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The Role of Mitonuclear Incompatibilities in the Process of Allopatric Speciation

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Running Title: Mitonuclear Incompatibilities and Speciation

Abstract: The mitochondrial oxidative phosphorylation (OXPHOS) system, vital to the function of most eukaryotic cells, requires intricate interactions between products of the nuclear and mitochondrial genomes. Rapid evolution of the mitochondrial genome creates an intrinsic selection pressure favoring compensatory mutations in interacting nuclear genes in order to maintain OXPHOS function. As this process of coadaptation occurs independently in allopatric populations, the resulting divergence between conspecific populations can subsequently manifest in mitonuclear incompatibilities in interpopulation hybrids; such incompatibilities can potentially restrict gene flow between populations, ultimately resulting in varying degrees of reproductive isolation. Evidence generally supports the model of fixation of deleterious mutations in the mitochondrial genome followed by compensatory nuclear gene evolution, but there is also strong evidence for adaptive evolution of the mitochondrial genome in response to extrinsic selection pressures, though support for adaptive mitonuclear differentiation is less clear. The fact that mitochondrial introgression occurs across species boundaries has raised questions regarding the efficacy of mitonuclear incompatibilities in reducing gene flow; several scenarios appear to satisfactorily explain this phenomenon and empirical evidence suggests that co-introgression of coadapted nuclear genes may support the function of introgressed mitochondria. The potential role of mitonuclear incompatibilities in speciation is enhanced where mtDNA substitution rates are elevated compared to the nuclear genome and where population structure maintains allopatry for adequate time to achieve mitonuclear coadaptation. Still, although mitonuclear incompatibility can reduce gene flow between taxa, there is little evidence supporting a primary role in the speciation process; current evidence suggests they play a supporting role, contributing in various degrees to the diverse set of barriers that lead to reproductive isolation and speciation.

Keywords: hybrid breakdown, allopatric speciation, reproductive isolation,

mitonuclear coadaptation

Introduction

The earth hosts a vast but unknown number of species. Mora et al (2011) estimated that there are ~8.7 (± 1.3) million species of eukaryotes on the planet, yet only ~2 million species have been described to date. How the process of speciation has led to this extant biodiversity remains rather poorly understood. The basic models of speciation - the allopatric and sympatric models and their variants - provide geographical context, but the mechanisms underlying the restriction of gene flow between populations leading to the evolution of reproductive isolation are rarely known in detail. Importantly, despite substantial effort and some significant progress, work to date has largely failed to give us a general model that identifies sets of genes that consistently contribute to the generation of new species. If anything, the pattern that seems to emerge is that there is no general pattern; rather, where it is known at all, the genes underlying reproductive isolation are unique to species pairs or, at best, to small branches of the evolutionary tree. This leads to an alternate view - that a great many genes are somehow involved in reproduction and the set that has become incompatible between any pair of taxa is simply the outcome of a stochastic process of mutation and drift, aided in some cases (perhaps a distinct minority), by adaptive divergence. Under such a scenario, we might indeed arrive at our current level of generalities - i.e., that different genes play key roles in reproductive isolation in different taxa, and other than characteristics such as "fast-evolving" or "located on the sex chromosome," we can't expect a more prescribed set of "speciation genes." Especially in the case of allopatric speciation where divergence between insipient species occurs in geographic isolation, this conclusion does not seem unreasonable.

However, there are fundamental processes that are part of the basic biology of eukaryotes that could readily be disrupted by hybridization between previously allopatric populations. Here I consider the hypothesis that a particular set of genes, consisting of both those located on the mitochondrial DNA (mtDNA) and those in the nuclear genome that closely interact with mtDNA and its protein and RNA products, define an important physiological genomic network that may frequently participate in the loss of fitness in hybrids and the subsequent evolution of reproductive isolation and speciation. This is not a new idea; mitonuclear interactions have been cited as playing a potential role in speciation in a broad range of eukaryotes, including plants (Levin 2003), yeast (Lee et al. 2008; Chou and Leu 2010), nematodes (Chang et al. 2016, Lamelza and Ailion 2017), copepods (Burton and Barreto 2012), wasps (Breeuwer and Werren 1995), fish (Bolnick et al. 2008), lizards (Bar-Yaacov et al. 2015) and birds (Trier 2014; Morales et al. 2018, Runemark et al. 2018) among others. Although still not widely acknowledged, there

has been increasing interest in the topic and Hill (2016, 2017, 2019) has gone so far as to suggest that species can (or should) be defined by the compatibility of their nuclear and mitochondrial genomes.

The goal of the current paper is to review some of the unique features of the interaction between nuclear and mitochondrial genomes and discuss how their pervasive coadaptation, typically achieved in allopatry, may set the stage for reproductive isolation when populations experience secondary contact. Although mitonuclear interactions are important to the function of all eukaryotes, here I restrict attention to metazoans where there is generally strong conservation of gene content of the mitochondrial genome. The primary focus of this review is to examine the role of mitonuclear incompatibilities in hybrid breakdown, reduction of gene flow between conspecific populations and closely related species, and the potential role of these incompatibilities in allopatric speciation.

Speciation and the formation of reproductive isolating barriers

For the sake of this discussion, we will define species as groups of organisms that interbreed in nature, produce fertile offspring and are reproductively isolated from other such groups, i.e., the biological species concept (Mayr 1942, Coyne 1994). Speciation, then, is the process by which reproductive isolation is established between formerly conspecific populations. This process most commonly takes place when populations become geographically isolated and genetically diverge under conditions of restricted gene flow. Such restriction can come at several stages of reproduction but are usually classified as coming before or after fertilization (pre- or post-zygotic).

This model of population divergence while in geographic isolation is the classic allopatric model of speciation and is generally viewed as a primary mode of taxon diversification. At first glance, it is intuitively attractive in its simplicity. In isolation or near isolation, populations genetically diverge due to the combined action of mutation, genetic drift and natural selection. However, it's not immediately obvious how evolution in allopatry might result in hybrid inviability or sterility upon secondary contact since the residents of each population seem fit. What sort of evolution within the subpopulations would lead to low fitness hybrids? A widely accepted solution to this problem was derived independently by Bateson (1909), Dobzhansky (1942) and Muller (1935). The basics of their models, reviewed in many papers on speciation, posits that different mutations occur and accumulate in isolated subpopulations; where they occur, the mutations may be neutral

or even slightly deleterious but fixed in the population primarily by genetic drift. Subsequently, when hybridization occurs, mutations at loci that have been fixed in one subpopulation now occur in the novel genetic background of the other subpopulation (where mutations at other loci have fixed); negative epistatic interactions may occur between the loci resulting in loss of hybrid fitness and potentially leading to reproductive isolation. Because these Bateson-Dobzhansky-Muller incompatibilities (BDMIs) can act independently of the external environment, they are termed "intrinsic," to distinguish them from incompatibilities that are a result of gene x environment or "extrinsic" selective forces. In reality, the intrinsic-extrinsic dichotomy quickly breaks down as many intrinsic incompatibilities vary in strength with external environmental parameters such as temperature or diet (e.g., Bundus et al. 2015, Rand et al. 2018, Camus et al. 2020). In any case, BDMIs that manifest as hybrid breakdown are usually best characterized as intrinsic post-zygotic isolating mechanisms arising in allopatry essentially as a by-product of largely random processes of population differentiation.

Coughlan and Matute (2020) observed that recent speciation research has shifted to a more ecological focus, emphasizing prezygotic and *extrinsic* postzygotic reproductive isolation mechanisms and a view that these mechanisms play a dominant role in the process of "ecological speciation." Although there are now several examples of ecological speciation, Coughlan and Matute (2020) point out that intrinsic postzygotic barriers are ubiquitous and, in fact, widely viewed as a “defining characteristic of good biological species.” Hence, while perhaps not the focus of much of current speciation research, intrinsic barriers likely play a significant role throughout the speciation process. BDMIs that result in reduced fitness of interspecific (or interpopulation) hybrids clearly restrict gene flow and can consequently contribute to multiple stages in the process of speciation, ranging from early establishment of reproductive barriers to reinforcement after initial barriers are formed.

The search for speciation genes

If BDMIs are responsible for the development of reproductive isolation, we should be able to map the interacting genes and determine their identities. While advances in molecular technologies have greatly expanded the taxonomic diversity of studies assessing the genetics of speciation, much of the work in this area has focused on the genetics of laboratory hybrids in model systems, seeking to determine if some genotypic combinations are sterile or inviable due to BDMIs.

Indeed, because of the availability of diverse genetic tools, much of our understanding of the genetic basis of speciation focuses on a few model systems, with *Drosophila* at the forefront. There are many studies mapping incompatibilities (see Blackman 2016), but here a single example will be illustrative of a broad swatch of speciation research. With the BMDI model as a starting point, Presgraves (2003) took advantage of deletion mapping to determine the number and location of loci involved in incompatibilities between *Drosophila melanogaster* and its sibling species *D. simulans*. This powerful approach is largely restricted to *Drosophila* where the required deletion stocks are available. The surprising result was that a screen of approximately 70% of the *D. simulans* autosomes revealed 40 genomic regions that were lethal or semilethal in the presence of the *D. melanogaster* X-chromosome. Importantly, replacement with the *simulans* X-chromosome "cured" all the incompatibilities; i.e., the BDMIs all involved negative epistasis between *simulans* autosomal genes and genes on the *melanogaster* X-chromosome. Recessive incompatibilities were found to be 8-fold more common than dominant ones. In short, these relatively closely related fruit fly species have accumulated a large number of mostly recessive lethal (or near lethal) incompatibilities. *Notably, in this extensive experimental study and many other influential papers on speciation, the potential role of the mitochondrial genome is not investigated*. In an interesting twist, Zhang et al. 2017 have documented a embryonic lethal mitonuclear incompatibility that can contribute to reproductive isolation between these same species. This is not to suggest that this mitonuclear incompatibility is any more or less important than the nuclear gene incompatibilities elegantly documented by Presgraves (2003); rather I only wish to point out that many excellent large-scale analyses of BDMIs in model systems are set up in a way that cannot assess the potential role of mtDNA and consequently, the potential role of the mitochondrial genome in hybrid incompatibilities did not receive widespread attention. In fact, as discussed by Hill (2019), *Drosophila* researchers in the early to mid 20th century largely dismissed the potential role of cytoplasmic elements in the process of speciation.

Despite the large number of incompatibilities identified by fine-scale mapping, few genes that contribute to reproductive barriers have been identified (Blackman 2016, Castillo and Barbash 2017). Perhaps what is most revealing aspect of the cases where genes have been identified is the broad diversity of function they represent, and in some cases, how minor their role appears to be in the parental species. For example, the homeobox gene, *Odysseus (OdsH)* produces hybrid male sterility in crosses between *Drosophila simulans* and *D. mauritiana* (Sun et al. 2004), making it clearly worthy of the designation as a "speciation gene." Yet surprisingly, knockout of this gene has little measurable phenotypic effect other than a minor impact on sperm production

in young male *D. melanogaster*. This suggests that it may often be hard to predict the genes that will have the greatest impact when hybridization places them in "foreign" genetic backgrounds.

In summarizing the state of the field some 17 years ago, Orr et al. (2004) noted that: 1) the factors that cause postzygotic reproductive isolation are often ordinary genes that have normal functions within species, 2) the identified incompatibility genes are evolving rapidly, and 3) this rapid evolution is driven by positive Darwinian selection (i.e., diversifying selection). However, the ecological factors driving rapid evolution are poorly known; importantly, Orr et al. (2004) noted that rather than adaptation to extrinsic environmental factors, "it is entirely possible that selection instead reflected adaptation to the internal genetic 'environment'. As discussed below, the Orr et al. (2004) summary is a pretty good fit for what we might expect if genes involved in mitonuclear coadaptation underlie hybrid incompatibilities that evolved in allopatry.

Mitonuclear coadaptation and BDMIs

It has been proposed that the capacity for increased complexity and diversity in eukaryotes is tied to their ability to generate high levels of energy, a result of the ancient acquisition of a bacterial symbiont by an archaeal cell (Lane and Martin 2015; Lane 2014, 2020). The resulting endosymbiosis ultimately led to the evolution of an intracellular organelle that is the site of the majority of cellular energy production - the mitochondrion. Over the course of millennia, the genome of the endosymbiont has been dramatically reduced, with many genes lost and many others transferred to the host genome leaving the mitochondria with only a remnant genome. In animals, this remnant genome (the mtDNA) is quite conserved, typically with only 13 protein coding genes, 22 tRNAs and two rRNAs and total genome size generally in the 14kb - 20kb range. A key feature of the mtDNA is that the proteins it encodes are all key components of the energy-producing pathway, oxidative phosphorylation (OXPHOS), while the RNAs are components of the mitochondrial ribosomes or the transfer RNAs that are all required for the translation of the mtDNA-encoded proteins in the mitochondria. Remarkably, none of the mtDNA-encoded elements can function in the absence of a host of nuclear genome-encoded partners. Indeed, over 1000 nuclear-encoded proteins are imported into the mitochondria and many of these (approximately 200) must interact intimately with the mtDNA encoded elements to achieve functional OXPHOS enzyme complexes (Burton and Barreto 2012). Despite the need for coordinated function, the nuclear and mitochondrial genomes are transmitted between generations completely independently, with the nuclear genome following biparental Mendelian inheritance while the mtDNA is (with few exceptions) maternally inherited in animals.

Consequently, the maintenance of functional OXPHOS necessarily requires ongoing coevolution of the two genomes.

Although BDMIs were first envisioned as involving interactions between nuclear loci, the potential for negative epistatic interactions between nuclear and mitochondrial genes has been pointed out by a small but growing number of researchers (e.g., Levin 2003, Rand et al. 2004, Gershoni et al 2009, Burton and Barreto 2012; Hill 2016) over the past two decades. In fact, mitonuclear interactions seem like a particularly likely place to look for BDMIs given the rapid evolution of mtDNA (see below), the intimate interactions between nuclear-encoded and mtDNA-encoded gene products, and the pervasive impacts to fitness expected if mitochondrial function is disrupted. With only 37 mtDNA genes versus 15-20,000 nuclear genes, logic might suggest the interactions involving the mitochondrial genome would be extremely rare compared to interactions among nuclear genes; however empirical observations have led to the suggestion that mitonuclear incompatibilities may play a disproportionate role in BDMIs (Burton and Barreto 2012), though there are no rigorous tests of this conjecture. Why might mitonuclear incompatibilities be more common than expected?

Incompatibilities are ultimately the result of interactions between mutations and the genetic environment in which they occur. Since novel mtDNA mutations occur in isolated populations within a species, the resulting trajectories of mitonuclear coadaptation will be unique to each population. In most animal lineages, rates of nucleotide substitution in mtDNA are significantly higher than those observed in the nuclear genome for a variety of reasons. First, given its haploid maternal inheritance in almost all animal species, the effective population size for mtDNA is reduced by four-fold compared to the nuclear genome, increasing the role of drift and consequently reducing the efficacy of selection in eliminating mildly deleterious mutations (though Sloan et al. (2017) have questioned this argument). Recombination is rare in animal mitochondrial genomes (Lunt and Hyman 1997) so DNA repair based on recombination mechanisms is therefore largely absent. Furthermore, mitochondria have a strongly oxidizing environment that could potentially lead directly to increased rates of DNA mutations. However, the spectrum of mtDNA mutations is not consistent with direct oxidative damage; rather that spectrum is more consistent with replication error potentially due to low fidelity of mitochondrial DNA polymerase (polymerase γ) (reviewed in DeBalsi et al. 2017). In particularly interesting recent work, Anderson et al. (2020) found that polymerase γ is, in fact, a high fidelity enzyme, with strong proof-reading function. However, oxidative damage specifically impacts this proof-

reading capability, resulting in a 10-100 fold decrease in fidelity; hence the impact of the oxidative environment may not be on the mtDNA directly but rather through its impact on the fidelity of polymerase γ. In any case, elevated mutations rates combined with increased opportunity for genetic drift results in mtDNA substitution rates that vary greatly among taxa, but are typically somewhere between 2 and 40-fold higher than substitution rates in the nuclear genome with a trend toward higher values in vertebrates compared to invertebrates (Allio et al. 2017; Duda 2021).

Mutation is, of course, the source of genetic variation required for evolution and the above discussion suggests that mtDNA has somewhat more than its share. Elevated mutation rates in mtDNA can have diverse effects. Several analyses of mtDNA variation in natural populations have concluded that populations harbor excess amino acid polymorphism that largely consists of mildly deleterious mutations, much of which is ultimately removed by purifying selection (Nachman 1998; Rand and Kann 1998). Of course mutations also provide the raw material for adaptive evolution; James et al. (2016) estimated that between 5-45% of nonsynonymous substitutions in mtDNA are fixed by adaptive evolution, and there are many studies that suggest that mtDNA variants play a role in adaptation to environmental conditions ranging from temperature to diet (e.g., see review in Hill 2019). Conversely, the estimate by James et al. (2016) also means that between 55-95% of nonsynonymous substitutions in mtDNA are fixed as a result of drift and a significant proportion of those are likely mildly deleterious. Because of its clonal maternal inheritance in most animals, mtDNA cannot be purged of these deleterious mutations; in fact, additional mutations will continue to accumulate in mtDNA lineages, likely further negatively impacting mitochondrial function and organismal fitness.

Regardless of the causes of the elevated mutation and substitution rates observed in mtDNA, they represent a clear challenge to the maintenance of mitochondrial function. Fixation of even a single mildly deleterious mutation causing minor mitochondrial dysfunction could result in reduced ATP synthesis and loss of fitness. Consequently, there will be strong selection favoring genetic variants that recover function. Such compensatory variants could be at other sites within the mtDNA itself - within the same gene (Neverov et al. 2021) or another subunit of the same OXPHOS complex (e.g., a subunit 1 mutation may possibly be compensated by a subunit 2 mutation in Complex IV). Since the entire mtDNA is inherited intact, such a new self-compensated mtDNA haplotype may be functionally neutral relative to the parental haplotype and elicit no evolutionary response in the nuclear genome.

On the other hand, many deleterious amino acid changes in mtDNA-encoded proteins may be compensated by mutations in nuclear-encoded subunits of the enzyme complex, giving rise to mitonuclear coadaptation (Burton and Barreto 2012, and see review by Hill 2020). Rand et al. (2004) described how amino acid changes in an mtDNA encoded subunit could select for a compensatory change at a specific contact amino acid site (i.e., where amino acids contact each other in the enzyme complex) in a nuclear subunit. In principle this process could be resolved temporally in phylogenetic trees showing first an mtDNA substitution in a lineage followed by appropriate nuclear substitutions at contact amino acids. Such an analysis has rarely been attempted, but was successfully achieved by Osada and Akashi (2012) on primate Complex IV (cytochrome c oxidase); they found that across primates, adaptive evolution of nuclear subunits was only observed at sites that interact with amino acid substitutions in mtDNA encoded subunits after those mtDNA substitutions occurred. Indeed, on a genome-wide basis, there is good evidence that nuclear proteins that interact with fast evolving mtDNA-encoded proteins consistently show elevated rates of evolution compared to proteins that do not have such interactions (Barreto et al. 2018).

The functional consequences of disrupting interactions between coadapted nuclear and mitochondrial genomes have been demonstrated in several systems, but the copepod *Tigriopus californicus* is a particularly well studied case. This species inhabits rocky intertidal pools along the Pacific coast of North America and shows strong genetic differentiation among populations along its range (Burton 1998, Edmands 2001, Willett and Ladner 2009). When geographic populations are mated in the lab, consistent patterns of hybrid breakdown are observed in the F2 generation. Ellison and Burton (2006) directly examined the impact of hybridization on the function of mitochondria isolated from hybrids. They found reduced activities of four of the five OXPHOS enzyme complexes; only Complex II did not show impaired function. Notably, Complex II is the only complex composed solely of nuclear gene products so it is unaffected by the disruption of mitonuclear coadaptation that occurs in hybridization. In sum, these data show a clear impact of hybridization on mitochondrial ATP production and, in turn, a loss of hybrid fitness. These results are not limited to *T. californicus* populations; hybrids between species of *Nasonia* wasps, for example, show the same pattern of reduced function of all OXPHOS complexes with the exception of Complex II (Ellison et al. 2008a).

Although hybrid breakdown of OXPHOS function typically receives the most attention in discussions of mitonuclear incompatibilities, other aspects of mitochondrial function also require interactions between mtDNA and nuclear genomes and are similarly susceptible to disruption of

mitonuclear coadaptation (Burton and Barreto 2012). One approach to identifying these interactions is to test for accelerated evolution in nuclear genes known to function via interactions with mtDNA genes. For example, mitochondria have ribosomes for the synthesis of the mtDNA encoded OXPHOS subunits. At the core of these ribosomes are the two mtDNA-encoded rRNAs (12S and 16S), which, like other mtDNA genes, evolves faster than nuclear ribosomal genes. Barreto and Burton (2013) reasoned that the large set of ribosomal proteins that are nuclear in origin but form the mito-ribosomes (with 12S and 16S rRNA) may show elevated rates of evolution compared to the ribosomal proteins that form the cytoplasmic ribosomes with slow evolving nuclear rRNAs. This prediction proved true and a similar result was documented in *Drosophila, Nasonia* (and yeast). Among *T. californicus* proteins that are imported into the mitochondria, Barreto et al. (2018) found elevated rates of evolution (specifically dN/dS ratios) in a set of 147 proteins that interact directly with mtDNA encoded proteins compared to 458 proteins imported into the mitochondrial but do not interact directly with mtDNA encoded proteins. Yan et al. (2019) took this approach further by examining evolutionary rates in both OXPHOS genes and ribosomal proteins across several orders of insects; they found that rates of evolution were strongly correlated between nuclear OXPHOS genes that had contact with mitochondrial OXPHOS genes and for amino acids in nuclear ribosomal genes that had contact with mitochondrial rRNAs (i.e., they looked at the specific amino acids involved in contact rather than whole proteins). The relationship was sufficiently strong that they suggest that evolutionary rate correlations could better predict which nuclear proteins interacted with mitochondrial proteins better than some programs designed to look directly for mitochondrial-targeting sequences!

Evolution of mitonuclear incompatibilities in allopatry

Allopatric conspecific populations diverge genetically through the combined action of mutation, drift and natural selection. While the populations will share ancestral polymorphisms (standing genetic variation) that predate the establishment of allopatry, mutational input will differ between the populations once they become isolated. The expected pattern of divergence between two allopatric populations is shown in Figure 1. Given its higher mutation and substitution rates, mtDNA divergence would be anticipated to occur somewhat before nuclear divergence, so the first stage of the process is shown as each population gaining its own novel mtDNA haplotype but sharing the ancestral standing genetic variation in the nuclear genome. Mitochondrial divergence continues with the fixation of different novel mtDNA mutations in the respective allopatric

populations. As cited above, some of these mutations are mildly deleterious but fixed by drift; the resulting degradation in mitochondrial performance is a potent selective force favoring nuclear alleles (or variants of the mtDNA itself) that partially (or wholly) recover performance. Initially, such variants might be sorted from within the standing variation that may have been selectively neutral with regard to the ancestral mitotype but are now favored in combination with one of the new variant mitotypes. Ultimately, novel compensatory mutations in nuclear genes will also be recruited as they become available. The key point here is that the mutational landscape (both in the mtDNA and the nuclear genome) differs between the populations and this will lead to unique coadapted mitonuclear complexes in each of the allopatric populations.

Here's where things get interesting. As pointed out above, the trajectories of mitonuclear coevolution will be unique in allopatric populations. When, in secondary contact, hybridization occurs, individual offspring will receive only the maternal mtDNA but a mixture of maternal and paternal nuclear genes. Mitonuclear coadaptation can be disrupted when the mitochondrial genome from one population must function in a “foreign” nuclear gene environment – a mitonuclear BDMI. In effect, mutations in mtDNA that led to the fixation of compensatory nuclear gene mutations may no longer have the benefit of the full complement of those nuclear mutations that “rescue” function. The resulting impact on mitochondrial function will vary. Where little mitonuclear coevolution has occurred, as expected in the early stages of population differentiation, populations will continue to be compatible for mitochondrial function and consequences might be negligible in terms of hybrid fitness. As differentiation continues, additional deleterious mtDNA mutations and subsequent compensatory nuclear variants will be fixed in each population. This process recruits a growing number of genes into the coadapted complexes within populations and results in an increasing number of incompatibilities (BDMIs) between populations. Orr (1995) and Orr and Turelli (2001) point out that the rate of accumulation of incompatibilities is likely to be non-linear, increasing as the square of divergence time, creating a "snowball" effect. The higher levels of divergence are, in turn, expected to result in increased loss of hybrid fitness, possibly reaching full reproductive isolation – an allopatric speciation event.

The above scenario predicts that populations with higher levels of mtDNA divergence will likely have fixed multiple deleterious mutations and potentially numerous compensatory nuclear mutations. Several recent studies provide ample evidence in support of these predictions. Healy and Burton (2020) used a novel experimental approach to investigate the extent of mitonuclear

interactions across the nuclear genome. The experiment was initiated by reciprocally mating two genetically divergent natural populations of the intertidal copepod *Tigriopus californicus*, a southern California population (SD) and a central California population (SC), approximately 750 km to the north. A substantial body of previous work on this species has shown that such crosses result in significant fitness reduction in F2 interpopulation hybrids that can be attributed to mitonuclear incompatibility (e.g., Ellison and Burton 2006, 2008). It was hypothesized that in a cohort of F2 hybrids, those with the best mitochondrial performance would achieve the fastest developmental rates. Each individual in the cohort was scored for development time and the fastest and slowest developing individuals were separately pooled for two analyses: first, rates of ATP synthesis in isolated intact mitochondria were determined; in both reciprocal crosses, fast developing individuals showed significantly higher ATP biosynthetic rates, indicating that sorting by development time successfully distinguished hybrids with high or low mitochondrial performance. Next, to determine if there was some pattern in the genomic composition of fast developing (i.e., high fitness) hybrids, a Pool-seq approach was used to determine allele frequency differences between the high and low fitness groups in the reciprocal crosses. Remarkably, in high fitness groups from both reciprocal crosses, elevated frequencies of alleles from the maternal parent were observed across multiple chromosomes (Figure 3); no such pattern was observed in the low fitness hybrid groups. In sum, the fitness of hybrids depended on the degree of mitonuclear coadaptation – or, viewed from a different angle, the successful avoidance of mitonuclear incompatibilities.

Several aspects of these results merit further consideration. First, it should be noted that the reciprocal F1 hybrids have identical genomic composition (*T. californicus* lacks sex chromosomes); they differ only in mtDNA haplotype. The results indicate that the mtDNA had a strong selective effect across much of the nuclear genome. To have high fitness, genes on five of the 12 chromosomes needed to match the mitotype - SC nuclear alleles were favored on the SC mitotype and SD alleles were favored on the SD mitotype. At each SNP, the neutral expectation is that genotypic proportions in the F2 are Mendelian 1:2:1 ratios. If we assume incompatibilities are recessive and that no homozygous recessives at incompatibility loci make it into our fast developing group, the expected increase in the frequency of the matching allele would be 0.67; as is apparent in Figure 3, some of the observed frequencies nearly reach that point (e.g., Chromosome 5). The high frequencies across multiple chromosomes suggest that matching must occur at many sites in order to avoid the reduced performance associated with any of multiple mitonuclear incompatibilities between the natural populations. In contrast, genomic patterns in

slow developers (low fitness) hybrids were much less clear. The authors interpret this as evidence that any single incompatibility may be sufficient to reduce performance; low fitness hybrids would likely have different incompatibilities so none reach an observable elevated frequency. Building on the above results, Han and Barreto (2021) followed a similar protocol, but used a different population pair; the same southern site (SD) was paired with an even more divergent Oregon population (SH). Results were qualitatively similar; mitochondria from fast developers had significantly higher performance in the ATP synthesis assay and a strong bias toward maternal alleles (i.e., matching the mtDNA) across the genome in both reciprocal crosses. As in the SD x SC cross, significant allele frequency effects were observed on Chromosomes 2, 4, and five; however the SD x SH cross showed strong effects on Chromosomes 8, 9, 10, and 11 that were absent in the former cross. These contrasting results lend clear support to the model that suggests that the evolutionary development of BDMIs follows a unique trajectory in each allopatric population. The somewhat greater mitochondrial and nuclear genome divergence between SD-SH compared to SD-SC likely represents a longer period of allopatry that facilitated the recruitment of BDMIs across more chromosomes. An important limitation of this approach is that although Pool-seq provides an allele frequency estimate for each population-diagnostic SNP (1.9 million SNPs were scored), but no genotypic data on individual hybrids is obtained. Given that the experiment includes only a single generation of recombination (and *Tigriopus* has no recombination in females), there is extensive linkage disequilibrium in the F2 hybrids and the actual number of genes involved in mitonuclear incompatibilities cannot be resolved much below the level of whole chromosomes. Future experiments could start with highly intercrossed hybrids (e.g., 20 or more generations) to increase the resolution to small genomic regions.

While the above studies use developmental rate as a proxy for fitness, additional studies have consistently found evidence for important viability effects of mitonuclear incompatibilities in the *Tigriopus* system. Foley et al. (2013) scored individual genotypes (73 SNP loci) of F2 hybrids between SD and SC populations at (same populations used by Healy and Burton 2020) and used a QTL approach to map incompatibility loci based on F2 hybrid viability. By first showing that F2 nauplii did not deviate significantly from expected Mendelian ratios, Foley et al. (2013) could attribute fitness effects to larval-to-adult viability. Results also showed that mitonuclear incompatibilities were stronger than nuclear-nuclear incompatibilities. In another demonstration of the unique nature of mitonuclear coadaptation in allopatric populations, Lima et al. (2019) reciprocally mated three different populations (SC, CAT and SD) of *T. californicus* to a single population from Los Angeles (AB). The F2 offspring of F1 hybrids were raised to maturity and

then sacrificed and scored using Pool-seq to estimate allele frequencies at each of > 1 million SNPs spanning the genome. Deviations from 0.5 reveal relative viabilities of the alleles on the respective mtDNA backgrounds. Experimental results provided evidence from all three sets of crosses that mitonuclear interactions were more common than nuclear-nuclear interactions; however, only one of 12 chromosomes showed a consistent pattern of mitonuclear coadaptation across the three populations. The variable results across the other chromosomes again exposes the different evolutionary trajectories realized among these allopatric populations. Notably, all three population pairs showed similarly high levels of mtDNA divergence (~20%) and similar numbers of chromosomes (4-5) involved in mitonuclear coadaptation; future work in this system could focus of pairs of populations with a range of levels of divergence in mtDNA to test for temporal patterns in the development of coadaptation and resulting mitonuclear BDMIs.

Pereira et. al. (in press) used an alternate strategy for identifying genomic regions involved in mitonuclear incompatibilities. They initiated replicate populations of *T. californicus* with low-fitness F2 hybrids between the SD and SC populations and followed the recovery of fitness over a 9 month period (approx. 9 generations). Replicate populations of each reciprocal cross showed some recovery of fitness and were then subjected to Pool-seq analysis. Changes in allelic frequencies in the populations could be due to both drift and selection against low-fitness hybrids, purging the populations of incompatibilities. By comparing the trajectories of populations with either SD or SC mitochondrial backgrounds, changes reflecting mitonuclear incompatibilities could be located. Although replicate populations showed variable responses, where signal across the replicates agreed, there was a predominant signal of mitonuclear (as opposed to nuclear-nuclear) interactions. As in the other studies discussed above, the incompatibilities were in very different locations depending on the mtDNA background.

Finally, in contrast to the experiments competing alternate nuclear alleles on a single mitochondrial background, Pritchard and Edmands (2013) used an approach more appropriate to addressing genome dynamics of secondary contact between divergent *Tigriopus* populations. By initiating replicate experimental populations with a mix of animals from the two parental populations and maintaining the hybrid populations over 21 months, they could examine how mitonuclear genotypes evolved through time. Although there was extensive variation among replicate populations, there was a general pattern of reduction of mitonuclear mismatches through time. These results again suggest that animals with mitonuclear mismatches were effectively removed from the populations by selection and represents strong evidence for mitonuclear

incompatibilities result in low fitness. In terms of examining the potential role of mitonuclear incompatibilities in speciation (rather than just demonstrating such incompatibilities exist), this direct mixing of populations is likely a more informative approach for future work.

Asymmetry in hybrid breakdown: Haldane's Rule and Darwin's Corollary

Among the few generalities observed in speciation research, Haldane's Rule (Haldane 1922) has survived repeated testing. It states that when one sex in interspecific F1 hybrids is sterile or infertile, that sex is almost always the heterogametic sex. This rule has proven true across diverse sex determination systems such as XY chromosomes (males are heterogametic) in organisms as divergent as mammals and *Drosophila* and ZW systems (females are heterogametic) found in birds, butterflies and a broad range of other taxa. (Of course this requires that the species involved have sex chromosomes; *Tigriopus* and a variety of diverse taxa lack heteromorphic sex chromosomes.) Beyond Haldane's Rule, another interesting "rule" of hybridizations between species is that reciprocal crosses often differ in the degree of intrinsic post-zygotic reproductive isolation; i.e., reciprocal F1 hybrids frequently differ in viability or fertility. In reference to Darwin's discussion of this in plants (although others reported it even earlier), Turelli and Moyle (2007) named the phenomenon "Darwin's corollary to Haldane's Rule." Incompatibilities between autosomal loci are expected to generate equal effects on sterility or viability in hybrids produced by reciprocal crosses, so the widely observed asymmetries must involve incompatibilities between and autosomal locus and a uniparentally inherited factor, which could be located on a hemizygous sex chromosome or mtDNA. Hence, the frequent occurrence of reciprocal asymmetries in hybrids likely implicates a role for mtDNA in hybrid breakdown, especially in taxa lacking sex chromosomes.

Several studies have examined the potential role of mtDNA in asymmetries in levels of reproductive isolation in reciprocal crosses. Bolnick and Near (2005) found that in freshwater sunfishes (Centrarchidae), 18 of 20 species pairs showed viability asymmetries in reciprocal crosses. These species lack heteromorphic sex chromosomes making mtDNA divergence a likely candidate for the asymmetries. Asymmetries in some cases were quite pronounced, for example, crosses between the congeners *Lepomis gulosus* and *L. macrochirus* gave average hybrid survival rates of 77% (*L. gulosus* mother) and 35% (*L. macrochirus* mother). Bolnick et al. (2008) note that substitutions leading to incompatibility between mtDNA and autosomal loci are expected to occur randomly, so which reciprocal cross is more affected will also be expected to be random.

Alternatively, if rates of mitochondrial evolution (expressed as the ratio of mtDNA to nuclear substitutions) differ between taxa, the lineage with faster evolution will have more substitutions and higher probability of incompatibility; this suggests that it may be possible to predict the direction of asymmetry - the mothers with higher mtDNA substitution rates will produce offspring with lower fitness (Turelli and Moyle 2007). The sunfish data set (after adjusting for phylogenetic non-independence) shows modest support for this prediction (13/18 cases fit the model prediction) leaving the role of evolutionary rate differences in determining the direction of asymmetry of incompatibilities involving the mtDNA somewhat unresolved. However, Brandvain et al. (2014) found that only 30 of 74 crosses between toads of the genus *Bufo* supported the prediction that species with higher mtDNA evolutionary rates were worse mothers in reciprocal crosses, clearly rejecting the hypothesis. Thus, it appears that stochastic mtDNA substitutions may play a greater role in determining hybrid asymmetries than lineage-specific rates of mtDNA evolution.

Can mitochondrial dysfunction contribute to reproductive isolation and speciation?

To summarize much of the discussion above, we have clear evidence that divergence in allopatry can result in the development of mitonuclear incompatibilities between conspecific populations, causing mitochondrial dysfunction and reduced fitness in hybrids. But the process of speciation needs to advance to reproductive isolation. Can mitonuclear incompatibilities underlie reproductive isolation?

Here we can envision two somewhat different but related scenarios. First, reduced mitochondrial function will typically mean reduced ATP production. Whether this is a result of an incompatibility that directly reduces the activity of an OXPHOS enzyme complex (e.g., Ellison and Burton 2006; Ellison et al. 2008) or in transcription or translation of a protein encoded in the mtDNA (e.g., Ellison and Burton 2008; Meiklejohn et al. 2013), the resulting energetic phenotypic may be similar. Given that most organismal activities require ATP, we can expect impacts on the broad range of phenotypes that could ultimately limit gene flow and lead to reproductive isolation. As a simple example, we would expect a reduction in ATP-requiring muscle contraction and hence, reduced motility. This would impact feeding, predator avoidance and mating activity, essentially attacking fitness at every turn. Hybrids with low fitness result in reduced gene flow between populations, and consequently contribute to reproductive isolation.

But we can also find examples where the effects of mitochondrial dysfunction directly impact reproductive isolation. These studies typically involve laboratory manipulations that move mitochondria from one population to the nuclear genetic background of other populations and then compare levels of hybrid viability or sterility. For example, Clancy et al. (2011) report on the results of an experiment where, using multiply inverted balancer chromosomes in *Drosophila melanogaster*, the nuclear genome was replaced in lines bearing mitochondria from different geographic populations. The line containing the mtDNA from Brownsville, TX, was found to produce sterile males. Sequencing showed that the mtDNA differed from another fully fertile mtDNA by a single amino acid substitution in cytochrome b and when the Brownsville mtDNA was on the Brownsville nuclear genetic background, flies were fully fertile. The nuclear gene responsible for the resulting sterility effect on the balancer chromosome background is unknown.

In an elegant study employing reciprocal mtDNA replacement between different subspecies of mice, Ma et al. (2016) found strong evidence for asymmetric effects; while *Mus musculus musculus* mtDNA on a *M.m. domesticus* nuclear background resulted in embryos with normal development but reduced male fertility (normal female fertility) in the F1 generation and normal F2 and F3 female fertility, the reciprocal cross yielded a high rate of embryonic loss, high stillborn rate and infertility in the F1 and consequently no F2 generation. However, when the nuclear background of the latter cross was made heterozygous (i.e., carrying chromosomes from both subspecies), the lost fertility and high stillborn rate was rescued (much as fitness was rescued by maternal backcrossing of *Tigriopus* interpopulation hybrids, Ellison and Burton 2008). Consistent with this rescue, Complex 1 activity also increased significantly. These results indicate that the presence of nuclear alleles from the mtDNA donor plays a key role in compatibility between nuclear DNA and mtDNA and further suggests that the underlying genetic incompatibilities are recessive. Although this is a compelling case for a mitonuclear contribution to species boundaries, it is important to note that nuclear-nuclear gene incompatibilities have also been identified as important reproductive isolating barriers in this pair of taxa (Mihola et al. 2009); the gene *Prdm9* contributes to hybrid sterility in male F1 mice from crosses between male *Mus musculus domesticus* and female *Mus musculus musculus,* but the specific gene incompatibilities involved are still not fully resolved (Gregorova et al. 2018).

Meiklejohn et al. (2013) reported perhaps the only case where an interspecific mitonuclear incompatibility has been resolved to the level of the specific genes involved in both genomes. Hybrids with the Ore-R *Drosophila melanogaster* nuclear-encoded mitochondrial tyrosyl–tRNA

synthetase are incompatible with a *D. simulans* (*simw*^{501}) mitotype containing a single base mutation in the mitochondrial-encoded *tRNA*^{Tyr}, this incompatibility is strain specific; the mutated tRNA has no detrimental effect in an alternate *D. melanogaster* strain. When the incompatible alleles are both present, flies showed decreased activity in respiratory Complexes I, III, and IV, but not Complex II which has no mitochondrial-encoded subunits. The incompatibility involves the charging of the tRNA by the tRNA synthetase which impacts the translation of the mtDNA encoded subunits of the mitochondrial electron transport system. Zhang et al. (2017) further examined this system and found that the incompatibility ultimately results in ovarian failure and embryonic lethality. Again, neither the nuclear nor the mtDNA mutations in this system have any apparent negative fitness consequence in their own genetic backgrounds, but when combined we have a mitonuclear incompatibility that is sufficiently severe that it can lead to complete reproductive isolation.

F_2 hybrid males of *Nasonia vitripennis* and *Nasonia giraulti* experience high larval mortality rates relative to the parental species. Gibson et al. (2013) mapped the nuclear incompatible allele to a region on chromosome 5; larvae with the allele halt growth early in their development and ~98% die before they reach adulthood. The mapped region contains an OXPHOS gene in Complex I, suggesting that the incompatible mitochondrial locus is one of the mitochondrial-encoded Complex I genes. Although not fully identified, the work suggests a strong effect on reproductive isolation likely involving a mitonuclear incompatibility in the OXPHOS system.

The above is a very incomplete list of known examples, but should adequately demonstrate that mitonuclear incompatibilities can produce the strong fertility or viability effects needed to be significant contributors to reproductive isolation among taxa.

Theoretical considerations

Another approach to understanding the potential role of mitonuclear incompatibilities in speciation is through modeling. Gavrilets (2003) provided an analytical model of BDMIs that provides insights into how the probability of speciation, the average waiting time to speciation, and the average duration of speciation depend on mutation and migration rates, population size, and selection for local adaptation. An important result is that under the model conditions, speciation can occur by mutation and random drift alone with no contribution from selection as different populations accumulate incompatible genes as apparent in the empirical data discussed above.

Two recent papers present models specifically addressing the potential role of mitonuclear BDMIs in speciation. Telschow et al. (2019) found that mitonuclear incompatibilities reduce gene flow stronger than nuclear-nuclear interactions when incompatibilities are recessive, but weaker when they are dominant. Furthermore, model results suggest that mitonuclear BDMIs are most effective in promoting speciation if females are the migrating sex. The authors note that there is some empirical support for this hypothesis in *Nasonia* parasitic wasps where mitonuclear incompatibilities have been well documented. Princepe and de Aguiar (2021) use a very different modeling approach to examine the role of mitonuclear interactions in speciation. The metric used was the accuracy of mtDNA barcoding in delineating newly evolved species in simulations where fitness either did or did not depend on mitonuclear interaction. Interestingly, they find not significant differences in barcode accuracy between the cases with and without the interaction. Rather, the spatial structure of the population played a more important role in species identification. While results with the current model parameterization did not find a significant role for mitonuclear interactions in the formation of new species, further development of this model may provide novel insights into conditions where mitonuclear interactions do play such a role.

mtDNA introgression - a problem for mitonuclear coadaptation's role in speciation?

A frequent point of contention for the role of mitonuclear coadaptation in speciation is the observation that mtDNA sometimes appears to freely introgress across species boundaries (Hill 2019). If mitochondrial function is dependent upon coadaptation with the nuclear genome, how could such introgression occur without negative fitness consequences for mitonuclear hybrids? In fact, mitochondrial introgression is not an uncommon observation; Toews Brelsford (2012) document over 120 cases in animals. In discussing some of those cases in *Bufo* toads, Brandvain et al. (2014) note that the deep discordance between mitochondrial and nuclear topologies clearly indicate that mitonuclear incompatibilities did not prevent interspecific gene flow or mtDNA introgression. They suggest probably the simplest viable solution: the contradiction between the expected mitonuclear barrier and the realized introgression may simply reflect the random nature of the mutation process and the resulting heterogeneity of phenotypic effects of mitochondrial introgressions into foreign genetic backgrounds, which may range from innocuous (or even favorable) to near lethal.

Although there are extensive literatures supporting both mitonuclear incompatibilities and mitochondrial introgression, few efforts have been made to reconcile the paradox between the two. Sloan et al. (2017) and Hill (2019) offer a number of possible solutions. One possible scenario is that the species that ultimately receives the introgressing mtDNA may have a mitochondrial genome with a high genetic load due to the fixation of multiple deleterious mutations (or mutations with strongly negative fitness consequences (Hill 2019). Here the introgressing mtDNA results in an increase in recipient fitness associated with the less load-ridden mtDNA despite losing the positive effects of coadaptation with its "native" nuclear background. This hypothesis has been suggested to explain the introgression of *Drosophila yakuba* mtDNA into the island endemic *D. santomea* (Llopart et al. 2014). The initial divergence between the two species has been estimated at ~400,000 years; *D. yakuba* has a larger N_e relative to that of *D. santomea*; this would potentially result in a decreased rate of accumulation of mildly deleterious mutations in its mtDNA compared to *D. santomea*. Interspecific hybridization after the secondary colonization of São Tomé by *D. yakuba* may have allowed the lower mutational load of the *D. yakuba* mitochondrial genome to be selectively favored and replace the *D. santomea* mtDNA, suggesting such selection was sufficient to overriding any mitonuclear incompatibilities between these sibling species.

Perhaps the simplest explanation for mtDNA introgression would be that the "invading" mtDNA might simply be better adapted to the local environment such that, even without nuclear coadaptation, it increases the fitness of hybrids and backcrosses such that it successfully displaces the native mtDNA (Blier et al. 2001). For example, Mishmar et al. 2003 found that regional patterns in mtDNA variation in humans likely resulted from selection by climatic conditions. Many studies have found environmental correlations with haplotype frequencies, but few have provided further evidence of actual fitness differences of mtDNA haplotypes (Sloan et al. 2017).

Camus et al. (2017) and Lajbner (2018) studied a latitudinal gradient in haplotype frequencies in natural populations of Australian *Drosophila melanogaster*, testing the hypothesis that the haplotype frequency cline reflects differences in thermal tolerance of the haplotypes. Despite the fact that the only SNPs in the coding regions of the dominant haplotypes were synonymous substitutions, they found that tolerance of thermal extremes (heat knock-down and chill coma) depends on mtDNA haplotype, and that the thermal performance associated with each haplotype corresponds with its latitudinal prevalence (Camus et al. 2017). Lajbner et al. (2018) found that

laboratory populations maintained in different thermal regimes exhibited changes in haplogroup frequencies across generations. Whether the observed SNPs confer adaptation by impacting gene expression or if variation in unsequenced non-coding regions of the mtDNA are responsible remains undetermined. Although there are not many studies confirming functional adaptations resulting directly from mtDNA variation, when extrinsic selection favoring introgression is sufficiently strong, it could reasonably be expected to overcome intrinsic coadaptation.

Immonen et al. 2020 provide a recent example of experimental evolution demonstrating the adaptive differences of mitonuclear genotypes with the thermal environment in the seed beetle, *Callosobruchus maculatus.* Using a set of lines where three different mtDNA haplotypes (Brazil, California and Yemen) were each introgressed (by backcrossing) onto the nuclear genetic backgrounds from the same three divergent geographic populations, experimental populations were constructed with the three mitotypes in equal proportions on each of the three nuclear backgrounds. Replicate populations were then grown at two stressful temperatures and after >30 generations Pool-seq was used to estimate haplotype frequencies. Results showed that at high temperature, all three native mitonuclear combinations (e.g., Yemen mtDNA on Yemen nuclear background) had the highest fitness (as opposed to the mismatched mitonuclear combinations). However, that was not the case at low temperature, where, for example, Yemen mtDNA had its highest fitness on the California nuclear background. The results show a strong signal for a genotype (nuclear) x genotype (mtDNA) x environment (G xGxE) interaction.

A more intriguing possibility is that the introgressing mtDNA might be accompanied by some of its complement of coadapted nuclear genes, essentially replacing the resident coadapted complex with an alternate set of coevolved mitonuclear genes. Beck et al. 2015, working on the *Drosophila yakuba* introgression in *D. santomea* discussed above, found evidence for elevated levels of introgression from *yakuba* to *santomea* for three nuclear genes encoding cytochrome c oxidase (COX, Complex IV) subunit V. As subunit V directly interacts with the mtDNA - encoded subunits of COX, the authors suggest this is a likely case of mitonuclear co-introgression and may help explain how the *yakuba* mtDNA crosses the species boundary.

In another case, Morales et al. (2018) describe apparent co-introgression in eastern yellow robins, *Eopsaltria australis* in Australia. This species split into northern and southern populations an estimated 2 million years ago resulting in substantial nuclear ($G_{ST} = 0.084$) and mtDNA (6.2%)

divergence (Morales et al. 2017). Examination of the complex pattern of introgression suggests that it is not compatible with a neutral explanation. Rather, introgression appears to be the result of adaptive introgression and mitonuclear co-introgression. This hypothesis is supported by several lines of evidence, but most importantly, there is strong sequence divergence in a large region (~15.4 megabases) of chromosome 1A that corresponds geographically with the pattern of mitochondrial DNA divergence. Furthermore, the specific chromosome 1A region is enriched for genes performing mitochondrial functions. These results suggest that at least in some cases, introgression of mtDNA across divergent populations can provide strong support for mitonuclear coadaptation rather than the opposite! One feature of this system is worth noting - the original divergence of the populations took place over the course of 2 million years and led to substantial divergence of both mtDNA and nuclear genomes. This relatively long history of allopatry may be a key feature required for the development of strong mitonuclear coadaptation within populations required to achieve some degree of reproductive isolation between populations.

Another compelling example of mitonuclear co-introgression in birds involves the hybrid Italian sparrow, *Passer italiae*. The Italian sparrow is a hybrid species between the Spanish and house sparrows. Trier et al. (2014) used cline analysis and found that among a relatively small set of species diagnostic SNPs identified from transcriptome data, those showing the sharpest clines (suggesting strong restrictions in gene flow) included a mitochondrial SNP and two nuclear-encoded mitochondrial genes. Hermansen et al. (2014) found that these candidate reproductive isolation genes are a subset of those found between the two parental species, which includes mitonuclear interacting loci. Taking the analysis to the whole genome level, Runemark et al. (2018) found that in four apparently independent lineages of hybrid Italian sparrows on Crete, Corsica, Sicily and Malta, specific genomic regions are invariably inherited from the same parent species and matching the mtDNA, from the house sparrow parent. These regions are over-represented on the sex chromosome (Z) and include candidate incompatibility loci, including DNA-repair and mitonuclear genes. In sum, it appears that viable hybrid species between Spanish and house sparrows can involve a variety of genomic mosaics (with phenotypic effects), but all those mosaics include the house sparrow mtDNA and regions of co-introgressed nuclear genes that function in the mitochondria. This repeated evolutionary outcome strongly implicates mitonuclear interactions as isolating barriers between the parentals and between the parental and hybrid species.

Although co-introgression seems a natural explanation for how mitochondria may introgress

across species boundaries, Sloan et al. (2017) raise important issues regarding the dynamics of the process that should be noted. Specifically, since mtDNA is not physically linked to coadapted nuclear genes, following an initial hybridization, both introgressing mtDNA and introgressing nuclear alleles will be at low frequency. In short order, the nuclear alleles will find themselves mostly in the presence of the foreign mitochondria where they lack the selective advantage they had on their own mtDNA background; consequently they are likely to be selected against, making successful introgression unlikely. As discussed by Hill (2019), this scenario can be circumvented if there is sequential introgression where initially the mtDNA introgresses successfully through some direct selective advantage (e.g., extrinsic environmental selection or lower mutational load relative to the resident mtDNA); subsequent hybridizations could then result in successful introgression of nuclear genes with high fitness on the introgressed mtDNA. This suggests that co-introgression of mtDNA and interacting nuclear genes is most likely to occur where there are repeated bouts of hybridization between taxa over a significant period of time.

This review is focused on the role of mitonuclear incompatibilities in the process of allopatric speciation. This perspective is not meant to imply that mitochondria are not potentially involved in speciation in other ways. Tobler et al. (2019) champion a different viewpoint. While agreeing that there is abundant evidence for mitonuclear incompatibilities reducing hybrid fitness via intrinsic selection, they expand the discussion to the possibility that reproductive isolating barriers can arise as a by-product of adaptive divergence of mitochondrial function. Such a role for mitochondria would be consistent with recent ideas about "ecological speciation" where extrinsic adaptations lead to population divergence even in the face of gene flow; furthermore such a scenario need not involve mitonuclear coadaptation. There is little doubt that mitochondrial genomes can be involved in adaptation to diverse environmental challenges and many examples exist (see citations in Tobler et al. 2019 and Hill 2019). However, how adaptation in mtDNA alone is translated into reproductive isolation is unclear. One possibility is that mitochondrial physiological adaptations lead to some sort of assortative mating leading to a pre-zygotic isolating barriers and reduction in gene flow; Tobler et al. (2018) suggest this might be the case for poeciliid fishes that have colonized hydrogen sulfide (H_2S) rich environments. In any case, it should be acknowledged that BDMIs are not the only mechanism where mitochondria may play a role in speciation.

In thinking about the nature of selection acting on the mitochondrial genome and its potential role in speciation, much of the literature tends to focus on one type of selection (intrinsic vs. extrinsic)

at a time, almost as if choosing a political party. In fact, both types of selective pressures are always operating simultaneously. Consider hypothetical allopatric populations experiencing different environments. Given its high rate of mutations and substitutions, it seems that the mtDNA will be host to a full range of mutations, both adaptive and deleterious. Bazin et al. (2006) provided strong evidence for recurrent bouts of adaptive mtDNA evolution as an explanation for why mtDNA variation is decoupled from population size across taxa, so it is appropriate to assume some proportion of the mutations are advantageous (recall that James et al. (2016) estimated that between 5-45% of nonsynonymous substitutions in mtDNA are fixed by adaptive evolution). However, it is also a safe assumption that the majority of mtDNA mutations are not sufficiently advantageous to drive selective sweeps; most fixed substitutions will be selectively neutral or mildly deleterious, and the latter will favor compensatory nuclear mutations leading to mitonuclear coadaptation. Consequently divergence between populations would be expected to include both adaptive variation and mitonuclear BDMIs. What happens during secondary contact? Because if its ecological relevance, incompatibility due to adaptive variation might win the most attention; however, intrinsic BDMIs might play an equal or even greater role in determining any resulting reproductive isolation.

So – can we point to examples of mitonuclear speciation?

Given all of the above discussion, it is fair to say that there is ample evidence that mitonuclear coadaptation is ubiquitous among eukaryotes and has been widely documented in diverse ways. It has also been repeatedly demonstrated that BDMIs can result in loss of fitness in hybrids which then leads to reduced gene flow between populations. Genome scans have shown that introgession across genomes in hybrids is not uniform and there are now at least a few well-documented cases where genomic regions with restricted gene flow are associated with nuclear loci that functionally interact with the mtDNA (or mtDNA gene products).

But more to the point, to my knowledge, there are no cases of mitonuclear incompatibilities giving rise to new species in allopatry or otherwise. Have we looked? Various studies have introgressed mitochondria across species boundaries and seen various levels of phenotypic effects, but Montooth et al. (2010) provide an interesting example. Placing nine mtDNAs from *D. melanogaster*, *D. simulans* and *D. mauritiana* into two *D. melanogaster* nuclear genetic backgrounds using crosses that prevent nuclear co-introgression allowed tests of the impact of highly divergent mtDNAs on common nuclear backgrounds. The striking result was

that the strongest effects were observed within species; there was no evidence that the more than 500 fixed differences between the mitochondrial genomes of *D. melanogaster* and the *D. simulans* species complex resulted in incompatibility with the *D. melanogaster* nuclear genome. As the authors point out, Drosophila species have relatively low mtDNA substitution rates so the genus may not be the best place to look for a mitonuclear role in speciation.

Based on the numerous studies described above, *Tigriopus californicus* has frequently been called the "poster-child" of mitonuclear incompatibilities among allopatric populations. Certainly this system meets the criteria that have been set for such events (Burton and Barreto 2012) - extremely high rates of mtDNA evolution and highly restricted gene flow among geographic populations at all spatial scales. Yet even here is no strong evidence for mitonuclear speciation events in *Tigriopus*. Laboratory crosses between highly differentiated populations consistently yield low fitness hybrids, yet these hybrids are neither sterile or inviable. Furthermore, over multiple generations, hybrid populations appear capable of purging mitonuclear incompatibilities (Pritchard and Edmands 2013, Pereira et al. 2021). Biogeographically, there is reproductive compatibility (production of at least some fertile F1 and F2 hybrids in both reciprocal crosses with no premating isolation) across a huge range of genetic divergence (over 23% sequence divergence in mtDNA) between at least San Diego, California and Friday Harbor, Washington, a span of 16 degrees latitude.

Interestingly, to the south, reproductive isolation is observed when *T. californicus* populations from central Baja California (Mexico) are crossed with northern Baja or southern California populations (Ganz and Burton 1995, Peterson et al. 2013). These populations show extreme mtDNA and nuclear genome differentiation (Barreto et al. 2018) and, given the strong evidence for mitonuclear incompatibilities in more northern populations, we might conclude that this apparent geographic speciation could be a case of mitonuclear speciation. Of course where no F1 or F2 offspring are produced, it is impossible to do the necessary genetic crosses to test the role of mitonuclear incompatibilities! But there are a few cases where populations are not completely reproductively isolated (Ganz and Burton 1995, Peterson et al. 2013). Hwang et al. (2012) studied one cross between two Mexican populations (Play Altamira to the south and Punta Morro to the north, separated by 3 degrees of latitude) that produced small numbers of fertile F1 and F2 offspring when the female was from Punta Morro (the reciprocal cross produced no offspring). Both F1 and F2 hybrids showed greatly reduced clutch sizes but parental-level survivorship. Recalling that backcrossing to the maternal line "cured" hybrid breakdown in crosses between

northern populations, we might expect backcrosses in the PA x PM cross might recover fitness if breakdown was due to mitonuclear incompatibility. However, this was not the case; neither backcross (maternal nor paternal) produced any offspring. Although this does not definitively exclude the possibility of mitonuclear speciation, it leaves us with no strong support for the known case of incipient allopatric speciation in *T. californicus*.

Summary

The effects of mitonuclear interactions on hybrid fitness and reproductive isolation between populations has been the subject of several extensive recent reviews and perspectives (e.g., Burton et al. 2013, Sunnucks et al. 2017, Sloan et al.2017, Hill et al. 2019, Hill 2019). All these reviews find ample evidence for mitonuclear coadaptation in a great diversity of organisms. Here I have tried to make some key points: 1) first and foremost is that the very nature of the mitonuclear coadaptation process in allopatry seems predisposed to the continuing development of BDMIs. 2) This process is ongoing in populations of the vast majority of eukaryotes. 3) Elevated mutation and substitution rates in mtDNA compared to the nuclear genome have been well documented. Furthermore, recent genome-scale data sets have consistently found the nuclear genes encoding the proteins that interact most closely with mtDNA products (both proteins and RNAs) also have elevated substitution rates, largely restricted to the sites of actual interaction. Hence the entire complex of mitonuclear interactions may often be among the first gene network to diverge when allopatric populations become established. As such, it is reasonable to suggest that mitonuclear incompatibilities may often be early contributors to the reduction of gene flow between incipient species. 4) These incompatibilities frequently arise as a by-product of intrinsic selection pressures favoring compensatory nuclear gene coadaptation; however, like all traits, the product of mitonuclear coadaptation is exposed to extrinsic selection, adding additional functional criteria that no doubt impacts the trajectory of coadaptation in some systems. Although there is much evidence for mitonuclear incompatibilities that could contribute to reproductive isolation, there is far less evidence that they "seal the deal" and result in species formation.

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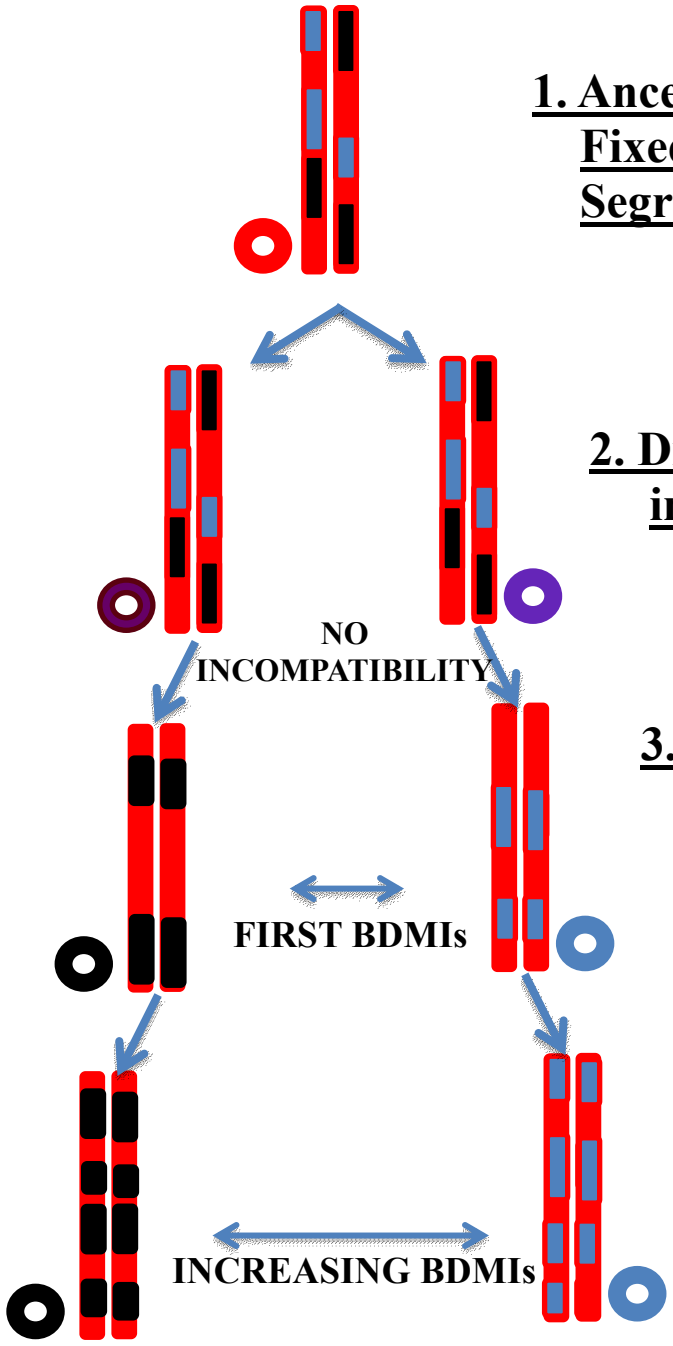
Figure legends

Fig 1 Evolution of mitonuclear incompatibilities in allopatric populations. Initial differentiation will typically be in the mtDNA followed by compensatory nuclear mutations. The figure represents each population's mtDNA (small circle) and one pair of autosomes with colored blocks representing nuclear genes that are coadapted to the mtDNA (if the same color as mtDNA)) or potentially incompatible (if different color)

Fig 2 Reciprocal hybridizations of divergent allopatric populations generating mitonuclear incompatibilities in the F2 generation (dashed elipses), followed by maternal and paternal backcrosses, with only the former "curing" the incompatibilities

Fig 3 Pool-seq results reciprocal F2 hybrids between SC and SD populations (from Healy and Burton 2020). In fast developing (high fitness) hybrids, SNP frequencies in several genomic regions show a strong bias toward "matching" nuclear alleles; i.e., SC alleles are favored in the presence of the SC mtDNA and SD alleles are favored on the SD mtDNA background, revealing the strong effects of mitonuclear coadaptation

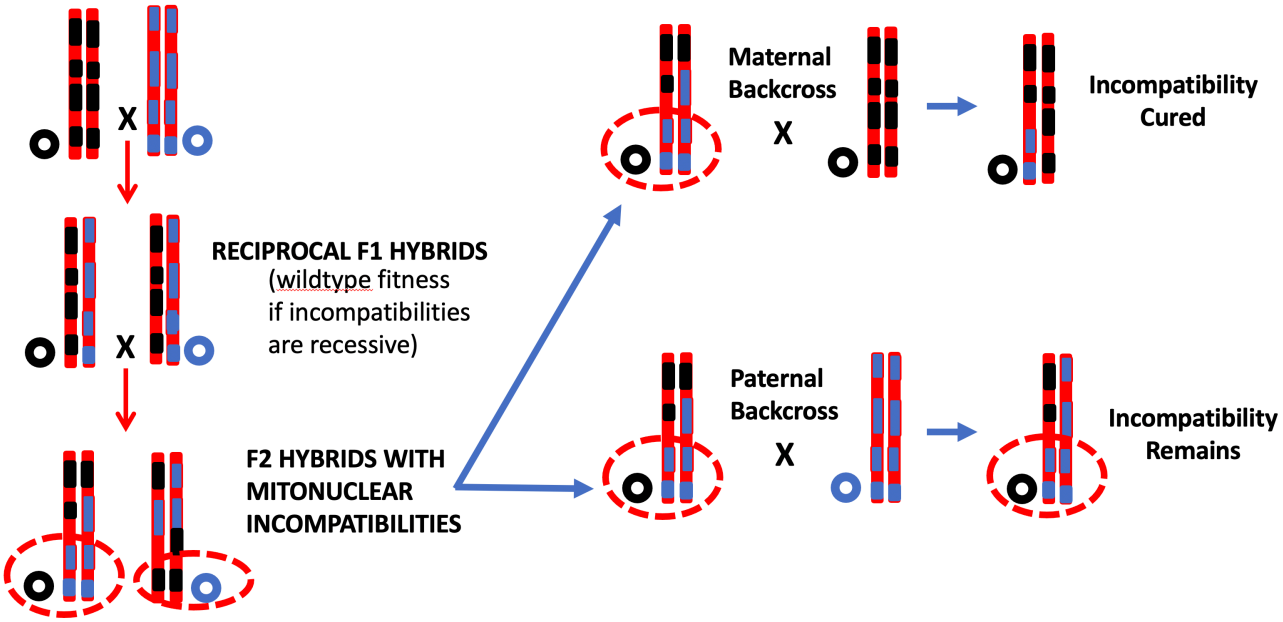


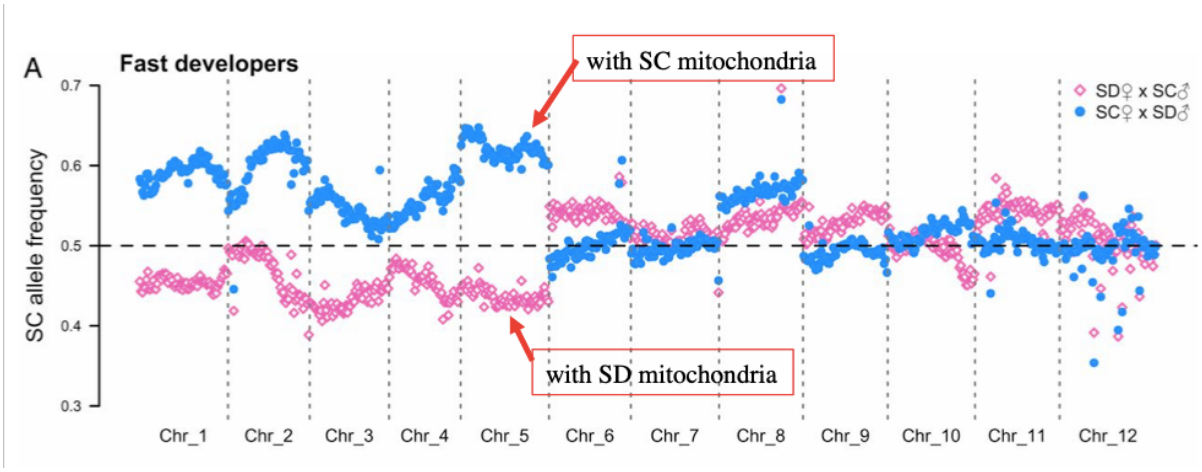
1. Ancestral population with
Fixed mt haplotype
Segregating nuc variation

2. Divergence of mtDNA
in isolated populations

3. Sorting of nuclear variation
with mtDNA mutations in each
population

4. Continued divergence
of both nuc and mtDNA


X
X
RECIPROCAL F1 HYBRIDS
(wildtype fitness
if incompatibilities
are recessive)
F2 HYBRIDS WITH
MITONUCLEAR
INCOMPATIBILITIES
Maternal
Backcross
X
Incompatibility
Cured
Paternal
Backcross
X
Incompatibility
Remains


A
Fast developers
with SC mitochondria
with SD mitochondria
SD♀ x SC♂
SC♀ x SD♂
SC allele frequency
0.7
0.6
0.5
0.4
0.3
Chr_1
Chr_2
Chr_3
Chr_4
Chr_5
Chr_6
Chr_7
Chr_8
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