

MITOCHONDRIAL–NUCLEAR EPISTASIS AFFECTS FITNESS WITHIN SPECIES BUT DOES NOT CONTRIBUTE TO FIXED INCOMPATIBILITIES BETWEEN SPECIES OF *DROSOPHILA*

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Efficient mitochondrial function requires physical interactions between the proteins encoded by the mitochondrial and nuclear genomes. Coevolution between these genomes may result in the accumulation of incompatibilities between divergent lineages. We test whether mitochondrial–nuclear incompatibilities have accumulated within the *Drosophila melanogaster* species subgroup by combining divergent mitochondrial and nuclear lineages and quantifying the effects on relative fitness. Precise placement of nine mtDNAs from *D. melanogaster*, *D. simulans*, and *D. mauritiana* into two *D. melanogaster* nuclear genetic backgrounds reveals significant mitochondrial–nuclear epistasis affecting fitness in females. Combining the mitochondrial genomes with three different *D. melanogaster* X chromosomes reveals significant epistasis for male fitness between X-linked and mitochondrial variation. However, we find no evidence that the more than 500 fixed differences between the mitochondrial genomes of *D. melanogaster* and the *D. simulans* species complex are incompatible with the *D. melanogaster* nuclear genome. Rather, the interactions of largest effect occur between mitochondrial and nuclear polymorphisms that segregate within species of the *D. melanogaster* species subgroup. We propose that a low mitochondrial substitution rate, resulting from a low mutation rate and/or efficient purifying selection, precludes the accumulation of mitochondrial–nuclear incompatibilities among these *Drosophila* species.

KEY WORDS: Epistasis, mtDNA evolution, polymorphism, X chromosome.

In many animals and fungi, the mitochondrial genome experiences significantly more mutations per base pair than the nuclear

genome (Lynch et al. 2008; Montooth and Rand 2008). Combined with a potentially reduced effective population size due to the effects of selection on this nonrecombining genome (Muller 1964; Hill and Robertson 1966; Maynard-Smith and Haig 1974; Charlesworth et al. 1993), the elevated mutation rate makes the mtDNA particularly prone to the fixation of deleterious mutations (Gabriel et al. 1993; Lynch 1996; Neiman and Taylor 2009). Additionally, cytoplasmic sweeps driven by selfish cytoplasmic

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elements can decrease diversity and increase the rate of deleterious substitution in the mtDNA (Shoemaker et al. 2004). The nuclear genome encodes many more genes necessary for mitochondrial function than does the mtDNA, making it a potentially large target size for mutations that rescue mitochondrial function. Stabilizing selection to preserve efficient mitochondrial function may result in compensatory evolution whereby fitness loss due to the fixation of slightly deleterious mutations in the mitochondrial genome is rescued by mutations in the nuclear genome (Rand et al. 2004; Dowling et al. 2008).

While the basic cellular function of the mitochondria has been conserved over long evolutionary timescales, this maintenance occurs across lineages that have diverged in their ecology, nutrient environment, behavior, and life history over shorter timescales. Positive selection may therefore act on mitochondrial and nuclear genomes in concert to adapt cellular metabolism to new physiologies and ecologies (Ballard and Rand 2005; Ihmels et al. 2005; Dowling et al. 2008). Thus, rapid mitochondrial divergence and coevolution between mitochondrial and nuclear genomes could in principle be driven by both positive and negative selection (Bazin et al. 2006; Meiklejohn et al. 2007; Oliveira et al. 2008). Either process could generate intergenomic incompatibilities between mitochondrial and nuclear genomes that have evolved in isolation from one another, resulting in decreased fitness in hybrids between isolated populations or closely related species (Burton et al. 2006).

Interpopulation hybrids of the marine copepod *Tigriopus californicus* have disrupted mitochondrial transcription and adenosine triphosphate synthesis, and lower fitness (Edmunds and Burton 1999; Ellison and Burton 2006, 2008a,b). Maternal backcrosses that reconstitute the parental mitochondrial–nuclear combinations restore fitness, highlighting the potentially important role of mitochondrial–nuclear interactions in hybrid breakdown (Ellison and Burton 2008a). Coevolution of mitochondrial and nuclear genomes results in sterile hybrids between the yeast species *Saccharomyces cerevisiae* and *S. bayanus* (Lee et al. 2008), and may underlie decreased hybrid fitness between species of the parasitic wasp *Nasonia* (Breeuwer and Werren 1995; Ellison et al. 2008; Niehuis et al. 2008). *Nasonia* and *Tigriopus* have high levels of mitochondrial divergence between species and among populations, respectively (Burton et al. 2006; Oliveira et al. 2008), and the regulatory regions of yeast mtDNAs evolve rapidly (Groth et al. 2000), raising the possibility that the accumulation of mitochondrial–nuclear incompatibilities may scale with mtDNA divergence.

In *Drosophila* the predominant evolutionary force shaping mtDNA divergence is purifying selection (Rand and Kann 1996, 1998; Ballard 2000; Montooth et al. 2009). However, evolutionary constraint varies across the mitochondrial proteome with particular oxidative phosphorylation complexes evolving

at significantly different rates (Ballard 2000; Montooth et al. 2009). These complex-specific evolutionary rates also vary across *Drosophila* lineages (Montooth et al. 2009). While mtDNAs within *D. simulans* are associated with differences in fitness, life-history traits, and mitochondrial physiology (James and Ballard 2003; Ballard et al. 2007) and may decrease cytochrome C oxidase activity in hybrids with *D. mauritiana* (Sackton et al. 2003), the extent to which molecular evolutionary forces lead to an accumulation of mitochondrial–nuclear incompatibilities for fitness between *Drosophila* species is unknown.

To test for within- and between-species mitochondrial–nuclear interactions, we generated strains of *D. melanogaster* that carry nine mtDNAs of varying molecular divergence from within the *D. melanogaster* species subgroup in combination with two *D. melanogaster* nuclear genomes. To avoid retaining nuclear variants from the maternal parent that may accompany mitochondrial introgression during repeated backcrossing (James and Ballard 2003; Dowling et al. 2008), the nuclear genomes were precisely introduced using balancer chromosomes. Testing each mtDNA in two nuclear genetic backgrounds allows quantification of mitochondrial–nuclear epistasis for fitness. The phylogenetic context of this experiment allows a test of whether mitochondrial–nuclear incompatibilities accumulate as mitochondrial and nuclear genomes diverge together along species lineages. We find no evidence that the accumulation of fixed differences in the mtDNAs gives rise to mitochondrial–nuclear incompatibilities between these sibling species. Instead, we find that particular mitochondrial and nuclear variants that segregate within species, potentially as neutral, mildly deleterious or population-specific polymorphisms, interact to generate the strongest epistatic effects on fitness.

Materials and Methods

GENERATING MITOCHONDRIAL–NUCLEAR HYBRIDS

We generated 35 *D. melanogaster* strains that combine nine mtDNAs from *D. melanogaster* and its sibling species (*ore*, *aut*, *zim*, *siI*, *sm21*, *sm22*, *sm38*, *simw*⁵⁰¹, *mau12*) with two *D. melanogaster* nuclear backgrounds (*Ore*, *Aut*) and two additional *D. melanogaster* X chromosomes (*P58*, *P89*) (Table 1, Fig. 1). Female offspring from crosses between *D. simulans* females and *D. melanogaster* males frequently die as embryos (Hadorn 1961), and those that survive are typically sterile. However, viable and weakly fertile F1 females can be obtained from crosses between *D. simulans* C167.4 females and *D. melanogaster* *In(1)AB* males (Davis et al. 1996), allowing the transfer of mtDNA from *D. simulans* into *D. melanogaster*. We backcrossed fertile F1 hybrid females to *D. melanogaster* males for multiple generations, after which we used balancer chromosomes to precisely replace the nuclear chromosomes with those from two

Table 1. Genotypes used to infer mitochondrial–nuclear and mitochondrial–X chromosome interactions for fitness.

Mitochondrial-X-nuclear genotype ¹	mtDNA haplotype	mtDNA source	X-chromosome genotype	Autosomal genotype
(<i>ore_t</i>); <i>Ore</i> ; <i>Ore</i> ; <i>Ore</i>	<i>mel</i>	<i>D. melanogaster OreR</i>	<i>OreR</i>	<i>OreR</i>
(<i>zim_t</i>); <i>Ore</i> ; <i>Ore</i> ; <i>Ore</i>	<i>zim</i>	<i>D. melanogaster Zim53</i>	<i>OreR</i>	<i>OreR</i>
(<i>siI_t</i>); <i>Ore</i> ; <i>Ore</i> ; <i>Ore</i>	<i>siI</i>	<i>D. simulans siI Hawaii</i>	<i>OreR</i>	<i>OreR</i>
(<i>sm21_t</i>); <i>Ore</i> ; <i>Ore</i> ; <i>Ore</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>OreR</i>	<i>OreR</i>
(<i>sm22_t</i>); <i>Ore</i> ; <i>Ore</i> ; <i>Ore</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>OreR</i>	<i>OreR</i>
(<i>sm38_t</i>); <i>Ore</i> ; <i>Ore</i> ; <i>Ore</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>OreR</i>	<i>OreR</i>
(<i>simw</i> ⁵⁰¹ _{<i>t</i>}); <i>Ore</i> ; <i>Ore</i> ; <i>Ore</i>	<i>siII</i>	<i>D. simulans simw</i> ⁵⁰¹	<i>OreR</i>	<i>OreR</i>
(<i>mau12_t</i>); <i>Ore</i> ; <i>Ore</i> ; <i>Ore</i>	<i>maI</i>	<i>D. mauritiana mau12</i>	<i>OreR</i>	<i>OreR</i>
(<i>aut_{tp}</i>); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>mel</i>	<i>D. melanogaster AutW132</i>	<i>AutW132</i>	<i>AutW132</i>
(<i>ore_{tp}</i>); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>mel</i>	<i>D. melanogaster OreR</i>	<i>AutW132</i>	<i>AutW132</i>
(<i>zim_{tp}</i>); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>zim</i>	<i>D. melanogaster Zim53</i>	<i>AutW132</i>	<i>AutW132</i>
(<i>siI_{tp}</i>); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siI</i>	<i>D. simulans siI Hawaii</i>	<i>AutW132</i>	<i>AutW132</i>
(<i>sm21_{tp}</i>); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>AutW132</i>	<i>AutW132</i>
(<i>sm22_{tp}</i>); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>AutW132</i>	<i>AutW132</i>
(<i>sm38_{tp}</i>); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>AutW132</i>	<i>AutW132</i>
(<i>simw</i> ⁵⁰¹ _{<i>tp</i>}); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans simw</i> ⁵⁰¹	<i>AutW132</i>	<i>AutW132</i>
(<i>mau12_{tp}</i>); <i>Aut</i> ; <i>Aut</i> ; <i>Aut</i>	<i>maI</i>	<i>D. mauritiana mau12</i>	<i>AutW132</i>	<i>AutW132</i>
(<i>aut_{tp}</i>); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>mel</i>	<i>D. melanogaster AutW132</i>	<i>P58</i> ²	<i>AutW132</i>
(<i>ore_{tp}</i>); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>mel</i>	<i>D. melanogaster OreR</i>	<i>P58</i>	<i>AutW132</i>
(<i>zim_{tp}</i>); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>zim</i>	<i>D. melanogaster Zim53</i>	<i>P58</i>	<i>AutW132</i>
(<i>siI_{tp}</i>); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siI</i>	<i>D. simulans siI Hawaii</i>	<i>P58</i>	<i>AutW132</i>
(<i>sm21_{tp}</i>); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>P58</i>	<i>AutW132</i>
(<i>sm22_{tp}</i>); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>P58</i>	<i>AutW132</i>
(<i>sm38_{tp}</i>); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>P58</i>	<i>AutW132</i>
(<i>simw</i> ⁵⁰¹ _{<i>tp</i>}); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans simw</i> ⁵⁰¹	<i>P58</i>	<i>AutW132</i>
(<i>mau12_{tp}</i>); <i>P58</i> ; <i>Aut</i> ; <i>Aut</i>	<i>maI</i>	<i>D. mauritiana mau12</i>	<i>P58</i>	<i>AutW132</i>
(<i>aut_{tp}</i>); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>mel</i>	<i>D. melanogaster AutW132</i>	<i>P89</i> ²	<i>AutW132</i>
(<i>ore_{tp}</i>); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>mel</i>	<i>D. melanogaster OreR</i>	<i>P89</i>	<i>AutW132</i>
(<i>zim_{tp}</i>); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>zim</i>	<i>D. melanogaster Zim53</i>	<i>P89</i>	<i>AutW132</i>
(<i>siI_{tp}</i>); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siI</i>	<i>D. simulans siI Hawaii</i>	<i>P89</i>	<i>AutW132</i>
(<i>sm21_{tp}</i>); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>P89</i>	<i>AutW132</i>
(<i>sm22_{tp}</i>); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>P89</i>	<i>AutW132</i>
(<i>sm38_{tp}</i>); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans C167.4</i>	<i>P89</i>	<i>AutW132</i>
(<i>simw</i> ⁵⁰¹ _{<i>tp</i>}); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>siII</i>	<i>D. simulans simw</i> ⁵⁰¹	<i>P89</i>	<i>AutW132</i>
(<i>mau12_{tp}</i>); <i>P89</i> ; <i>Aut</i> ; <i>Aut</i>	<i>maI</i>	<i>D. mauritiana mau12</i>	<i>P89</i>	<i>AutW132</i>

¹The mitochondrial genotype is given in parentheses, followed by the X, 2nd, and 3rd chromosome genotypes. Subscript “*t*” indicates that the stock has been cleared of *Wolbachia* using tetracycline and “*p*” indicates a P cytoplasm.

²Two *D. melanogaster* X chromosomes (*P58* and *P89*) from Davis, CA (S. Nuzhdin, USC) were also precisely substituted into the *AutW132* panel of mitochondrial–nuclear genotypes.

D. melanogaster inbred lines. While this design avoids retention of nuclear variants from the maternal parent that may accompany mitochondrial introgression during backcrossing (James and Ballard 2003; Dowling et al. 2008), it does restrict tests for inter-specific mitochondrial–nuclear interactions to *D. melanogaster* nuclear backgrounds.

Females of *D. simulans Hawaii* (mitochondrial haplotype *siI*) and *D. mauritiana mau12* (mitochondrial haplotype *maI*, which

differs from the *D. simulans siIII* haplotype by one nucleotide substitution) (Ballard 2000) were repeatedly backcrossed to *D. simulans C167.4* males. Subsequently, females carrying the *siI* or *maI* mtDNAs were crossed to *D. melanogaster In(1)AB,w* males. The *In(1)AB* chromosome carries a mutation that rescues F1 hybrid female viability and fertility (Hutter et al. 1990; Aruna et al. 2009). *D. simulans simw*⁵⁰¹ (mitochondrial haplotype *siII*) females were crossed directly to *In(1)AB,w* males, as F1 female viability and

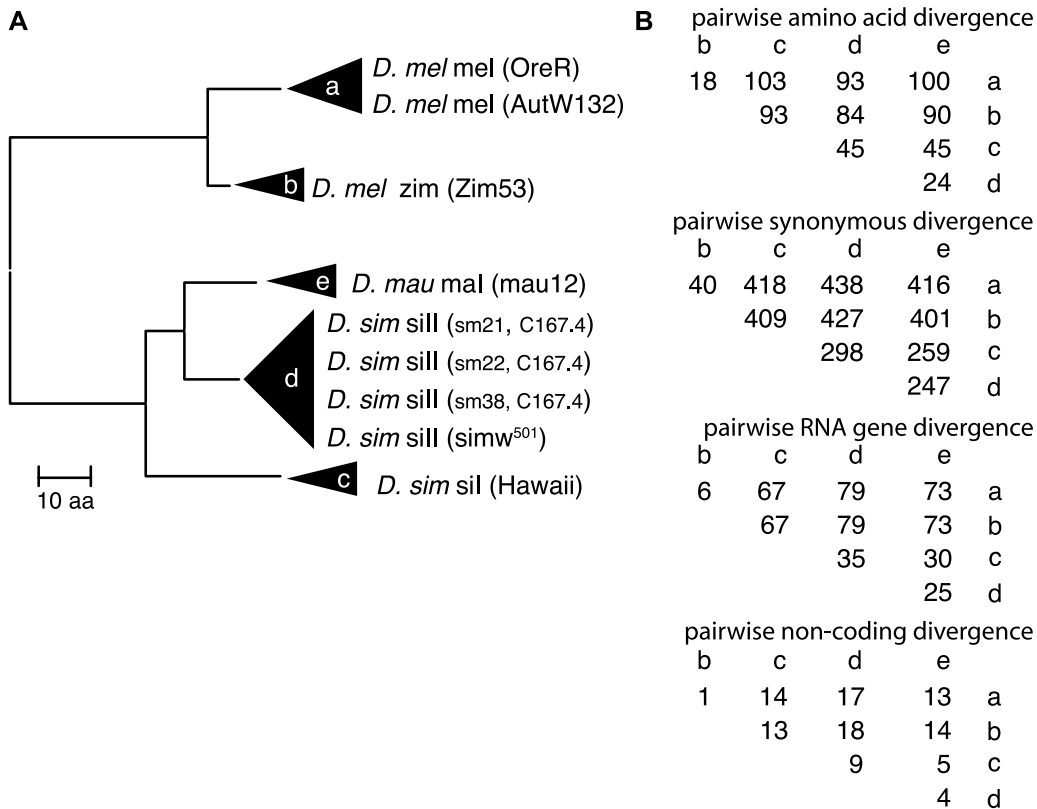


Figure 1. Phylogenetic relationship between the five mitochondrial haplotype groups used to study mitochondrial–nuclear epistasis for fitness. (A) Neighbor-joining tree using a concatenated proteome amino acid sequence (length = 3725 aa). Species names are followed by the mitochondrial haplotype and the line from which the mtDNA was isolated in parentheses. sm21, 22, and 38 are replicate mitochondrial introgressions from *D. simulans* strain C167.4. (B) Pairwise divergence between the five mitochondrial haplotypes. The numbers of pairwise differences were estimated from 3724 codons for amino acid and synonymous site divergence, 3606 nucleotide sites for tRNA and rRNA gene divergence, and 147 noncoding sites. The difference in substitutions between branches d and e when compared to outgroups a, b, or c is due to homoplastic substitution. Whole mtDNA sequence data from Ballard (2000) were aligned to the reference *D. melanogaster* and *D. simulans sill* genomes as in Montooth et al. (2009). NCBI accessions: a, NC_001709; b, AF200829; c, AF200835; d, AF200840; e, AF200831.

fertility are also rescued in this cross. In addition, three strains of *D. melanogaster* (*sm21*, *sm22*, *sm38*) that carry the *D. simulans* C167.4 mtDNA (mitochondrial haplotype *siII*) (Sawamura et al. 2000), and a strain from the *D. melanogaster* Zimbabwe race (*Zim53*) were also used as a source of mtDNA. We backcrossed females carrying target mtDNAs to *D. melanogaster Oregon-R* (*OreR*) males for three generations, replacing much of the nuclear genome with *D. melanogaster* material.

To eliminate any *D. simulans* or *D. mauritiana* genomic regions that might have been retained during the introgression, we precisely replaced the nuclear genomes of all hybrid strains with chromosomes from either a *D. melanogaster Oregon-R* (*Ore*) strain that was inbred for two generations via full-sib mating or from an isofemale line of *D. melanogaster* from Austria, *AutW132* (*Aut*), obtained from Christian Schlötterer (Institut für Populations-genetik, Veterinärmedizinische Universität Wien, Vienna, Austria). To simultaneously replace the autosomes we used a strain of *D. melanogaster* carrying the

second-chromosome balancer *CyO* and the third-chromosome balancer *TM6B,Tb,Dr*. To replace the X chromosome we used strains that carried the *FM7c,B,sn* balancer chromosome in an otherwise *Ore* or *Aut* genetic background. The mtDNA haplotypes of the constructed genotypes were confirmed by direct sequencing of mtDNA using the primer pairs, 3593F 5'-gaacagtcccgccttaggag/4528R 5'-gcagttaatcggacagctaatgtccc and 5314F 5'-gctccattactattcgggactc/6195R 5'-cattaacagtgtatgcctc. Before the fitness assays, mitochondrial haplotypes were re-confirmed using PCR-RFLP analysis with the 3593/4528 primer pair and the *Alu* I, *Dra* I, and *Rsa* I restriction enzymes.

CONTROLLING THE CYTOPLASM

The intracellular endosymbiont *Wolbachia* is cotransmitted with the mtDNA through the maternal cytoplasm of many *D. melanogaster* and *D. simulans* strains and can have myriad phenotypic effects, including cytoplasmic incompatibility (Merçot and Charlat 2004; Clark et al. 2005; Ikeya et al. 2009). To avoid

confounding mtDNA and *Wolbachia* effects, we cured all cytoplasms of *Wolbachia* infection prior to the replacement of the nuclear chromosomes by rearing larvae on instant Carolina media mixed with a 0.03% tetracycline solution for two generations. Successful clearing of *Wolbachia* was confirmed by failure to amplify a PCR product using *Wolbachia*-specific primers (F 5'-tggtccaataagtgatgaagaaac; R 5'-aaaattaacgctactcca). All PCRs were run alongside a positive *Wolbachia*-infected control.

The ability to suppress mobilization of the *P* transposable element is also maternally inherited (Brennecke et al. 2008). When *P* element naïve (M cytotype) females mate with *P* element containing (P cytotype) males, mobilization of *P* elements can occur in offspring genomes (Kidwell et al. 1977; Brennecke et al. 2008). Failure to PCR amplify *P* element sequence (F 5'-taaaaggaggcactcaacg; R 5'-ctcagctgctctaaacg) indicated that the balancer stocks to be used for replacing the nuclear genome and the *Oregon-R* stock that provided the nuclear genome were M cytotype. However, the wild lines that provided the *Aut* nuclear genome and the X chromosomes were P cytotype. To prevent mobilization of *P* elements during the substitution of *Aut* nuclear background chromosomes, the balancer strains and all mitochondrial–nuclear hybrid lines to be used for *Aut* chromosomal substitution were backcrossed to *AutW132* males for multiple generations to establish a P cytotype.

TESTING FOR MITOCHONDRIAL–NUCLEAR INTERACTIONS AFFECTING FITNESS

We used a chromosome segregation assay modified from Rand et al. (2001) to quantify fitness effects of mitochondrial–nuclear interactions (Fig. 2). In this assay, individuals competed in the same vial with siblings carrying a visibly marked X chromosome

(*FM6*) that confers a Bar eye phenotype. Relative fitness was measured as the egg-to-adult viability of wild-type individuals relative to their *FM6*-bearing siblings across the 35 mitochondrial–nuclear genotypes (Table 1). Differences between *nuclear genotypes* in the relative competitive viability of wild-type flies compared to their *FM6* siblings result from viability effects of the X chromosome directly competing with *FM6*, and genetic interactions between the wild-type or *FM6* X chromosomes and the autosomes. Differences between *mitochondrial–nuclear genotypes* in relative competitive viability result from interactions between the mitochondrial genome and the wild-type or *FM6* X chromosomes, or more complex interactions between the X chromosome, the autosomes, and the mtDNA. The advantages of this assay are that competing individuals share the same common rearing environment, and genotypes are easily inferred from the Bar phenotype. In males, differences in relative competitive viability arise from the hemizygous effects of X-linked variants in combination with the mitochondrial–nuclear genotype. In females, viability effects of wild-type X-linked variants that are completely dominant to *FM6* when combined with particular mitochondrial–nuclear genomes will not be detected in this assay.

We generated strains carrying an *FM6* chromosome in both the *Ore* and *Aut* nuclear backgrounds. Females of each mitochondrial–nuclear genotype were mated to males carrying the *FM6* chromosome in the same autosomal background. For the segregation assay, the resulting heterozygous females (*Aut/FM6*; *Aut*; *Aut* or *Ore/FM6*; *Ore*; *Ore*) were crossed to *Aut* or *Ore* males, respectively (Fig. 2A). All offspring from these crosses inherit the mtDNA from the initial female. Female offspring were scored as either wild-type homozygotes or *Bar* heterozygotes, and male offspring were either wild-type or *Bar* hemizygotes.

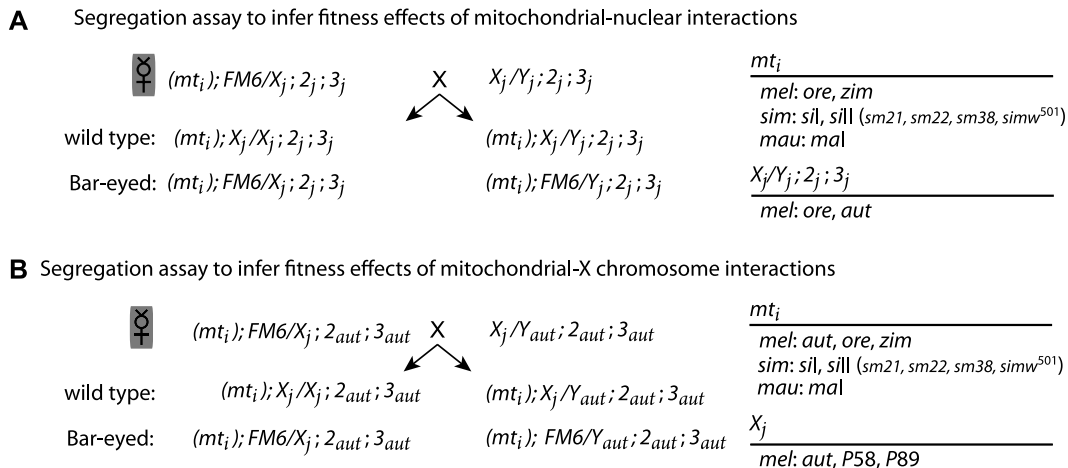


Figure 2. The segregation assay used to measure relative fitness. (A) The crossing design used to test for fitness effects of mitochondrial–nuclear interactions. (B) The crossing design used to test for fitness effects of mitochondrial–X chromosome interactions. *FM6* is a non-recombining X chromosome that results in a dominant, visible Bar-eyed phenotype. The mitochondrial genotype is given in parentheses followed by the X/Y, second and third chromosome genotypes.

Relative competitive viability was calculated for each sex in each vial as the number of wild-type offspring divided by one plus the total progeny of that sex emerging from that vial (Haldane 1956), and is the measure of relative fitness used throughout.

ISOLATING MITOCHONDRIAL–X CHROMOSOME INTERACTIONS AFFECTING FITNESS

In order to test specifically for fitness effects of interactions between the X chromosome and the mtDNA, we combined the nine mtDNAs with three X chromosomes (*Aut*, *P58*, and *P89*) in the *Aut* nuclear background (Table 1). Differences between mitochondrial–X chromosome genotypes in these strains result only from direct interactions between the mitochondrial genome and the wild-type or *FM6* X chromosomes, as the autosomal genetic background is held constant. The *P58* and *P89* X chromosomes were derived from a *D. melanogaster* population in Davis, CA collected by Sergey Nuzhdin (University of Southern California). The *P58* and *P89* X chromosomes were substituted into the *Aut* autosomal background using balancer chromosomes. For the segregation assay, females heterozygous for *FM6* and either the *P58*, *P89*, or *Aut* X chromosome in an *Aut* autosomal background were mated to males carrying the corresponding *P58*, *P89*, or *Aut* X chromosome in an *Aut* autosomal background (Fig. 2B). All offspring from this cross inherit the mtDNA from the initial female. Female offspring were scored as either wild-type homozygotes or *Bar* heterozygotes for the *Aut*, *P58*, or *P89* X chromosomes. Male offspring were either wild-type *Aut*, *P58*, or *P89* hemizygotes or *Bar* hemizygotes. Relative fitness for each X-mitochondrial genotype was calculated as described above.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Relative fitness of each genotype was measured in six replicate vials in which two males and two females were allowed to mate continuously and lay eggs for 3 days. Parents were transferred to a second vial to mate and lay a second brood of eggs for an additional 3 days. Wild-type and *Bar*-eyed progeny emerging from these vials were counted every other day for 10 days. The entire experiment was conducted in two complete and independent blocks. The full design measured fitness for 35 mitochondrial–nuclear genotypes tested in six replicate assays for each of two replicate broods in two replicate blocks, with 118,932 individuals scored.

On average, 145 offspring emerged from a single vial. We eliminated observations where fewer than 10 flies of a given sex emerged from a vial. This removed less than 5% of the data, leaving 797 observations of male fitness and 807 observations of female fitness. The effects of mtDNA, nuclear genotype, and the interaction between mtDNA and nuclear genotype were tested using mixed model analysis of variance (ANOVA) for each sex

separately, due to the hemizygous versus heterozygous effects in the two sexes (see “Methods” above). The complete ANOVA model was $y_{ijklm} = \mu + M_j + N_k + MN_{jk} + B_l + b_i + Px_{ijklm} + \epsilon_{ijklm}$, where y_{ijklm} is male or female relative fitness estimated from a single vial, M_j is the fixed effect of mtDNA, N_k is the fixed effect of either the nuclear or X-chromosome genotype, MN_{jk} is the interaction between these factors, B_l is the fixed effect of brood, and b_i is the random effect of block. The total number of offspring emerging from each vial (“vial productivity”) was used as a covariate in the model (Px_{ijklm}) to control for any larval density effects on relative fitness. Separate slopes for the regression of fitness on vial productivity were fit for each nuclear or X-chromosome genotype. A significant mtDNA \times nuclear interaction effect in the model was used to infer the presence of mitochondrial–nuclear epistasis for fitness. The biological interpretation of this interaction is that the fitness effect of substituting a particular mtDNA variant is conditional on the nuclear genetic background. Linear mixed models were performed using the nlme library in the software package *R* version 2.6.1 and verified using the mixed procedure in SAS version 9.1.3 (SAS Institute Inc., Cary, NC).

We employed two approaches to quantify the contributions of fixed interspecific mitochondrial differences and segregating intraspecific mitochondrial variation to mitochondrial–nuclear epistasis for fitness. Due to its similarity with the *D. simulans* *siIII* haplotype (Ballard 2000), we grouped the *D. mauritiana* *maI* haplotype with *D. simulans* for this analysis. First, we modified the linear mixed model above to test for a fixed effect of species mtDNA (*D. melanogaster* or *D. simulans*), within which we nested the random effect of within-species mitochondrial genotype. The absence of a species mtDNA effect in this model would indicate that, on average, there is no differential effect on fitness caused by substituting a *D. simulans* versus a *D. melanogaster* mtDNA into a *D. melanogaster* nuclear background. Additionally, we used the varcomp procedure in SAS to partition the variance in fitness into the between-species mtDNA effect and the within-species mitochondrial genotype effect using restricted maximum likelihood.

Three of the *D. simulans* *siII* mtDNAs (*sm21*, *sm22*, *sm38*) originated from replicate mtDNA introgressions from a single *D. simulans* strain. While these mtDNAs could potentially have diverged in sequence after the initial introgression, they are clearly not independent. When only lines containing these mtDNAs were analyzed, there was no significant difference in relative fitness between the three replicate *siII* mtDNAs (females: $F = 1.08$, $P = 0.34$, males: $F = 1.74$, $P = 0.18$) and no interaction with the nuclear genome (females: $F = 0.58$, $P = 0.56$, males: $F = 1.09$, $P = 0.35$). In the X chromosome experiment, there was no main effect of the three *siII* mtDNAs on relative fitness in females ($F = 1.01$, $P = 0.37$), only a marginally significant effect in males

($F = 3.28$, $P = 0.04$), and no significant interaction with the X chromosome (females: $F = 0.26$, $P = 0.90$, males: $F = 0.81$, $P = 0.52$). Data for these three *siII* mtDNAs were therefore pooled in our analyses.

Results

MITOCHONDRIAL-NUCLEAR FITNESS INTERACTIONS

We assayed 12 genotypes that combined six mtDNAs (*ore*, *zim*, *siI*, *siII*, *simw⁵⁰¹*, *mau12*) with two *D. melanogaster* nuclear backgrounds (*Ore*, *Aut*) to determine whether interactions between

the mtDNA and the nuclear genome generate epistasis for fitness (Table 1). We used an X-chromosome segregation assay that competes individuals of each mitochondrial–nuclear genotype against competitors carrying a visibly marked *FM6* X chromosome emerging from the same cross in replicate vials (see Methods, Fig. 2A). The *FM6* chromosome has low fitness, and the fitness of wild-type individuals relative to *FM6* competitors was, on average, greater than 0.5. Male relative fitness was greater than female relative fitness for all mitochondrial–nuclear genotypes (Fig. 3), consistent with the presence of recessive deleterious mutations on the *FM6* X chromosome that are masked in female heterozygous *FM6* competitors.

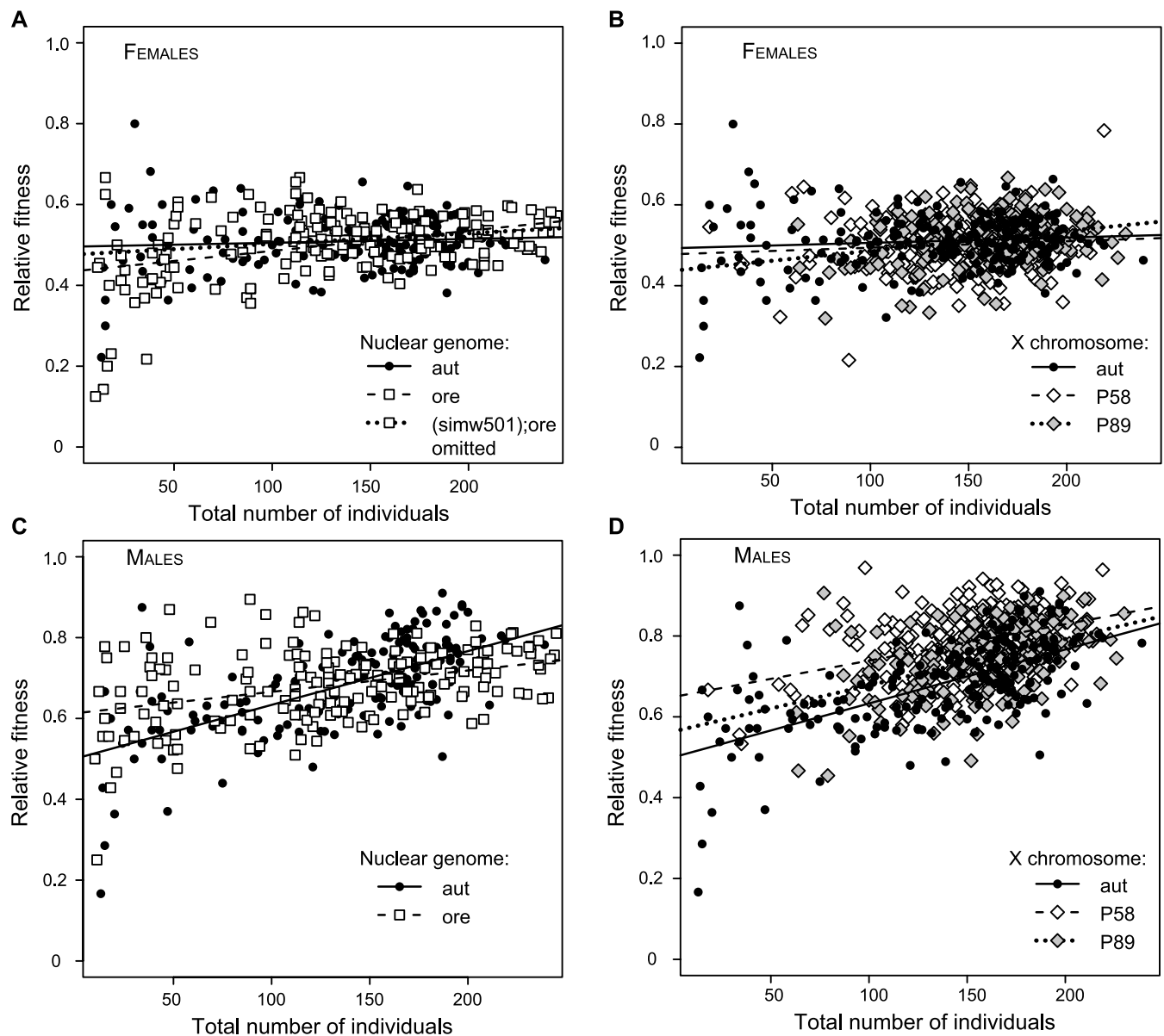


Figure 3. Positive relationship between relative fitness estimates and the total number of individuals emerging from a vial for females and males in the mitochondrial–nuclear (A, C) and mitochondrial–X chromosome (B, D) experiments. Regression lines reveal a tendency for the slope to differ between autosomes and to differ between X chromosomes.

Table 2. Mixed model ANOVA for effects of the mtDNA, nuclear genotype, and the mitochondrial–nuclear interaction on relative fitness.

Dependent variable	Fixed effect	num df	den df	F-value	P-value	Random effect	σ^2
Female fitness	mtDNA	5	346	6.727	<0.0001	Block	8.17e-7
	Nuclear	1	346	0.779	0.3781	Residual	4.82e-3
	mtDNA × nuclear	5	346	4.242	0.0009		
	Brood	1	346	0.002	0.9646		
	Vial productivity × nuclear ¹	2	346	5.138	0.0063		
Male fitness	mtDNA	5	346	0.324	0.8982	Block	5.02e-4
	Nuclear	1	346	0.028	0.8666	Residual	6.45e-3
	mtDNA × nuclear	5	346	1.559	0.1710		
	Brood	1	346	17.008	<0.0001		
	Vial productivity × nuclear	2	346	39.995	<0.0001		

¹The number of flies emerging from each vial was used as a linear covariate with different slopes estimated for the two nuclear genetic backgrounds.

Relative fitness was weakly positively correlated with the total number of individuals emerging from a vial (Fig. 3A, C), suggesting that *FM6* flies are less competitive in crowded rearing conditions. This relationship was stronger in males than in females (Table 2), and the slope of the relationship differed between nuclear backgrounds, particularly in males (Fig. 3C). We therefore included the number of individuals emerging from a vial (“vial productivity”) as a covariate in the mixed model ANOVA, allowing different slopes for the *Ore* and *Aut* nuclear backgrounds. Including vial productivity greatly increased the fit of the model for male relative fitness ($LR = 56.78$, $P < 0.0001$), but only mildly for female relative fitness ($LR = 4.73$, $P = 0.03$).

For male relative fitness, there was no main effect of mtDNA or interaction between mitochondrial and nuclear genotype (Fig. 4, Table 2). However, for female relative fitness, there was a significant interaction effect between mitochondrial and nuclear genotypes for relative fitness (Fig. 4, Table 2). Several mitochondrial–nuclear interactions contribute to female relative fitness. For example, while the *D. melanogaster zim* mtDNA has greater relative fitness than the *D. melanogaster ore* mtDNA in an *Ore* nuclear background, the fitness effects of these mtDNAs are reversed in an *Aut* nuclear background.

The strongest interaction results from the reduced relative fitness of females that have a *D. simulans simw⁵⁰¹* mtDNA paired with the *D. melanogaster Ore* nuclear genome, but not with the *D. melanogaster Aut* nuclear genome, providing strong evidence of mitochondrial–nuclear epistasis. The (*simw⁵⁰¹*);*Ore* genotype also has lower mean vial productivity than other genotypes, although not outside the range observed for other genotypes (Fig. 5A). To confirm that (*simw⁵⁰¹*);*Ore* individuals have low relative fitness, even for their low level of productivity, we analyzed only vials producing fewer than 62 individuals, which was

the maximum productivity of (*simw⁵⁰¹*);*Ore* parents. Even with the reduced number of observations in this range, we found that (*simw⁵⁰¹*);*Ore* females have significantly lower relative fitness than all other vials producing 62 or fewer offspring (Fig. 5B).

MITOCHONDRIAL–X CHROMOSOME FITNESS INTERACTIONS

To isolate two-way fitness interactions between mtDNA and X chromosomes, we combined seven mtDNAs (*aut*, *ore*, *zim*, *sil*, *siII*, *simw⁵⁰¹*, *mau12*) with three *D. melanogaster* X chromosomes (*Aut*, *P58*, *P89*) in an *Aut* autosomal background (Table 1). As in the segregation assays above, there was a significant relationship between vial productivity and relative fitness that was stronger in males than in females and was dependent on the X-chromosome genotype (Fig. 3B, D, Table 3). We treated vial productivity as a covariate in the analysis with separate slopes for the different X chromosomes. Including vial productivity increased the fit of the model for both male ($LR = 156.9$, $P < 0.0001$) and female viability ($LR = 11.4$, $P = 0.0007$).

In contrast to the mitochondrial–nuclear segregation assay, male, but not female, relative fitness was significantly affected by interactions between the X chromosome and the mitochondrial genome (Fig. 6, Table 3). The epistatic interactions are particularly evident in the changing rank order of male relative fitness values between X-chromosome genotypes among the *D. melanogaster* mtDNAs (Fig. 6). Additionally, the fitness effects of *D. simulans* and *D. mauritiana* mtDNAs were dependent on the X chromosome. For example, there was no difference in relative fitness between males carrying the *siII* and *simw⁵⁰¹* mtDNAs in an *Aut* X-chromosomal background, but the *siII* mtDNA conferred higher relative fitness than the *simw⁵⁰¹* mtDNA in both the *P58* and *P89* X-chromosomal backgrounds (Fig. 6).

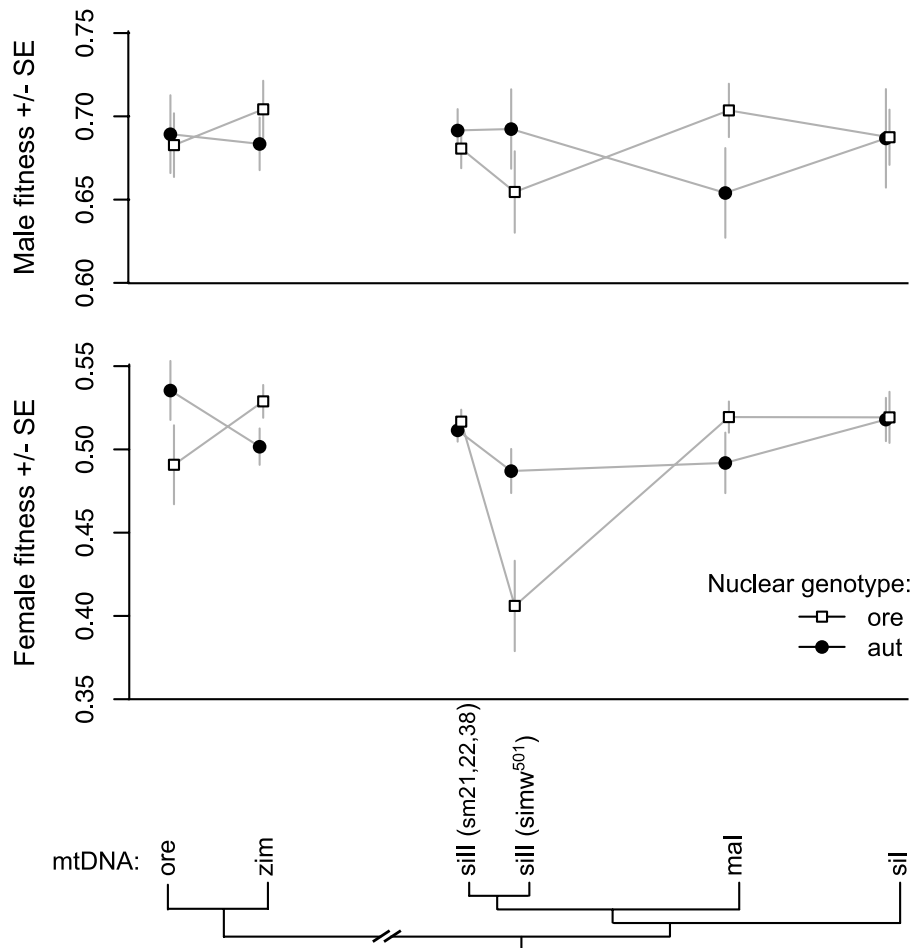


Figure 4. Fitness values for males and females from 12 mitochondrial–nuclear genotypes relative to visibly marked sibling competitors reveal significant mitochondrial–nuclear epistasis for fitness in females. The mtDNAs are arranged along the x-axis by phylogenetic distance, highlighting the lack of relationship between mitochondrial molecular divergence and the fitness effect of mitochondrial substitution.

WITHIN- VERSUS BETWEEN-SPECIES EFFECTS ON FITNESS

D. simulans and *D. mauritiana* mtDNAs have effects on relative fitness similar to those caused by *D. melanogaster* mtDNAs when combined with *D. melanogaster* nuclear genomes (Figs. 4 and 6). To quantify this observation, we compared the variance in relative fitness explained by variation among mitochondrial genotypes within *D. melanogaster* and within the *D. simulans* species complex to the variance in relative fitness explained by differences between these species mtDNA lineages.

Fitness consequences of mtDNA interactions with the *Ore* and *Aut* nuclear genomes were significant only in females (Table 2). Among females, 5.7% of the variance in relative fitness was explained by variation among mitochondrial genotypes within species, and 9.2% of the variance in relative fitness was attributed to within species mitochondrial genotypes interacting with the two nuclear genomes. However, 0% of the variance in relative fitness could be attributed to between-species differences in

mtDNA. Mitochondrial–X chromosome interaction effects on relative fitness were significant only in males (Table 3), with 21.3% of the variance explained by variation among *D. melanogaster* X chromosomes. This X-linked variation interacted with within-species mitochondrial genotypes to explain 2.23% of the variance in relative fitness. However, none of the variance in male relative fitness could be explained by mtDNA differences between *D. melanogaster* and *D. simulans*.

Mixed models incorporating a random effect of mitochondrial genotype nested within a fixed effect of species mtDNA revealed no effect of species mtDNA on female fitness in the mitochondrial–nuclear experiment ($F = 1.24$, $P = 0.47$) and no effect of species mtDNA on male fitness in the mitochondrial–X chromosome experiment ($F = 0.01$, $P = 0.94$). On average, substituting a *D. melanogaster* mtDNA into a *D. melanogaster* nuclear background has as great of an effect on relative fitness as substituting a *D. simulans* mtDNA into a *D. melanogaster* nuclear background. Furthermore, there is no tendency for *D. simulans*

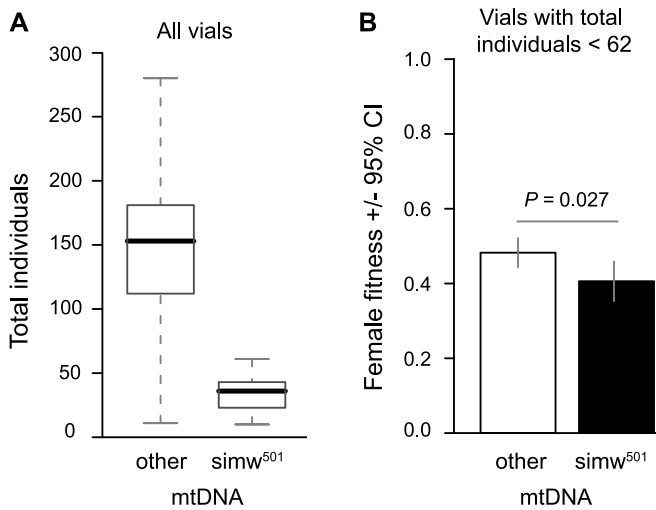


Figure 5. The *(simw⁵⁰¹);Ore* mitochondrial–nuclear genotype confers low vial productivity, but also has low relative fitness given its productivity. (A) Boxplots for *(simw⁵⁰¹);Ore* compared to all other genotypes in the mitochondrial–nuclear interaction dataset demonstrate the low median number of total individuals emerging from vials of this genotype (bold horizontal bars), but also show that this level of productivity is within the range of productivity (dotted lines) of other genotypes. (B) Relative fitness of *(simw⁵⁰¹);Ore* females is significantly less than that observed for all other genotypes when only vials producing less than 62 individuals were analyzed.

or *D. mauritiana* mtDNA introgression to consistently increase or decrease fitness relative to *D. melanogaster* mtDNAs. These results suggest that mitochondrial–nuclear incompatibilities affecting egg-to-adult competitive viability do not scale with molecular divergence and have not accumulated between these species.

Table 3. Mixed model ANOVA for effects of the mtDNA, X-chromosome genotype, and the mitochondrial–X chromosome interaction on relative fitness.

Dependent variable	Fixed effect	num df	den df	F-value	P-value	Random effect	σ^2
Female fitness	mtDNA	6	613	1.11	0.3574	Block	4.9e-13
	X chromosome	2	613	2.73	0.0663	Residual	3.80e-3
	mtDNA \times X chrom	12	613	1.34	0.1922		
	Brood	1	613	5.44	0.0200		
	Vial productivity \times X chrom ¹	3	613	5.59	0.0009		
Male fitness	mtDNA	6	613	3.383	0.0027	Block	7.47e-5
	X chromosome	2	613	98.191	<0.0001	Residual	5.12e-3
	mtDNA \times X chrom	12	613	2.534	0.0029		
	Brood	1	613	88.847	<0.0001		
	Vial productivity \times X chrom	3	613	62.557	<0.0001		

¹The number of flies emerging from each vial was used as a linear covariate with different slopes estimated for the three X-chromosome genetic backgrounds.

MALE AND FEMALE MITOCHONDRIAL–NUCLEAR EPISTASIS

The segregation assay used here has greater power to detect relative fitness effects in males than in females, as males experience the full effects of all interactions between the mtDNA and the wild-type or *FM6* X chromosomes regardless of dominance. For example, a completely recessive synthetic lethal interaction between a *D. simulans* mtDNA and the *FM6* chromosome would appear to be a male-specific mitochondrial–nuclear interaction in this assay. Despite this bias, we detected significant effects of mitochondrial–nuclear interactions on female fitness but not in males (Table 2). Although these interactions are stronger in females, Figure 4 reveals a consistent pattern of mitochondrial–nuclear genotype effects on male and female fitness. These effects are magnified in females, particularly the severe effect of the *(simw⁵⁰¹);Ore* genotype. Comparing male and female fitness values reveals little sexual antagonism across mitochondrial–nuclear genotypes (Fig. 7B). Mitochondrial–nuclear genotypic combinations that confer low fitness in females also confer low fitness in males, generating a significant correlation between male and female fitness values across the 12 mitochondrial–nuclear genotypes (Pearson's $r = 0.71$, $P = 0.009$).

In contrast, the X-mitochondrial segregation assay indicated that some mitochondrial–X chromosome genotypes have different fitness effects in males and females. For example, the *Aut* mtDNA confers the lowest relative fitness in males but the highest relative fitness in females (Fig. 8B). There is more male than female fitness variation among mitochondrial–X chromosome genotypes, and there is little congruence between the male and female fitness effects of any given genotype (Fig. 8C). In contrast to the mitochondrial–nuclear dataset, there is no correlation among male and female mean fitness values across mitochondrial–X

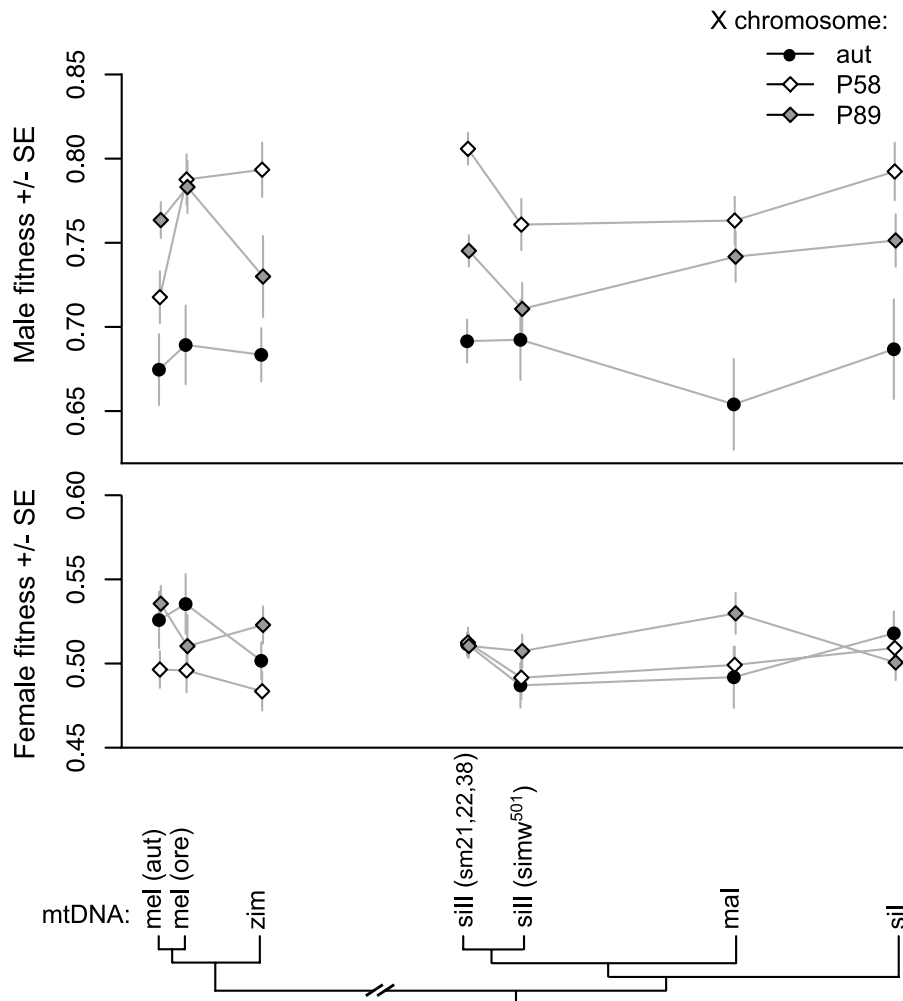


Figure 6. Fitness values for males and females from 21 mitochondrial-X chromosome genotypes relative to visibly marked sibling competitors reveal significant mitochondrial-X chromosome epistasis for fitness in males. The mtDNAs are arranged along the x-axis by phylogenetic distance, highlighting the lack of relationship between mitochondrial molecular divergence and the fitness effect of mitochondrial substitution.

chromosome genotypes (Pearson's $r = -0.12$, $P = 0.60$). However, this absence of a correlation may be driven by the strong sex-specific main effects of the X chromosomes (Fig. 8A).

Discussion

MITOCHONDRIAL-NUCLEAR INTERACTIONS FOR FITNESS RESULT FROM SEGREGATING VARIATION RATHER THAN FIXED DIFFERENCES BETWEEN SPECIES

Our results provide evidence for epistatic fitness interactions between mitochondrial polymorphisms segregating within *D. melanogaster* subgroup species and nuclear polymorphisms segregating in *D. melanogaster*. The strongest interactions in males were between *D. melanogaster* mtDNAs and *D. melanogaster* X chromosomes (Fig. 6), two of which were sampled from the same population. In females there was a strong

epistatic interaction between two *D. simulans* *siII* mtDNAs and the two *D. melanogaster* nuclear backgrounds (Fig. 4). Unique among the *D. simulans* species complex mtDNAs, the *simw*⁵⁰¹ mtDNA strongly impacts fitness when combined with the *D. melanogaster* *Ore* nuclear genetic background. The *D. simulans* *simw*⁵⁰¹ mtDNA differs from the other *D. simulans* *siII* mtDNAs by only three nucleotide substitutions (Meiklejohn, Montooth, and Rand, unpubl. result). Thus, this case of intergenomic epistasis does not arise from the more than 500 substitutions that have fixed between the mtDNAs of *D. melanogaster* and the *D. simulans* species complex. Rather, a small number of polymorphic sites that distinguish the *D. simulans* *simw*⁵⁰¹ mtDNA from other *D. simulans* *siII* mtDNAs interact epistatically with nuclear alleles that are segregating within *D. melanogaster* as potentially neutral, slightly deleterious or population-specific polymorphisms.

In both the mitochondrial-nuclear and mitochondrial-X chromosome experiments we observed no consistent increase

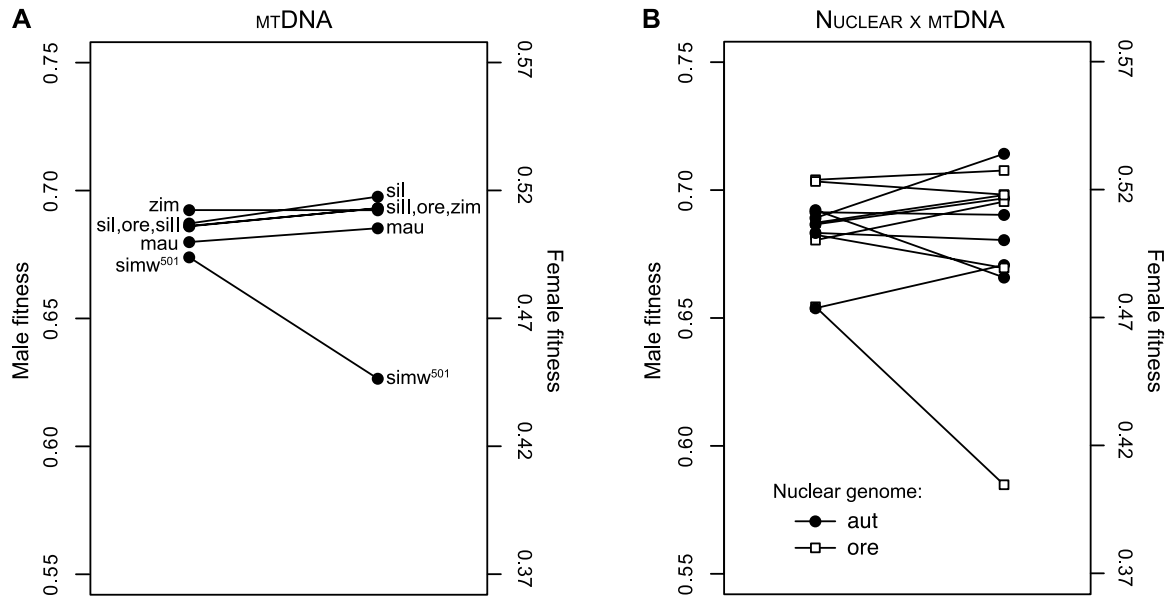


Figure 7. Comparisons of male and female relative fitness effects of mitochondrial (A) and mitochondrial–nuclear (B) genotypes. The y-axes are shifted relative to one another, but remain on the same scale. The nuclear genome has no main effect on relative fitness in either sex and is not shown.

or decrease in relative fitness associated with substituting the *D. melanogaster* versus the *D. simulans* species complex mtDNAs (Figs. 4 and 6). As a result, 0% of the variance observed in relative fitness can be attributed to an effect of the species from which the mtDNA was derived. These results suggest that the fixed differences between the mtDNAs of these species are largely neutral and fully compatible with the *D. melanogaster* nuclear

genome, although we cannot rule out strong effects of individual mtDNA substitutions that were subsequently compensated by other mitochondrial substitutions. This result is inconsistent with the accumulation of mitochondrial–nuclear incompatibilities that would arise from a process of divergent coevolution between these mitochondrial and nuclear lineages. However, we tested for mitochondrial–nuclear effects on egg-to-adult competitive

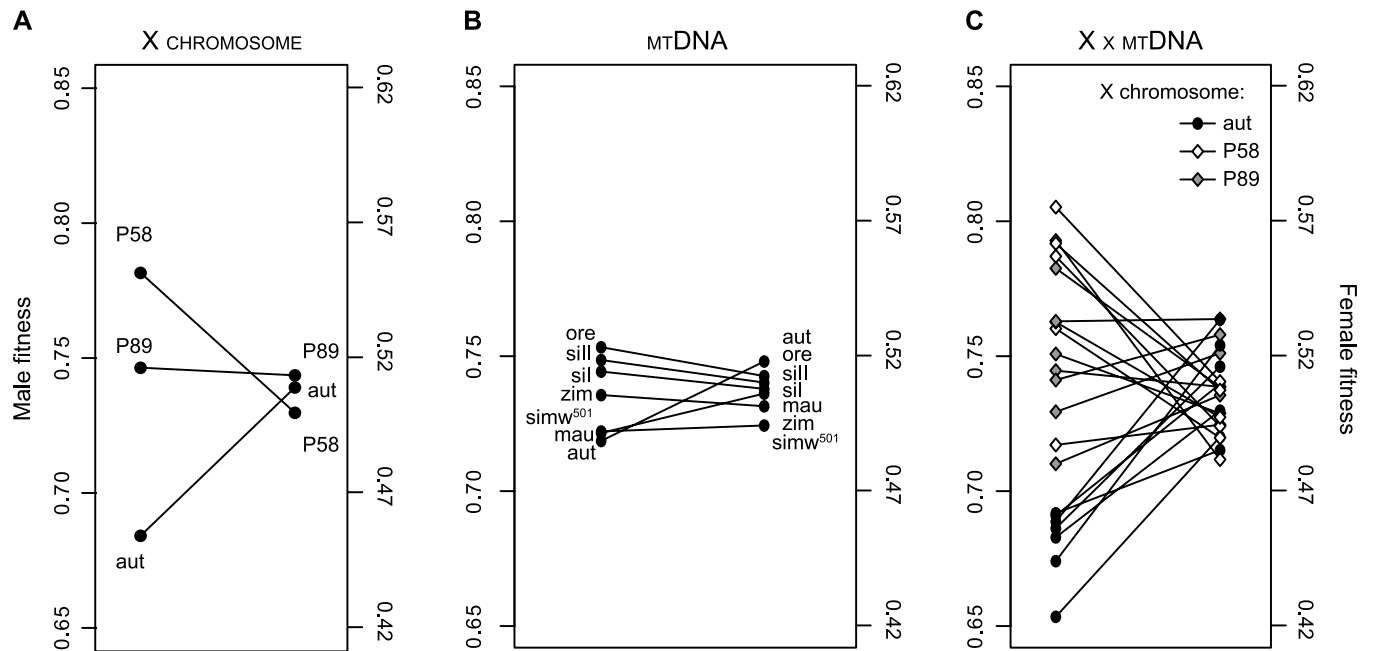


Figure 8. Comparisons of male and female relative fitness effects of X chromosome (A), mitochondrial (B), and mitochondrial–X chromosome (C) genotypes. The y-axes are shifted relative to one another, but remain on the same scale.

viability in a single environment. It remains possible that components of adult fitness are more sensitive to mitochondrial–nuclear incompatibilities and that these incompatibilities experience environment- or sex-specific effects (e.g., Chippindale et al. 2001).

D. melanogaster is strongly reproductively isolated from *D. simulans*, *D. mauritiana*, and *D. sechellia*, as interspecific crosses involving *D. melanogaster* normally produce sterile and/or inviable hybrid progeny (Sturtevant 1920; Lachaise et al. 1986). Despite this, our experiments show that the approximately 100 nonsynonymous and 400 synonymous fixed differences between *D. melanogaster* and its sibling species mtDNAs do not cause lethal or sterile incompatibilities in combination with the *D. melanogaster* nuclear genome, consistent with prior experimental introgressions of *D. simulans* mitochondria into *D. melanogaster* (Sawamura et al. 2000). Although we did not introgress mtDNAs in the reciprocal direction, the fact that viable hybrids of both sexes and fertile female hybrids can be recovered from *D. melanogaster* mothers and sibling species fathers (Hutter and Ashburner 1987; Barbash and Ashburner 2003) indicates that the *D. melanogaster* mtDNA is also largely compatible with the *D. simulans* nuclear genome. Mitochondrial–nuclear compatibility may be common among related *Drosophilids*, as mitochondrial introgression has occurred in nature between closely related species of *Drosophila* (Powell 1983; Machado and Hey 2003; Bachtrog et al. 2006).

Expression of X-linked variation and its interaction with the mtDNA was more pronounced in males than in females (Fig. 8). However, due to the design of the segregation assay, any completely dominant alleles that differed between the wild-type X chromosomes would be detected in males but not in females. The male-specific effects of the X chromosome observed here should therefore be interpreted cautiously. In contrast, fitness effects in the mitochondrial–nuclear experiment were significant only in females. In particular, the (*simw*⁵⁰¹);*Ore* genotype has a pronounced effect on female relative fitness, but little effect in males. This genotype exhibits a number of other deleterious phenotypes, including reduced female fecundity, and increased development time and compromised bristle development in both sexes (Meiklejohn, Montooth, and Rand, unpubl. results). These data suggest that nuclear and mitochondrial genomes in *Drosophila* can segregate variants, which alone may have little effect on fitness, but when combined result in strong epistatic effects that may be expressed differently in males and females.

Natural selection is not expected to maintain joint mitochondrial–autosomal polymorphism in the absence of frequency-dependent selection or differential selection in the sexes (Clark 1984; Gregorius and Ross 1984). However, the co-transmission of the mtDNA with the X chromosomes through females can maintain joint mitochondrial–X chromosome poly-

morphisms, particularly when these polymorphisms have sex-specific effects (Rand et al. 2001; Dowling et al. 2008). Thus, while the interactions we observed might result from segregating deleterious variants that have not yet been removed by purifying selection, it is also possible that they reflect variation maintained by sex-dependent fitness effects of mitochondrial–X chromosome interactions. The observed phenotypic effects of *D. simulans* mitochondrial haplotypes (James and Ballard 2003; Ballard et al. 2007), and the fact that interactions between nuclear and mitochondrial genomes are stronger between populations than within populations of *D. melanogaster* (Clark and Lyckegaard 1988; Rand et al. 2001; Dowling et al. 2007b) raises the possibility that local adaptation and population structure may also maintain mitochondrial variation within these species.

POPULATION GENETICS OF MITOCHONDRIAL–NUCLEAR INTERACTIONS

Efficient purifying selection prevents the fixation of deleterious mutations, but, until removed, mildly deleterious polymorphisms will segregate within populations. *D. melanogaster* and *D. simulans* populations harbor an excess of nonsynonymous mitochondrial polymorphism and these polymorphisms segregate at lower frequencies relative to the neutral expectation, consistent with a slightly deleterious model of molecular evolution (Ballard and Kreitman 1994; Rand and Kann 1996, 1998). The frequency and fate of such variants depends on their fitness effects and the effective population size (N_e). Complete linkage and uniparental inheritance reduces mtDNA N_e relative to nuclear loci and reduces the efficacy of natural selection, making the mtDNA particularly prone to the fixation of deleterious polymorphisms (Gabriel et al. 1993; Lynch 1996; Neiman and Taylor 2009).

Compensatory evolution at nuclear-encoded loci that recovers fitness loss due to high-frequency or fixed deleterious mtDNA variants has the potential to result in the accumulation of mitochondrial–nuclear incompatibilities between diverging lineages (Rand et al. 2004; Dowling et al. 2008). We find no evidence that these incompatibilities have accumulated within the *D. melanogaster* species group, consistent with strong purifying selection acting on the *Drosophila* mitochondrial genome (Ballard 2000; Montooth et al. 2009). In the *D. melanogaster* species subgroup, it appears that deleterious mitochondrial variants are efficiently removed from populations before compensatory mutations that might resolve any deleterious mitochondrial effects are fixed in the nuclear genome.

The compatibility between the mitochondrial and nuclear genomes of the *D. melanogaster* species group contrasts with the results from three opisthokont experimental systems. First, the inability of a *S. bayanus* nuclear-encoded protein to translate the *S. cerevisiae* mitochondrial *OLII* mRNA is responsible for a sporulation defect in hybrids (Lee et al. 2008). This

incompatibility appears to have been driven by rapid divergence between the *S. cerevisiae* and *S. bayanus* mitochondrial *OLH1* 5' UTR (Lee et al. 2008). Such rapid evolution is characteristic of yeast mitochondrial intergenic sequences (Groth et al. 2000). Second, mitochondrial–nuclear incompatibilities have been identified among the parasitic wasps of the *Nasonia* species complex (Breeuwer and Werren 1995; Ellison et al. 2008; Niehuis et al. 2008). *Nasonia* has a mitochondrial substitution rate that is 30 times higher than the nuclear substitution rate (Oliveira et al. 2008). This substitution rate is hypothesized to have driven evolution of the nuclear-encoded genes of the oxidative phosphorylation pathway and the accumulation of mitochondrial–nuclear incompatibilities among *Nasonia* species that decrease hybrid fitness (Oliveira et al. 2008; Gibson et al. 2010; The *Nasonia* Genome Working Group 2010). Third, populations of the marine copepod *T. californicus* have mitochondrial substitution rates that are 25-fold higher than nuclear rates and experience mitochondrial–nuclear incompatibilities that decrease hybrid fitness (Burton et al. 2006; Ellison and Burton 2008a). In contrast, across *Drosophilids*, the mitochondrial synonymous substitution rate is only 2.75 times the nuclear substitution rate (Montooth et al. 2009).

These studies suggest that the rate of substitution in the mtDNA may be a critical parameter for the accumulation of incompatibilities driven by compensatory evolution between the mitochondrial and nuclear genomes. There are a number of possible reasons why *D. melanogaster* and *D. simulans* might have a lower ratio of mitochondrial to nuclear substitution rates than other taxa. First, mitochondrial mutation pressures could be reduced in *Drosophila*. In *D. melanogaster* mtDNA mutations occur almost exclusively at G:C base pairs and convert G:C to A:T (Haag-Liautard et al. 2008). This biased mutational process, combined with the low G+C content in the *Drosophila* mtDNA, may be why *D. melanogaster* has a lower ratio of mitochondrial to nuclear mutation rates than *S. cerevisiae*, *C. elegans*, and humans (Lynch et al. 2008; Montooth and Rand 2008). Second, *Drosophila* effective population sizes may simply be large enough that purifying selection can efficiently prevent the fixation of deleterious mtDNA mutations.

Mitochondrial evolutionary dynamics are also influenced by cytoplasmically inherited endosymbionts that sweep through populations via their effects on host reproduction (Hoffmann and Turelli 1997), and are posited to have driven mitochondrial–nuclear coevolution in *Nasonia* (Oliveira et al. 2008; Raychoudhury et al. 2009, 2010). Cytoplasmic incompatibility from *Wolbachia* infection is currently weak and uncommon in *D. melanogaster* (Fry et al. 2004; Merçot and Charlat 2004). However, there are at least five systems of *Wolbachia*-induced cytoplasmic incompatibility known from *D. simulans* (Clancy and Hoffmann 1996), suggesting that endosymbiont-driven cytoplas-

mic sweeps are likely to have occurred in *Drosophila* as well as *Nasonia*. *Wolbachia* has been implicated in mtDNA evolution in other *Drosophila* species groups (Shoemaker et al. 2004; Bachtrog et al. 2006), providing a comparative context in which to explore the relationship between cytoplasmic sweeps, mtDNA substitution rates, and the accumulation of mitochondrial–nuclear incompatibilities.

CONTEXT DEPENDENCE OF INTERGENOMIC EPISTASIS

If females and males are considered to constitute different cellular and physiological environments in which genes function, then the sex-dependence of mitochondrial–nuclear interactions reflect the complex interactions that exist between genetic and environmental factors (e.g., Bergland et al. 2008), which may be common for mitochondrial–nuclear interactions (Rand et al. 2001; Dowling et al. 2007a). The existence of this complexity highlights the need for fitness studies of mtDNA to include multiple nuclear backgrounds and rigorous genetic controls, as fitness differences in one background could be reversed in other genetic backgrounds. Resolving the relative contributions of mutation and substitution rates, genetic drift and genetic draft (Gillespie 2000; Bazin et al. 2006; Meiklejohn et al. 2007), and complex epistasis remains a significant challenge for the understanding of the evolution of the mtDNA and its interaction with nuclear loci. However, the mitochondrial–nuclear genotype as a unit of selection, particularly in species where this genotype can be manipulated, offers the potential to explore how complex genetic interactions influence the evolutionary process (Gillespie and Turelli 1989; Whitlock et al. 1995; Barton and Turelli 2004; Phillips 2008). The well-characterized physiological function encoded by the mitochondrial–nuclear genotype provides the opportunity to functionally dissect how genetic interactions are expressed in an environment- and sex-dependent fashion to influence metabolic fitness. This will be an important step in characterizing how genomic loci cooperate and conflict, as well as how metabolic physiologies evolve.

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