: Nevado *et al.* 2011; Hofman *et al.* 2012; many others) suggesting, at least superficially, that mitonuclear co-adaptation is not pervasive and that mtDNA can readily function on foreign genetic backgrounds. There are a variety of explanations for these observations and the simplest, of course, is that co-adaptation has not evolved, perhaps due to either insufficient time or lack of relevant genetic variation. Another possibility is that co-adaptation does in fact exist and the required nuclear alleles have co-introgressed. Such nuclear introgression may often not be detected in studies that only survey a relatively small number of randomly selected nuclear markers. Finally, the dynamics of mtDNA introgression are often not consistent with neutral expectations; combinations of intrinsic and extrinsic natural selection (e.g., Bachtrog *et al.* 2007) or sexual selection (Chan & Levin 2005) likely contribute to the introgression, making it difficult to assess the magnitude of mitonuclear co-adaptation.

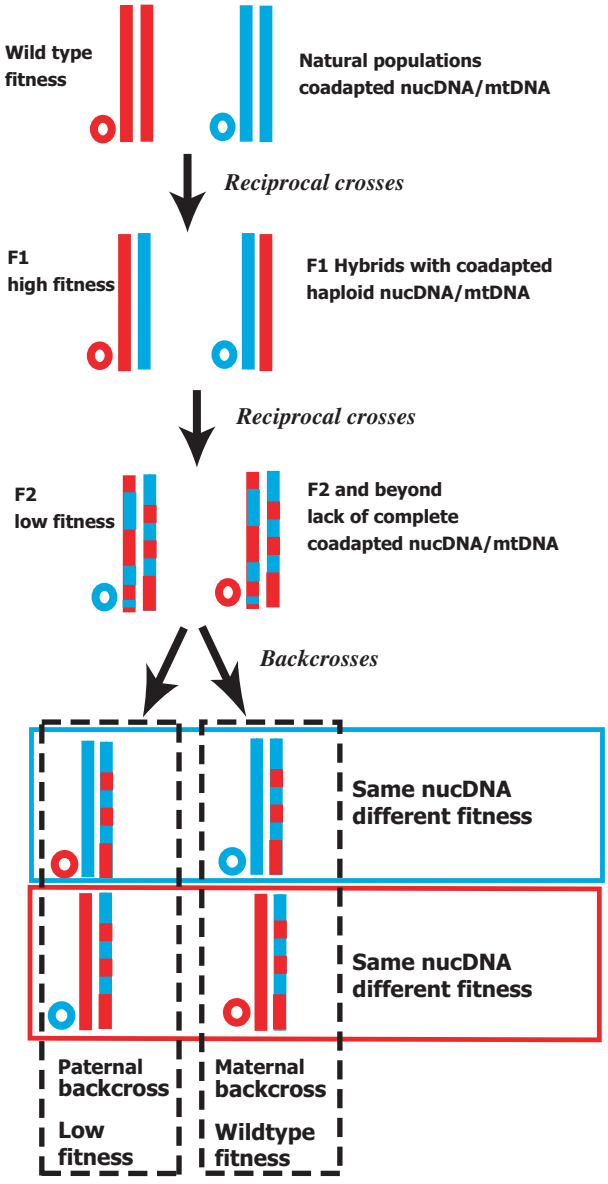

Fig. 3 Prediction from mitonuclear incompatibility. If mitonuclear interactions account for the low fitness of F_2 (and later) hybrids, then backcrosses to paternal and maternal parental lines will yield different results. By re-establishing the full nuclear and mtDNA genomic complement of a parental line, only the maternal backcross is expected to result in wild-type fitness. The high fitness of F_1 hybrids indicates that co-adapted act as dominant alleles.

The diversity of mitonuclear interactions

Interactions between nuclear and mitochondrial genomes are extensive and diverse in nature. The most discussed interactions are between protein gene products that are limited to the OXPHOS enzyme complexes (see below). However, this is not the only site of potential mitonuclear co-adaptation. Cells must coordinate the expression of nuclear-encoded genes, which are typically present in one or a few copies per cell, with the expression of mitochondrial genes, which are present in several hundred or thousands of copies per cell (Leon *et al.* 1998). Overproduction of mitochondrial OXPHOS

subunits relative to nuclear OXPHOS subunits, for example, could represent a serious drain on cellular resources, so just as the *structural* subunits for these enzyme complexes show evidence for co-adaptation, *regulatory* interactions between the nuclear and mitochondrial genomes have co-adapted over evolutionary time. These interactions can be disrupted when, by natural hybridization or laboratory manipulation, alien nuclear or cytoplasmic genes are introduced into a cell. Consequently, organelle divergence can act as an effective post-zygotic barrier to gene flow and may promote speciation (Levin 2003).

An alternate characterization of nuclear/mitochondrial interactions can focus on the types of macromolecules involved rather than their direct function. For example, interactions between OXPHOS subunits are, of course, protein–protein interactions (see Table 1). In contrast, many of the regulatory functions involved in mtDNA replication and transcription involve protein–DNA interactions. Like the protein–protein interactions, protein–DNA interactions require intergenomic co-adaptation, as a protein of nuclear origin typically needs to bind specific sequence elements in the mtDNA, such as transcriptional promoter sites or origin of replication motifs; like other parts of the mtDNA, these non-coding regions appear to evolve quickly, so disruption of mitonuclear function might be expected in hybrids (Ellison & Burton 2008b, 2010).

Finally, an additional class of interactions is protein–RNA interactions. Numerically, the protein–RNA class provides the greatest number of interactions between genomes and yet it is the most overlooked amongst investigators interested in intergenomic co-adaptation. A quick survey of sets of nuclear proteins that interact with mtDNA-encoded RNAs include: (i) proteins (approximately 80) that combine with the 12*S* and 16*S* rRNAs to form the mitochondrial ribosomes required for translation of the 13 mtDNA-encoded OXPHOS proteins, (ii) proteins functioning directly in mtDNA translation, such as initiation and elongation factors, and (iii) proteins responsible for charging mtDNA-encoded tRNAs with specific amino acids (aminoacyl tRNA synthetases, aaRS). In this latter class, there is a unique set of at least 17 mitochondrial aaRSs, separate from the 20 aaRSs that charge the cytoplasmic tRNAs, each catalyzing the esterification of a specific amino acid or its precursor to one or all its compatible cognate tRNAs to form an aminoacyl-tRNA (Bonnefond *et al.* 2005).

Subunit incompatibilities in the OXPHOS system

As this topic has been widely discussed elsewhere (e.g., Blier *et al.* 2001; Rand *et al.* 2004; Ballard & Melvin 2010;

Table 1 Summary of mitonuclear interactions. Although there are many examples of disrupted interactions impacting fitness in the context of human diseases, the potential role of protein–DNA and protein–RNA mitonuclear interactions in Dobzhansky–Muller interactions remains understudied

Mitochondrial function	Dominant interactions	mtDNA- encoded genes	nucDNA- encoded genes	Example studies
ATP production	Protein-protein	13 protein subunits	~75 protein subunits	Many studies of evolutionary rate interactions (e.g., Osada & Akashi 2012), functional interactions and fitness consequences (reviewed by Ballard & Melvin 2010)
Transcription	Protein-DNA	Non-coding control regions (promoters and terminators)	mtRPOL, TFAM, TFB1, TFB2	Functional interactions between mtRPOL and mtDNA: human/mouse (Gaspari *et al.* 2004); copepod populations (Ellison & Burton 2008b)
Replication	Protein-DNA	Non-coding origin of replication	DNA polymerase, mtRPOL, TFAM, helicase, ligase	mtDNA copy number in hybrids: Ellison & Burton (2010)
Translation	Protein-RNA	12S and 16S rRNAs 22 tRNAs	~80 ribosomal proteins 17 aminoacyl tRNA synthases, initiation factors, elongation factors	Translation deficiency in hybrids: Lee *et al.* (2008); poor intron excision: Chou *et al.* (2010); Ribosomal protein divergence: Matthews *et al.* (1978); Pietromonaco *et al.* (1986)

Castellana *et al.* 2011), our treatment here will not be comprehensive. Evidence for subunit incompatibilities has come from a variety of sources and the implicated structural genes are components of Complexes I, III, IV and the ATP synthase (Complex V). Attention has been focused on these interactions because the functional OXPHOS system requires all 13 of the mtDNA-encoded proteins and approximately 73 nuclear proteins, giving a large target for mitonuclear co-adaptation and a potentially high susceptible system for breakdown in interpopulation hybrids. As this system is responsible for generating the majority of ATP consumed in most eukaryotic cells, it is likely that small changes in flux through the OXPHOS system will have a significant impact on cellular metabolism and ultimately fitness.

A variety of approaches have been used to investigate the co-adaptation of nuclear and mtDNA-encoded OXPHOS subunits. Computational methods, focusing on rates of evolution of specific subunits along branches of a phylogenetic tree have now been combined with detailed knowledge of the three-dimensional structure of OXPHOS complexes in efforts to: (i) identify specific amino acid residues involved in interactions between nuclear and mitochondrial gene products and (ii) assess the timing of compensatory mutations. In a particularly elegant analysis, Osada & Akashi (2012) have combined phylogenetic and 3D structural data on cytochrome oxidase (COX, Complex IV) and found intriguing evidence for compensatory evolution in nuclear subunits of COX. Despite a higher rate of synonymous substitutions in mtDNA vs. nucDNA (20–40-fold in mammals), no evidence for adaptive evolution in the mtDNA-encoded subunits was found. In contrast, elevated rates of amino acid substitution were found in nucDNA-encoded subunits and specifically in those positioned where they interact with variable mtDNA-encoded subunits. Further, using a phylogenetic analysis, Osada and Akashi were able to infer temporal sequences of changes; a pattern of mtDNA changes followed by compensatory nucDNA changes was well supported. However, not all studies agree on this temporal pattern of mtDNA mutation followed by nuclear compensatory response; Azevedo *et al.* (2009) argue that the compensatory nuclear state may already exist in a population, making it pre-adapted to an otherwise deleterious mtDNA mutation. This latter idea of pre-adapted states already existing in the nuclear genome gains some support from the work of Dowling *et al.* (2007) that shows significant variation in fitness of mtDNA haplotypes when expressed on different nuclear genotypes extracted from within a panmictic population. At this point, the number of detailed examples of compensatory evolution is probably too low to determine if one dynamic is more common than the other.

Another approach for identifying the specific amino acids responsible for incompatibilities involves direct *in vitro* functional analyses. Rawson & Burton (2002) found functional evidence for nuclear-mitochondrial incompatibilities between cytochrome *c* (CYC) and Complex IV. CYC is a small nucDNA-encoded protein that functions by transferring electrons from Complex III to Complex IV in the OXPHOS system. In this work, the CYC protein from two different populations of *T. californicus* was cloned and expressed in *Escherichia coli*. The isolated proteins were chemically reduced and then used as substrate for Complex IV enzyme assays. CYC from a San Diego population was oxidized significantly faster by mitochondria from the San Diego (rather than a Santa Cruz) population; CYC from Santa Cruz showed faster oxidation by Santa Cruz mitochondria. Harrison & Burton (2006) took this approach a step further: using site-directed mutagenesis, the effect of each of the three amino acid differences in the two CYC variants was determined. Notably, Willett & Burton (2004) found sequence-based evidence for divergent (positive) selection for CYC between these two populations; the functional studies support that this selection is consistent with intergenomic co-adaptation.

In addition to reducing ATP synthesis, incompatibilities between subunits in OXPHOS enzyme complexes may exacerbate fitness problems by increasing cellular oxidative stress. As a by-product of aerobic metabolism, reactive oxygen species (ROS) are formed when electrons passing through the OXPHOS system are leaked prematurely and react with molecular oxygen before reaching Complex IV. The resulting superoxide anion ($O_2^{-}\bullet$) is a precursor to powerful oxidants such as hydroxyl radical (OH•) and hydrogen peroxide (H_2O_2). Oxidative stress occurs when antioxidant enzymes are unable to scavenge all ROS, resulting in a net increase in ROS production. If unquenched for long enough, ROS can cause damage to DNA, lipids and proteins and thereby significantly impair cellular functions and incur significant costs to repair (Turrens 2003; Murphy 2009).

Impaired activity of OXPHOS enzymes, often due to mutations to certain interacting subunits, are known to result in unbalanced production of ROS and oxidative damage (Zuin *et al.* 2008; García-Ruiz *et al.* 2010; reviewed in Lin & Beal 2006). In mammalian systems, rampant oxidative damage stemming from somatic mtDNA mutations are implicated in several neurodegenerative diseases (Lin & Beal 2006) and in metastatic tumour growth (Ishikawa *et al.* 2008). In the yeast *Schizosaccharomyces pombe*, Zuin *et al.* (2008) identified 12 deletion mutants, including nuclear- and mtDNA-encoded genes, which lacked components of the OXPHOS system. They showed that most of these

mutants, when compared with wild-type strains, displayed reduced oxygen consumption and increased intracellular ROS levels in association with growth defects. The authors suggest that such deletions in OXPHOS subunits likely disturbed the flow of electrons between enzyme complexes, exacerbating electron leakage and greatly increasing accidental ROS production.

In the same way that *de novo* mutations in OXPHOS genes may increase ROS overproduction, outcrossing between genetically divergent species or populations is expected to impair OXPHOS enzyme activities through the breakup of mitonuclear co-adaptation (Rand *et al.* 2004; Burton *et al.* 2006). The reduction in enzyme activity in OXPHOS complexes and in ATP production rate observed in F_2 hybrids in *T. californicus* (Ellison & Burton 2006) and *Nasonia* (Ellison *et al.* 2008) is evidence that impedance to electron flow can occur through hybridization. Hence, it is likely that electron leak, and subsequent ROS overproduction, is exacerbated in such systems.

The compounded effects of reduced ATP synthesis and elevated oxidative stress provide a clear link between mitonuclear co-adaptation and organismal fitness. To our knowledge, no explicit tests of this hypothesis have been performed in systems with F_2 hybrid breakdown.

Hybrid breakdown in mitochondrial transcription

Ellison & Burton (2006) assayed individual complexes in the OXPHOS system in recombinant inbred lines and parental controls of *T. californicus* derived from natural populations and found that all but Complex II showed, on average, reduced activity in hybrids. Although these results could be explained by the incompatibilities amongst subunits in each of the complexes, Ellison & Burton (2008b) proposed an alternative hypothesis: the across-the-board reductions of OXPHOS enzyme activities might be more easily explained by a systemic failure that affected the expression of all the mtDNA-encoded subunits (as Complex II has no mtDNA-encoded subunits, it was unaffected). Such a systemic failure could be at the level of transcription or translation of the mtDNA. Mitochondrial gene expression involves more than 100 nuclear genes, including a dedicated mitochondrial RNA polymerase and associated transcription factors, RNA processing machinery, and a large number of proteins involved in mitochondrial translation (Shutt *et al.* 2010).

The molecular machinery required for mitochondrial transcription in metazoans appears to be relatively simple, consisting of a single subunit (phage T7-related) RNA polymerase and one or more transcription factors. These components are all encoded in the nuclear genome. Despite the fundamental importance of understanding mitochondrial transcription, the manner in which these components interact in the process is incompletely understood. However, work on mammalian systems reconstructed *in vitro* demonstrated that a three-component system (mtRPOL, TFAM, and TFB2) was required for activity and that mouse and human systems were unable to initiate transcription from the heterologous promoter (Gaspari *et al.* 2004). More recently, Shutt *et al.* (2010) concluded that TFAM was not essential, whereas Malarkey *et al.* (2012) report that TFAM binding to the mitochondrial promoter dramatically enhances transcription from the light strand promoter in human mtDNA.

Although all the details have yet to be worked out, the observed mtDNA promoter-specificity of mitochondrial transcriptional machinery suggests that it might be a potential site of hybrid incompatibilities. Ellison & Burton (2008b) explored this possibility in the copepod *T. californicus* by examining the effect of mtRPOL genotype on transcript levels in reciprocal hybrids. Quantitative PCR was used to assay paired nuclear/mitochondrial genes; for example, changes in the mtDNA-encoded COI gene were compared with changes in the nucDNA-encoded COVa (both are components of Complex IV). Although all genes studied were up-regulated under salinity stress, only expression of mtDNA-encoded genes differed amongst hybrid vs. parental lines. Lines bearing certain mtDNA-mtRPOL genotypic combinations showed a diminished capacity to up-regulate mitochondrial genes in response to hypoosmotic stress. Effects on the transcriptional profile depended on the specific interpopulation cross and were correlated with viability effects; in six interpopulation crosses amongst three natural populations, homozygotes for the mtRPOL allele derived from the same population as the mtDNA were favoured in four crosses whereas a mismatch was favoured in two crosses. In both of the latter crosses, the maternal line was the SD (San Diego) population, suggesting that a 'weak' mtRPOL may have been fixed in that population, presumably via drift. In any event, the strong effect of mtDNA on mtRPOL genotypic fitness is consistent with the hypothesis that disruption of the mitochondrial transcriptional system in interpopulation hybrids may play a central role in hybrid breakdown.

This hypothesis requires that in addition to variation in mtRPOL, there is genetic variation in the control region of the mtDNA where transcriptional machinery binds. Burton *et al.* (2007) sequenced the complete mitochondrial genomes from three *T. californicus* populations and found extensive variation in the control region adjacent to the transcription initiation site (the actual promoter site has not yet been mapped).

Mitochondrial DNA replication

Just as mtDNA transcription has a dedicated RNA polymerase, replication of mtDNA is carried out by a dedicated DNA polymerase γ (polg) encoded by a nuclear gene, *POLG*; an accessory subunit, p55, is encoded by *POLG2*. Additional proteins involved include replication factors such as the mitochondrial single-stranded DNA binding protein and the mtDNA helicase (Twinkle). Copeland (2012) points out that a number of human mitochondrial diseases are not due to the enzymes directly functioning in the replication itself—rather they are involved in mitochondrial nucleotide metabolism which provides the DNA building blocks required for replication. However, we hypothesize that only population differences in the former enzymes are likely to result in hybrid breakdown as they require direct interaction between the nuclear-encoded enzyme and the mtDNA sequences recognized in the replication process.

It is interesting to note that mtRPOL plays key roles in both mtDNA transcription and mtDNA replication. In the latter, mtRPOL synthesizes the RNA primer that is required for POLG DNA replication. Ellison & Burton (2010) examined mtDNA copy number and transcription in laboratory hybrids between divergent populations of the copepod *T. californicus*. Lines having different combinations of mtRPOL and mtDNA showed a genotype-dependent negative association between mitochondrial transcriptional response and mtDNA copy number. The authors hypothesize that an observed increase in mtDNA copy number and reduced mtDNA transcription in hybrids reflects the regulatory role of mtRPOL; depending on the mitonuclear genotype of mtRPOL, hybridization may disrupt the normal balance between transcription and replication of the mitochondrial genome.

Mitochondrial translation

Perhaps the clearest molecular mechanisms so far elucidated for mitonuclear incompatibilities can be found in hybrids between certain species of *Saccharomyces* yeasts. Mating between *S. cerevisiae* (Sc), *S. bayanus* (Sb), and *S. paradoxus* (Sp) can occur freely in laboratory conditions, suggesting prezygotic barriers to genetic exchange have not yet evolved (Chou & Leu 2010). Although diploid hybrids can reproduce asexually without fitness deficits, their gametes (spores) have highly compromised fertility, with only ~1% germinating and forming colonies (reviewed in Greig 2009).

Suspecting a form of recessive incompatibility, Lee *et al.* (2008) generated hybrid lines by systematically replacing individual *S. cerevisiae* chromosomes with their *S. bayanus* homologs and measured the viability of spores produced from homozygous diploids. They found that *S. bayanus* chromosome 13 caused 0% sporulation in a *S. cerevisiae* background, and that this deficiency was only rescued by the mitochondrial-targeting *AEP2* gene located on chromosome 13. The Aep2 protein interacts with the 5′-UTR region of the mitochondrial gene *OLI1* and facilitates its translation. Given the high divergence in *OLI1* 5′-UTR sequence between *S. cerevisiae* and *S. bayanus*, and the lack of Oli1 product in the sterile hybrid line, the authors suggested the incompatibility is caused by the failure of Sb-Aep2 to properly function in the translation of Sc-*OLI1* (Lee *et al.* 2008).

Chou *et al.* (2010) discovered an additional mitonuclear incompatibility in yeast hybrids. After generating hybrids between *S. cerevisiae* × *S. bayanus* and *S. cerevisiae* × *S. paradoxus*, they screened DNA libraries for genes that could rescue F_2 hybrid sterility. They found a mitochondrial-targetting, nuclear-encoded gene (*MRS1*) as a major component in the incompatibility. Functional assays revealed that Sc-*MRS1* was incompatible with *S. bayanus* and *S. paradoxus* mitochondria, resulting specifically in a lack of *COXI* mature mRNA. Mrs1 is a protein necessary for the excision of certain *COXI* introns. In *S. bayanus* and *S. paradoxus*, Mrs1 excises two introns, but in *S. cerevisiae*, a more derived species, one of those introns was lost. Sc-*MRS1* has hence likely co-evolved to excise only one *COXI* intron, generating incompatibility between Sc-*MRS1* and Sb- and Sp-*COXI* mRNAs.

Although different molecular mechanisms (intron splicing and translation activation) were shown to cause mitonuclear incompatibility in yeast hybrids, two important patterns are common between these cases. As predicted by the DM model, incompatibility between the interacting genes was asymmetrical (see Orr 1995), meaning that fitness loss was not as extreme in the respective reciprocal crosses. Finally, evolutionary sequence analyses revealed no evidence for positive selection acting on these genes during divergence of *S. cerevisiae* from the other species, suggesting the incompatibilities likely evolved as a result of the high mutation rates in the mitochondrial genome (Lee *et al.* 2008; Chou & Leu 2010; Chou *et al.* 2010). Lynch *et al.* (2008) estimated that the mutation rate in *S. cerevisiae* mtDNA is 37-fold higher than in the nuclear genome.

Intergenomic interactions in mitochondrial translation are many and the potential for disruption seems great. A large proportion of these interactions can be found in the structure and function of mitochondrial ribosomes, which are responsible for translation of all 13 mtDNA protein-coding genes. Ribosomes, both cytosolic and mitochondrial, are composed of a core of ribosomal

RNAs (rRNA) surrounded by ribosomal proteins (RPs). Ribosomal proteins are essential for proper ribosome assembly and function, as they bind rRNA directly and interact with other translation components such as elongation and initiation factors (Moore 1998). Recent technical advances allow three-dimensional crystal structures of ribosomes to be visualized (Fig. 1), highlighting the intimate association of RPs and rRNAs (Klinge *et al.* 2011). All RPs (~160 total in eukaryotes) are encoded in the nucleus, but approximately half of them are imported exclusively into mitochondria and associate closely with mtDNA-encoded 12*S* and 16*S* rRNAs. We can hypothesize that such mitochondrial RPs (mRPs) are under stronger diversifying selection than RPs acting in cytosolic ribosomes (cRPs). Electrophoretic assays of cRPs and mRPs from rat and cow gave strong support to this hypothesis, with homologous mRPs, but not cRPs, showing large differences in mobility between the species (Matthews *et al.* 1978; Pietromonaco *et al.* 1986). To our knowledge, no further tests of this hypothesis have been published.

Despite the fundamental role of mitochondrial ribosomes in the translation of mtDNA proteins, only recently have associations been made between mRPs and mitochondrial fitness. Many human mRPs have been mapped to chromosomal locations associated with metabolic, morphological, or sensory disorders (Kenmochi *et al.* 2001). For instance, *mRPL53* and *mRPS15* are candidate genes for the multiple mitochondrial dysfunctions syndrome and Stuve–Wiedemann syndrome, respectively, based on their chromosomal locations and the mitochondrial nature of these diseases (i.e. activity of some OXPHOS complexes are depressed; reviewed in O'Brien *et al.* 2005). Interestingly, non-syndromic hearing loss, thought to result from diminished OXPHOS activity and inadequate ATP production, maps to 18 loci that also contain different mRPs (Sylvester *et al.* 2004). More direct evidence comes from studies that detect responsible point mutations in mRPs in patients with pronounced OXPHOS deficiencies. For instance, Miller *et al.* (2004) reported on a newborn patient with fatal lactic acidosis and traced the condition to a C-to-T mutation in *mRPS16* that created premature termination and truncated the protein by 26 amino acids. Another fatal neo-natal mitochondrial disease was traced to an amino acid substitution in mRPS22 (Saada *et al.* 2007). In both cases, the abundance of 12*S* rRNA, a core component of the small subunit of mito-ribosomes, was reduced to ~10% of normal levels. Moreover, activities of OXPHOS complexes I, III, IV and V, but not of complex II, were greatly reduced in those patients, which is consistent with findings of mitonuclear incompatibility described above (Ellison & Burton 2006).

It is important to note that mRP-caused dysfunctions are only relevant to the topic of mitonuclear co-adaptation when they involve protein–ribosome interactions. Many mRPs are known to have extra-ribosomal functions as independent peptides (Zimmermann 2003). MRPL12, for example, is the initial binding site for elongation factors when bound to mito-ribosomes (O'Brien *et al.* 2005); as a 'free' protein, it also associates with human mtRPOL to activate mtDNA transcription (Surovtseva *et al.* 2011). Multifunctional RPs in eukaryotes are evidence that some of these proteins evolved either through gene duplication or by recruitment of pre-existing proteins. A striking example of the latter process is the acquisition in humans of mRPL39, which is hypothesized to have been originally co-opted by ribosomes as a threonyl aaRS, later losing its central and C-terminal domains by adaptive evolution (Spirina *et al.* 2000; O'Brien *et al.* 2005).

Mitochondrial tRNAs

Animal mtDNAs typically encode 22 tRNAs that are required for the translation of the 13 mtDNA-encoded proteins. As the function of tRNAs is highly dependent on secondary structure, mutations in these genes result in a diversity of functional deficiencies. Suzuki *et al.* (2011) point out that over 200 mitochondrial tRNA point mutations are associated with human pathologies. Kern & Kondrashov (2004) have shown that there is extensive intramolecular compensatory evolution; i.e., mutations in tRNAs that subsequently result in selection for mutations within the tRNA that restore structure and function. Although interesting, the non-recombining haploid nature of animal mitochondrial genomes prevents this sequential mutation scenario from contributing to DM incompatibilities.

Here we are interested in the interactions between mitochondrial tRNAs and the nuclear-encoded proteins with which they interact. For example, Hino *et al.* (2004) found that a mutation in human $tRNA^{Ile}$ alters the T-stem structure and decreases the binding affinity for elongation factor Tu. Moreno-Loshuertos *et al.* (2011) provide a careful analysis of the impact of a different mutation in the $tRNA^{Ile}$ gene in mouse cell lines. A single nucleotide substitution in the anticodon loop (two bases downstream from the anticodon) results in reduced OXPHOS capacity. As in the Ellison & Burton (2006) study, mitochondrial enzymes with no mtDNA-encoded subunits are not affected by the tRNA mutation, but all the OXPHOS complexes with mtDNA-encoded subunits show significant reductions in activity. The tRNA mutation appears to result in inefficient aminoacylation—i.e., the $tRNA^{Ile}$ fails to get

charged, resulting in reduced translation of mtDNA-encoded OXPHOS subunits. Moreno-Loshuertos *et al.* (2011) noted that OXPHOS Complex II, which lacks mtDNA-encoded subunits, shows *elevated* activity in response to the $tRNA^{Ile}$ mutation. This suggested that there was an increase in mitochondrial mass, presumably resulting from increased mitochondrial biogenesis. This was confirmed by determining that mutant cells have almost double the amount of mtDNA observed in control cells. The authors hypothesize that dysfunction of the OXPHOS system results in elevated H_2O_2 by mitochondria, triggering the signalling cascade that adapts mitochondrial biogenesis to cell demands.

Although the proposed mechanism is quite different, the phenotypic consequences of pathological tRNA mutations are similar to the phenotypes observed in interpopulation hybrids of *T. californicus* (Ellison & Burton 2006, 2010): this not only includes the reduction of OXPHOS complex activities, but also the increase in mtDNA content of cells as they apparently attempt to compensate for lost ATP synthesis capacity by increasing mitochondria number. It appears that disruption of a large range of mitonuclear interactions which result in reduction of OXPHOS activity will have varying fitness consequences depending on the success of compensatory mitochondrial biosynthesis. Consequently, elevated mtDNA content may be a valuable biomarker for assessing the effects of hybridization on OXPHOS function.

Genomic level perspectives

From a molecular evolutionary perspective, if rapid evolution of the mtDNA results in significant selection pressure for compensatory evolution amongst interacting nuclear genes, we might expect to see elevated rates of evolution amongst the many nuclear genes that produce proteins that function within the mitochondria. Although some studies have found evidence for elevated rates of evolution in single nuclear genes involved in OXPHOS enzyme complexes (discussed in the previous section), we hypothesize that a general pattern could be apparent across the full set of nuclear genes with mitochondrial function. Mitonuclear co-adaptation would then produce a signal of elevated ratios of non-synonymous to synonymous substitution rates (d_N/d_S) in nuclear-encoded proteins that interact with mtDNA itself or with its encoded components.

Recent advances in whole genome sequencing make testing the above hypothesis possible. Werren *et al.* (2010) searched for rapidly evolving genes amongst the whole genomes of three *Nasonia* (parasitoid wasps) congeners and found the nuclear genes that interact with mitochondria had significantly elevated d_N/d_S when compared with other classes of genes. The effect was apparent specifically in those genes encoding mitochondrial RPs and complexes I and V. The availability of whole genome sequences, the rapid evolutionary rate of *Nasonia* mtDNA (van Opijnen *et al.* 2005; Oliveira *et al.* 2008), and existing evidence implicating nuclear-mitochondrial incompatibilities in F_2 hybrid breakdown (e.g., Niehuis *et al.* 2008) make the *Nasonia* system particularly attractive for future dissection of the role of mtDNA in DMI and speciation.

Although the number of systems with complete genomes is still limited (but rapidly expanding!), the work on *Nasonia* suggests exciting new directions for examining the impact of rapid mtDNA evolution across the entire nuclear genome. In fact, such an approach can efficiently be undertaken by comparing transcriptome data obtained using RNA-seq (i.e., characterization of the mRNA pool through next-generation sequencing of cDNA). A recent study by Gagnaire *et al.* (2012) used a transcriptomic screen to identify regions of high non-synonymous divergence between hybridizing *Anguilla* eel species. Amongst 87 divergent nuclear genes, they detected an overrepresentation of exonic regions involved in ATP biosynthetic processes, with the highest d_N/d_S attributed to *atp5c1*, a gene encoding a key protein that interacts with the mtDNA-encoded ATP synthase 6 in the OXPHOS complex V. The authors increased taxon sampling for the two interacting genes, and using the McDonald–Kreitman test (McDonald & Kreitman 1991), they showed that amino acid replacements between species were likely driven by diversifying selection in both proteins.

Another approach to looking for genome-wide impacts of mitonuclear interactions is to sort nuclear genes into two classes—those whose products are imported into the mitochondria and those that are not. In a preliminary study, Barreto and Burton (unpublished) sorted the set of orthogolous genes from two *T. californicus* population (Barreto *et al.* 2011) into these two groups using BLAST annotations and MITOPRED (Guda *et al.* 2004) to predict cellular localization. Putative identifications grouped 6327 genes into the cytosolic-acting category and 1296 genes targeted to the mitochondria (mTPs). Results showed a small but significantly elevated d_N/d_S when mTP are compared with cytosolic-acting proteins. Although many nuclear-encoded mTPs are as conserved as cytosolic proteins ($d_N/d_S = 0$), certain functional groups within the mTP category are likely to reveal the strongest signals of compensatory co-adaptation. Given the numerous pathways in which the two genomes interact (Table 1), research on these systems should now focus on elucidating the contributions of specific genetic complexes.

Mitonuclear interactions, hybrid breakdown, and speciation

In reviewing the work on mitonuclear incompatibilities, we can arrive at some perspectives about why such a tiny piece of extranuclear DNA can have such pervasive effects on hybrids between divergent populations. First, as has been noted by many researchers, the mtDNA encodes 13 proteins that are essential for the OXPHOS system that is the key to eukaryotic energy metabolism and that all these proteins require intricate interactions with nuclear gene products to achieve their function. Less widely appreciated are the functions of the rest of the mtDNA, which includes both the RNA-coding regions and the non-coding regions. Both the rRNA and tRNAs are essential in the translation of the mtDNA proteins, and again, they cannot function without interactions with nuclear-encoded proteins. The replication and transcription of mtDNA are also essential for cell viability and these functions require recognition of sequences in non-coding regions of the mtDNA by nuclear-encoded DNA and RNA polymerases and their accessory factors. So in sum, the entire mtDNA molecule is replete with sites that dictate mitonuclear interactions.

Despite the very large number of mitonuclear interactions, it is worth noting that many proteins that function in the mitochondria are not involved in such interactions. Somewhere between 1000 and 1500 nuclear proteins function in the mitochondria. Although mutations in any can result in mitochondrial dysfunction, only those which directly interact with mtDNA elements or products lead to the mitonuclear breakdown that is our focus here. For example, enzymes in the citric acid cycle exist in the mitochondrial matrix and are wholly nuclear in origin. We expect that deleterious mutations in such enzymes are not subject to the compensatory co-adaptation observed in the interacting systems outlined for OXPHOS, mtDNA replication, transcription and translation. Still, our examination here has identified on the order of 200 nuclear proteins that do participate in these processes and are subject to co-adaptation. This is roughly on the order of 1% of the genes in a eukaryotic genome—a significant target of natural selection.

On average, nucleotide substitution rates in mtDNA are much higher than in nuclear DNA. Many factors likely contribute to this difference—poor fidelity of DNA polymerase γ, lack of recombination and DNA repair, increased exposure to ROS—but the result is clear. Regardless of whether compensatory mutations create a selective force on existing nuclear variation or on new nuclear gene mutations, the high rate of mtDNA evolution suggests that mitonuclear interactions may evolve more quickly than nuclear-nuclear interactions; while both have been observed to play roles in hybrid breakdown and speciation (e.g., Presgraves 2010), we believe that mitonuclear interactions may be disproportionately represented given the taxonomic bias of work to date. Most cases of DMI reported to date are from studies of *Drosophila* species that have anomalously low rates of mtDNA evolution relative to the nuclear genome. For mtDNA to figure prominently in incompatibilities, high rates of evolution, as observed in most taxa studied to date, may well be required (Montooth *et al.* 2010).

The evolution of co-adapted mitochondrial and nuclear genomes typically proceeds in allopatry (but see Barton & de Cara (2009) for parapatric and sympatric scenarios). Reaching a degree of population differentiation that will ultimately lead to hybrid breakdown will often require a high degree of population structure so that mtDNA variants are contained geographically as coadapted compensatory variants evolve. The copepod *T. californicus* shows highly restricted gene flow that is stable through time (Burton 1997) and the rate of mtDNA evolution in *T. californicus* is 55-fold higher than in the nuclear genome (Willett 2012). Combined, these factors set the stage for mitonuclear incompatibilities and perhaps not surprisingly, this system provides some of the best evidence for widespread DM incompatibilities involving mtDNA. However, rapid rates of mtDNA evolution and strong population structure are not uncommon amongst natural systems; the frequency with which mitonuclear DM incompatibilities result awaits further study.

Conclusions

Our title raises a hypothesis that is difficult to address in a rigorous quantitative manner. With <20 kb of DNA (typically $\ll$0.01% of a metazoan's total genetic composition) and limited gene content (typically <0.1% of the total genes), the mtDNA would not seem to be a significant target for the random accumulation of incompatibilities. Given that the genetic basis of DMI has been determined in relatively few cases, we find it remarkable that several cases of mitonuclear incompatibilities have been found. Our goal here is to point out that there are many types of mitonuclear interactions beyond the often-cited protein–protein interactions and these may help account for the apparent disproportionate role of mtDNA in DM incompatibilities.

In reviewing the available literature, we find evidence for co-adaptation in mtDNA replication, transcription and translation. Replication and transcription involve interactions between nuclear proteins and binding motifs in the mtDNA itself. Translation requires

extensive interactions amongst nuclear proteins and the rRNAs and tRNAs encoded in the mtDNA. Combined with the protein–protein interactions in the OXPHOS system, these processes involve a non-trivial portion of the nuclear transcriptome and suggest that mutations occurring almost anywhere on the mtDNA molecule can impact physiology and fitness in hybrids where mitonuclear co-adaptation has not had a chance to evolve.

Given the extent of these interactions, it is perhaps surprising that mitonuclear co-adaption has not been more widely reported in natural systems. As the extent of the disruption to mitochondrial physiology is typically correlated with the extent of divergence amongst hybridizing lineages, the magnitude of hybrid breakdown and potential role in speciation is not uniform across taxa. Co-adaptation will be most pronounced when rates of mtDNA evolution are especially high or when restricted gene flow results in strong population structure. As recognition of the potential role of mitonuclear interactions in hybrid breakdown increases, we suspect that more examples will be discovered in natural systems. However, making a strong case for mitonuclear incompatibilities typically requires multiple generations of laboratory crosses that are not practical for many organisms.

Finally, the past decade has witnessed rather dramatic changes in the ways in which evolutionary biologists have viewed mtDNA. It is important to keep these changes in perspective. Initial use of mtDNA as a powerful phylogeographic (and phylogenetic) marker was largely based on the assumption that mtDNA serves as a selectively neutral marker. Indeed, where the variation within and amongst populations is restricted to synonymous substitutions, this assumption will generally be valid. Studies highlighting the key attributes of mtDNA —generally non-recombining, high mutation rate, reduced effective population size and maternal inheritance—will continue to provide powerful insights into natural history and evolution. However, it has become increasingly clear that other aspects of mtDNA, and in particular its extensive interactions with the nuclear genome, can result in non-neutral evolution; as in the nuclear genome, favourable mutations in mtDNA can promote local adaptation (Grossman *et al.* 2001; Mishmar *et al.* 2003; Fontanillas *et al.* 2005; Kivisild *et al.* 2006; Ruiz-Pesini & Wallace 2006). But perhaps more interesting is the propensity for fixation of mildly deleterious mutations in mtDNA; these result in intrinsic selective forces that may play important roles in hybrid breakdown and speciation that clearly merit further investigation.

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R.S.B. is Professor of Marine Biology interested in evolutionary genetics and molecular ecology of marine organisms. F.S.B. is a postdoctoral scholar in the Burton laboratory, and his research interests include speciation, phylogeography, and reproductive behaviour in marine animals.
