Similar efficacies of selection shape mitochondrial and nuclear genes in *Drosophila melanogaster* and *Homo sapiens*

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ABSTRACT

Deleterious mutations contribute to polymorphism even when selection effectively prevents their fixation. The efficacy of selection in removing deleterious mitochondrial mutations from populations depends on the effective population size (N_e) of the mtDNA, and the degree to which a lack of recombination magnifies the effects of linked selection. Using complete mitochondrial genomes from *Drosophila melanogaster* and nuclear data available from the same samples, we re-examine the hypothesis that non-recombining animal mtDNA harbor an excess of deleterious polymorphisms relative to the nuclear genome. We find no evidence of recombination in the mitochondrial genome, and the much-reduced level of mitochondrial synonymous polymorphism relative to nuclear genes is consistent with a reduction in N_e. Nevertheless, we find that the neutrality index (*NI*), a measure of the excess of nonsynonymous polymorphism relative to the neutral expectation, is not significantly different between mitochondrial and nuclear loci. Reanalysis of published data from *Homo sapiens* reveals the same lack of a difference between the two genomes, though small samples in previous studies had suggested a strong difference in both species. Thus, despite a smaller N_e, mitochondrial loci of both flies and humans appear to experience similar efficacies of selection as do loci in the recombining nuclear genome.

20 INTRODUCTION

The effective size of a population (N_e) impacts how effectively selection removes deleterious mutations and fixes advantageous mutations. The unique genetics of the mitochondrial genome (mtDNA) are thought to reduce its N_e relative to the nuclear genome, via haploid, uniparental inheritance, the mitochondrial bottleneck in the maternal germline, and a lack of recombination
25 that decreases N_e via selection on linked sites (Hill and Robertson 1966; Maynard Smith and Haigh 1974; Gillespie 2000; Meiklejohn *et al.* 2007; White *et al.* 2008; Charlesworth 2012). In addition, cytoplasmic transmission can link the mtDNA to selfish cytoplasmic elements (e.g., *Wolbachia* in insects) that may sweep through populations, further decreasing mitochondrial N_e and possibly increasing mitochondrial substitution rates via the fixation of slightly deleterious
30 mutations (Shoemaker *et al.* 2004). For these reasons it has been widely hypothesized that selection is less effective in mitochondrial genomes than in their nuclear counterparts, and that mitochondrial genomes may accumulate greater numbers of deleterious substitutions (Lynch 1996; Lynch 1997). Analyses of sequence data in *Drosophila* and mammals have largely supported the conclusion that mtDNA harbor significant levels of slightly deleterious
35 polymorphism (Ballard and Kreitman 1994; Rand and Kann 1996; Nachman 1998; Rand and Kann 1998; Weinreich and Rand 2000).

N_e is not the only evolutionary parameter that distinguishes mitochondrial and nuclear genomes. The distinct functional landscape of the mitochondrial genome likely affects the distribution of selective effects (s) of mutations that arise in this genome. Animal mitochondrial
40 genomes typically encode regulatory information for replication and transcription nested within a hypervariable region (also known as the D-loop, control, or A+T-rich region), 22 tRNAs, 2 ribosomal components, and 13 protein-coding genes—all core components of oxidative

phosphorylation (OXPHOS). Outside of the control region, there is little noncoding DNA in animal mtDNAs. In Drosophilids, 99% of the genome outside of the control region encodes DNA and RNA genes with highly conserved sequences that function in mitochondrial protein synthesis and aerobic respiration (Clary and Wolstenholme 1985; Wolstenholme and Clary 1985; Ballard 2000; Montooth *et al.* 2009). This suggests that the distribution of selective effects in the mtDNA may be shifted towards larger negative effects on fitness.

The mutational landscape of the mtDNA also differs from the nuclear genome. In most animal taxa the mitochondrial mutation rate greatly exceeds that of the nuclear genome (Lynch *et al.* 2008), and the mitochondrial mutational process is also highly biased (Montooth and Rand 2008). For example, nearly all mitochondrial mutations in *D. melanogaster* change a G:C base pair to an A:T (Haag-Liautard *et al.* 2008). When combined with the strong A+T-bias in this mitochondrial genome, where 95% of third codon positions are an A or a T (Montooth *et al.* 2009), this indicates that the most commonly occurring mutations in protein-coding loci of the *Drosophila* mtDNA will change an amino acid. Relative to the nuclear genome, animal mitochondrial genomes thus experience a greater mutational pressure that can also be biased in some taxa towards nonsynonymous mutations; these are likely to have deleterious effects in a molecule that encodes such highly conserved functions.

Some of the strongest population genetic patterns in support of distinct selective pressures acting on mitochondrial and nuclear genomes come from analyses of the neutrality index (*NI*) (Rand and Kann 1996; Nachman 1998; Rand and Kann 1998; Weinreich and Rand 2000). *NI* is a summary statistic of the deviation from the neutral expectation in the McDonald-Kreitman test (Mcdonald and Kreitman 1991; Rand and Kann 1996), and is calculated from counts of synonymous and nonsynonymous polymorphic and fixed sites within and between

related species. Weakly deleterious nonsynonymous mutations that segregate in the population but that will not contribute to divergence lead to a value of *NI* greater than 1. When the efficacy of selection is decreased, the expectation is that the number of segregating weakly deleterious polymorphisms will increase; this is the pattern that has been observed in mtDNA. Meta-
70 analyses of McDonald-Kreitman tables and their associated *NI* values for mitochondrial and nuclear loci in animals have concluded that *NI* is predominantly greater than the neutral expectation of 1 for the mtDNA (Rand and Kann 1996; Nachman 1998; Rand and Kann 1998; Betancourt *et al.* 2012) and exceeds the average *NI* of the nuclear genome (Weinreich and Rand 2000). Although the relative sparseness of the data was recognized early on (Nachman 1998),
75 and the conclusions were largely limited to how selection shapes animal mtDNA, these patterns are often taken as evidence that selection is largely ineffective in the mtDNA because of its reduced N_e, and that mitochondrial genomes are expected to harbor more deleterious polymorphisms than do their nuclear counterparts.

Here we revisit this pattern using new, complete mitochondrial genomes from *D.*
80 *melanogaster* that we compare with published nuclear data from the same samples (Langley *et al.* 2012) and with available human data from both nuclear and mitochondrial genomes (Bustamante *et al.* 2005; Just *et al.* 2008; Rubino *et al.* 2012). We find little evidence that the effects of selection differ on average between mitochondrial and nuclear genomes of flies or humans, despite evidence that there is much reduced N_e due to a lack of recombination and
85 linkage with the cytoplasm. We discuss reasons why *NI* is, on average, similar between mitochondrial and nuclear loci, despite the distinct population genetic properties of these two genomes.

MATERIALS AND METHODS

90 ***D. melanogaster* mtDNA assembly, annotation, and estimates of sequence diversity**

Raw sequence read files from 38 genetic lines of *D. melanogaster* from Raleigh, NC, USA (Mackay *et al.* 2012) sequenced by the 50 Genomes subproject of the *Drosophila* Population Genomics Project (DPGP) (Langley *et al.* 2012) were downloaded from the NCBI Sequence Read Archive. We used the Burrows-Wheeler Aligner (BWA), and specifically the fast and

95 accurate short read alignment with Burrows-Wheeler Transform (Li and Durbin 2009), to map sequence reads to the *D. melanogaster* mitochondrial reference genome (NC_001709). We allowed up to 5 gaps, 5 gap extensions, and 5 mismatches per aligned read, but few reads needed such flexibility and most were filtered out in later steps. Using SAMtools, we post-processed the alignments to filter out low-quality alignments and to detect single nucleotide polymorphisms

100 (SNPs) (Li *et al.* 2009). SNPs with a quality score greater than 20 and indels with a quality score greater than 50 were kept for further analyses. We then generated a consensus sequence for each of the *D. melanogaster* mtDNAs listed in Table S1. Due to high variance in coverage across the A+T-rich D-loop, we did not include this region in our final assemblies or analyses.

We annotated the consensus sequence for each mtDNA using the GenBank annotation of

105 the *D. melanogaster* reference sequence (NC_001709). Using ClustalW (Larkin *et al.* 2007), we performed a whole genome alignment, as well as gene-specific alignments, of each consensus sequence to the reference sequence and to the outgroup species *D. yakuba* (NC_001322). There are very few indels in the coding regions of *Drosophila* mtDNA (Montooth *et al.* 2009), making alignment straightforward. From these alignments we calculated expected heterozygosity (π), the

110 number of segregating sites (S), and Watterson's θ_W (Watterson 1975) as measures of sequence diversity. The mitochondrial haplotype network was inferred from 80 segregating sites in the

coding region of the mtDNA for which there were no missing or ambiguous data using TCS version 1.21 (Clement *et al.* 2000).

115 **Tests for recombination in the *D. melanogaster* mtDNA**

We estimated linkage disequilibrium (LD) between all pairs of mitochondrial SNPs using the statistic *D'* (Lewontin 1964), where *D*' = 0 indicates no LD and |*D*'| = 1 indicates perfect LD. Because recombination erodes LD as a function of distance, a negative correlation between |*D*'| and genetic distance between pairs of SNPs has been used as evidence for recombination

120 (Awadalla *et al.* 1999). To test this prediction we looked for significant negative correlations between |*D*'| and genetic distance. We also conducted these same tests using another statistical measure of association, r^2 (Hill and Robertson 1966), which is more robust to variation in mutation rates (Awadalla *et al.* 1999; Meunier and Eyre-Walker 2001; Innan and Nordborg 2002). We calculated these correlations using a variety of minor allele frequency cutoffs (Table

125 S2). We also tested for the presence of all four genotypes at pairs of SNPs (the "four-gamete test"; Hudson and Kaplan 1985) using DNAsp version 5 (Rozas *et al.* 2003).

Neutrality tests

Using π and θ_W, we calculated Tajima's *D* (Tajima 1989), which is expected to be 0 for a

130 neutrally evolving locus. Demographic effects will skew the site-frequency spectrum of both synonymous and nonsynonymous polymorphisms at a locus. Contrasting Tajima's *D* between nonsynonymous and synonymous polymorphisms therefore tests whether nonsynonymous alleles experience a greater skew in frequency relative to putatively neutral synonymous alleles, indicative of selection (Rand and Kann 1996). We implemented this analysis using the

135 heterogeneity test (Hahn *et al.* 2002), which simulates 10,000 genealogies with no recombination using the values of synonymous and nonsynonymous *S* calculated from the data and compares the estimated difference in Tajima's *D* to the random distribution of differences between synonymous and nonsynonymous polymorphisms. We calculated several other summaries of the site-frequency spectrum, including Fu and Li's *D*, which characterizes the proportion of
140 mutations on external and internal branches of a genealogy (Fu and Li 1993) and Fay and Wu's *H*, which tests for an excess of high-frequency derived alleles in a sample relative to the neutral expectation (Fay and Wu 2000). These latter statistics were calculated using a set of 80 segregating sites in the coding region of the mtDNA for which there were no missing or ambiguous data. Significance was determined using 10,000 coalescent simulations as
145 implemented in DNAsp version 5 (Rozas *et al.* 2003).

We conducted McDonald-Kreitman (MK) tests (Mcdonald and Kreitman 1991) using Fisher's exact tests of the two-by-two contingency tables of counts of nonsynonymous and synonymous polymorphisms (P_N and P_S) within *D. melanogaster* and nonsynonymous and synonymous fixed differences (D_N and D_S) between *D. melanogaster* and *D. yakuba*.
150 Polymorphic sites within *D. melanogaster* only contributed to fixed differences if the allele in *D. yakuba* was not present in *D. melanogaster.* For codons with more than one change, we calculated the number of nonsynonymous and synonymous differences as the average over all possible mutational pathways between codons (Nei and Gojobori 1986). For any gene with a count of 0 in any cell of the MK table, we added a count of 1 to all cells (Sheldahl *et al.* 2003;
155 Presgraves 2005). 23% of thirteen *D. melanogaster* mitochondrial genes, 9.5% of 6,113 *D. melanogaster* nuclear genes, 0% of thirteen *H. sapiens* mitochondrial genes, and 73% of 11,624

H. sapiens nuclear genes required these additional counts. Fisher's exact tests were performed using R version 2.15.1 (R Core Team 2012).

We calculated *NI*—the ratio of P_N/P_S to D_N/D_S—as a summary statistic of the MK table (Rand and Kann 1996). Assuming that selection is constant, the neutral expectation is that D_N/D_s will equal P_N/P_S (Kimura 1983; Mcdonald and Kreitman 1991), and *NI* is expected to be 1. When the MK test is significant, an *NI* less than 1 indicates a significant excess of nonsynonymous fixed differences and an *NI* greater than 1 indicates a significant excess of nonsynonymous polymorphisms. We calculated several modified statistics similar to *NI*, including $Z^* = Log_{10}\left(\frac{(D_N+1)(P_S+1)}{(D_S+1)(P_N+1)}\right)$ (Presgraves 2005) and the direction of selection $DoS = \frac{D_N}{D_N+D_S} - \frac{P_N}{P_N+P_S}$, which is an unbiased summary statistic of the two-by-two MK table (Stoletzki and Eyre-Walker 2011). The sign of these latter two statistics is more intuitive; negative values are consistent with an excess of weakly deleterious (negatively selected) polymorphisms and positive vales are consistent with an excess of advantageous (positively selected) substitutions.

The short length and reduced polymorphism of mitochondrial genes decreases the power of the MK test and upwardly biases *NI* (Weinreich and Rand 2000). Because of this, we also performed MK tests and calculated *NI* using the summed counts of polymorphic and divergent sites for each of the OXPHOS complexes: Complex I (NADH dehydrogenase, ND), Complex IV (cytochrome *c* oxidase, CO) and Complex V (ATP synthetase, ATPase). Cytochrome B (CytB) is the only Complex III gene encoded by the mtDNA. Stoletzki and Eyre-Walker (2011) emphasize that contingency data should not generally be summed, particularly when there is heterogeneity among contingency tables, and they provide an unbiased estimator of overall *NI* for combining counts, $NI_{TG} = \frac{\sum D_{Si}P_{Ni}/(P_{Si}+D_{Si})}{\sum P_{Si}D_{Ni}/(P_{Si}+D_{Si})}$. We calculated this statistic and used the DoFE software package (Stoletzki and Eyre-Walker 2011) to calculate bootstrap confidence intervals

180 and to conduct Woolf's tests of homogeneity (Woolf 1955). The only data set with significant heterogeneity was the *D. melanogaster* nuclear gene set ($P<0.0001$). The same approaches were used to analyze polymorphism and divergence in the human data sets (see below), as well as in a subset of the mitochondrial haplotypes reported in our study that were independently sequenced and assembled by Richardson et al. (2012) (Table S3).

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Comparisons of mitochondrial and nuclear *NI* in flies and humans

To compare patterns of polymorphism and divergence between mitochondrial and nuclear genomes, we obtained existing data for nuclear genes in *D. melanogaster* and for nuclear and mitochondrial genes in *Homo sapiens.* Counts of polymorphic and divergent sites for *D.*
190 *melanogaster* nuclear genes were obtained from the DPGP analysis of the same 38 genomes from Raleigh, NC, USA, using *D. yakuba* as the outgroup species (Langley *et al.* 2012). The human nuclear data from Bustamante et al. (2005) included counts of polymorphic and divergent sites from 19 African Americans and 20 European Americans, using the chimpanzee *Pan troglodytes* as an outgroup species. We calculated the number of polymorphic and divergent sites
195 for human mitochondrial genes using mtDNA sequences from 19 African Americans (Just *et al.* 2008), 20 European Americans (Rubino *et al.* 2012), and the chimpanzee mitochondrial reference genome D38113.1 (Horai *et al.* 1995). We also analyzed subsets of the human mitochondrial sequence data to characterize the sensitivity of *NI* to sampling (Table S4). Table S5 provides accession numbers for the human mtDNA sequences used. Comparisons of the
200 distributions of *NI* between data sets were performed using Mann-Whitney *U* tests in R version 2.15.1 (R Core Team 2012).

RESULTS

An excess of low- and high-frequency derived mitochondrial polymorphisms

205 We assembled 14,916 bp of sequence containing the transcribed regions of the mtDNA with a median coverage of 32x for 38 genetic lines sampled from a single population of *D. melanogaster* in Raleigh, NC (Langley *et al.* 2012; Mackay *et al.* 2012)(Table S1). Over 98% of these nucleotides encode the 13 protein-coding genes, 22 tRNAs and two ribosomal RNAs. The per-site expected heterozygosity in this region (π) of the mtDNA was 0.0008. We identified 137

210 segregating sites in this population sample, 103 of which were in protein-coding genes. Median heterozygosity in protein coding genes was 0.0023 per synonymous site and 0.0002 per nonsynonymous site. Silent site heterozygosity was significantly lower in mitochondrial genes relative to nuclear genes (Mann-Whitney U, mtDNA v. X chromosome, $P_{MWU} = 0.00002$; mtDNA v. autosomes, $P_{MWU} < 0.00001$) and was only 0.16 times that of the autosomes (Figure

215 1A), lower than what is expected if the mtDNA has an effective population size that is one-quarter that of the autosomes.

In addition to there being very few segregating sites in the *D. melanogaster* mtDNA, polymorphisms at these sites were skewed towards low frequencies (Figure 2A,B), as evidenced by consistently negative values of Tajima's D (Table 1). Tajima's D across the mtDNA was -

220 2.607 and differed significantly from the neutral expectation of 0 (S=80 for coding sites with no missing data, $P<0.0001$), as did Fu and Li's D ($D = -2.67$, $P<0.05$). The minor allele frequency for unpolarized synonymous polymorphisms was always less than 11%, and all but one of the nonsynonymous polymorphisms was a singleton (Figure 2A,B). Using *D. yakuba* as an outgroup revealed that the derived allele was nearly fixed for 44% of segregating synonymous sites, while

225 there was only a single derived nonsynonymous polymorphism at high frequency (Figure 2C,D).

Thus, the mitochondrial genome was essentially devoid of intermediate frequency polymorphisms, with derived synonymous mutations at either very high (greater than 89%) or very low (less than 11%) frequencies and nearly all derived nonsynonymous polymorphisms at frequencies less than 5%. This skew towards high-frequency derived alleles resulted in a
230 significant negative value of Fay and Wu's H statistic (H = -41.2, P = 0.005).

A partial selective sweep in the *D. melanogaster* mtDNA

The large fraction of derived alleles at high frequencies is a consequence of the haplotype structure of this sample (Figure 3). Nearly 30% of individuals in this population shared an
235 identical mitochondrial haplotype and an additional 66% of individuals differed from this haplotype by only one to five mutations. The two remaining haplotypes (RAL-639 and RAL-335) were highly divergent from this common haplotype group, contributing nearly half of the segregating sites to the population sample. These two haplotypes shared the ancestral state with *D. yakuba* at 17 of the 23 derived high-frequency synonymous polymorphisms (i.e., they have
240 the low-frequency ancestral allele; Figure 2C). When these two haplotypes were removed from the analysis, there remained a strong skew toward rare alleles (Tajima's D = -2.31, $P < 0.01$; Fu and Li's D = -3.14, $P < 0.02$), but Fay and Wu's H, which is sensitive to the number of high-frequency derived alleles, was only weakly significant (H = -10.34, P = 0.043). The remaining six derived, high-frequency synonymous polymorphisms, as well as the single derived, high-
245 frequency nonsynonymous polymorphism, were the result of single mtDNAs within the common haplotype group having the same allelic state as *D. yakuba*. Given the lack of recombination in the mtDNA (see below), these are likely new, rather than ancestral, mutations. Six of these seven

mutations would have changed a C or G to a T or A, consistent with the mutation bias in the *D. melanogaster* mtDNA (Haag-Liautard *et al.* 2008).

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No evidence for recombination in the *D. melanogaster* mtDNA

We tested for a negative correlation between linkage disequilibrium (LD) and the distance between each pair of polymorphic sites in the *D. melanogaster* mitochondrial genome, as a signature of the decay of LD over distance via recombination (Awadalla *et al.* 1999). There was

255 no evidence for a decrease in LD with increasing distance between sites, regardless of the measure of LD or the minor allele cutoff used (Table S2). There were no pairs of polymorphic sites for which all four gametes were present (Hudson and Kaplan 1985; Bruen *et al.* 2006), further supporting an absence of effective recombination.

260 **Weakly deleterious polymorphism in the *D. melanogaster* mtDNA**

The skew in the site-frequency spectrum toward rare alleles (Figure 2) resulted in negative values of Tajima's *D* across the entire mtDNA (Table 1). There was no evidence that the skew toward rare alleles differed between synonymous and nonsynonymous polymorphisms (Figure 2A,B): heterogeneity tests (Hahn *et al.* 2002) of Tajima's *D* between synonymous and

265 nonsynonymous sites were never significant ($P > 0.35$ for all genes and complexes). However, unfolding the frequency spectra revealed that the large number of high-frequency derived sites were nearly all synonymous (Figure 2C,D). This would indicate that the haplotype that has increased in frequency carried many more synonymous than nonsynonymous polymorphisms. Given that the mutation bias in the *D. melanogaster* mtDNA greatly favors nonsynonymous

270 mutations (Haag-Liautard *et al.* 2008), this pattern suggests a history of effective purifying

selection removing mitochondrial haplotypes that contain nonsynonymous polymorphisms. Furthermore, all nonsynonymous polymorphisms that have arisen on the common mitochondrial haplotype are present at very low frequencies.

The current distribution of polymorphisms relative to divergence showed little evidence
275 for an excess of segregating deleterious polymorphisms. No single gene and only one OXPHOS complex significantly departed from the neutral expectation after Bonferroni correction (Table 2 and Table 3); the MK test was significant for Complex V (ATPase), with an excess of nonsynonymous polymorphism (Fisher's exact test, $P_{FET} = 0.007$). For the entire set of protein-coding mitochondrial genes, there was a slight excess of nonsynonymous polymorphism relative
280 to the neutral expectation that was not significant after Bonferroni correction ($P_{FET} = 0.041$). Using the unbiased estimator NI_{TG} (Stoletzki and Eyre-Walker 2011) did not change this inference: NI_{TG} for mitochondrial-encoded proteins was 1.67, but the confidence intervals on NI_{TG} nearly included the neutral expectation of 1 (Table 3).

These patterns were verified using 36 of the 38 mitochondrial haplotypes in our sample
285 that were independently sequenced and assembled by Richardson et al. (2012). No single mitochondrial gene rejected the neutral expectation and the MK table counts for Complex V (ATPase) were exactly the same in the two data sets (Table S3). When counts of polymorphism and divergence differed between our datasets, they typically differed by only a single count. The only exception was in several Complex I (ND) genes, for which our assembled mtDNAs had a
290 small number of additional nonsynonymous polymorphisms relative to the Richardson et al. (2012) data set (ND genes, $df = 6$, $P_{MWU,\ paired} = 0.021$; all other genes, $df = 5$, $P_{MWU,\ paired} = 1$) that resulted in slightly higher values of NI (ND included, $df = 12$, $P_{MWU,\ paired} = 0.016$; all other genes, $df = 5$, $P_{MWU,\ paired} = 1$). This was not due to exclusion of two mitochondrial haplotypes in

the Richardson et al. (2012) data set, and the additional polymorphisms in our data were not
295 clustered on any single haplotype. The reduced number of nonsynonymous polymorphisms in the Richardson et al. (2012) data provided even less support for an excess of nonsynonymous segregating variation in the mitochondrial genome. Patterns of polymorphism and divergence for the entire set of mitochondrial-encoded proteins in this dataset did not deviate from the neutral expectation (P_{FET} = 0.423), and the confidence intervals on NI_{TG} for mitochondrially
300 encoded proteins contained the neutral expectation of 1 (NI_{TG} = 0.821, 95% CI = 0.386 to 1.90).

On average, *NI* is the same for mitochondrial and nuclear genes in flies

The distribution of *D. melanogaster* mitochondrial gene *NI* values was contained within that of the 6,151 nuclear genes for which we had MK table counts, with many nuclear genes having
305 both more positive and more negative values of *Z** and *DoS* (Figure 1 and Figure 4). The median value of *NI* was 1.97 (NI_{TG} = 1.67) for the 13 mitochondrial genes and 1.48 (NI_{TG} = 1.35) for 6,113 nuclear genes, and did not differ significantly between genomes (P_{MWU} = 0.278). Confidence intervals for NI_{TG} were overlapping for the two genomes (Table 3). Median values of *Z** and *DoS* were negative for both genomes (Figure 1 and Figure 4), consistent with the
310 presence of a slight excess of deleterious polymorphisms in both genomes. *Z** for *D. melanogaster* mitochondrial genes did not differ significantly from nuclear genes, independent of nuclear location (mtDNA vs. X chromosome, P_{MWU} = 0.065; mtDNA vs. autosomes, P_{MWU} = 0.325) (Figure 1C). *DoS* was modestly significantly different between mitochondrial and X-chromosome genes (mtDNA vs. X, P_{MWU} = 0.044), but not between the mtDNA and autosomal
315 genes (mtDNA vs. autosomes, P_{MWU} = 0.126). Nevertheless, there was a trend for mitochondrial genes to differ more from X-linked loci than they did from autosomal loci for both *Z** and *DoS*

(Figure 1C,D). Analysis of assembled mtDNAs from Richardson et al. (2012) (see above) yielded qualitatively similar results; *NI* did not differ between mitochondrial and nuclear genes (P_{MWU} = 0.500), and neither *Z** or *DoS* differed significantly between the mitochondria and any
320 nuclear location (*Z**, P_{MWU} > 0.471 for both comparisons; *DoS*, P_{MWU} > 0.726 for both comparisons).

_NI_ does not differ between mitochondrial and nuclear genes in humans

Summary statistics of the MK table also did not differ between mitochondrial and nuclear genes
325 in *H. sapiens* (*NI,* P_{MWU} = 0.657; *Z**, P_{MWU} = 0.243; *DoS,* P_{MWU} = 0.700)(Figure 4), nor did the site-frequency spectrum differ between non-synonymous and synonymous mitochondrial polymorphisms (heterogeneity test, *P* > 0.36 for all genes). Values of *Z** and *DoS* for mitochondrial genes in humans were well within the distribution of the 11,624 nuclear genes examined, and the confidence intervals of NI_{TG} were overlapping for the mitochondrial and
330 nuclear genomes (Table 3). The distributions of mitochondrial gene MK summary statistics were also largely overlapping and did not differ significantly between *D. melanogaster* and *H. sapiens* (*NI,* P_{MWU} = 0.545; *Z**, P_{MWU} = 0.441; *DoS,* P_{MWU} = 0.310)(Figure 4), despite differing nuclear N_e between these species. This further supports the idea that the efficacy of selection in the mitochondrial genome is largely independent of N_e.

335 We also found that *NI* is very sensitive to sampling (Table 4). When only a few individuals are sampled, the choice of genomes can lead to high variability and extreme values in *NI*, potentially as a result of single haplotypes that may carry multiple polymorphisms, as appears to be the case for human *ND6* (Table 4). For example, depending on which Japanese individual we included in our analyses, *NI* for *ND6* takes on values of 30.71 (MK, P_{FET} = 0.001),

340 5.50 (MK, P_{FET} = 0.31), or 1.79 (MK, P_{FET} = 0.16) when sampling only 3 mtDNAs. As more mtDNAs are sampled, point estimates of *NI* and *Z** for each mitochondrial gene become more similar to the neutral expectation (Table 4) and average values of *NI* and NI_{TG} no longer show strong evidence for deviations from the neutral expectation.

345 DISCUSSION

Using a large sample of whole-genome sequence data, we have tested a number of hypotheses about mtDNA evolution, and about differences in the efficacy of selection on mitochondrial versus nuclear genes. Our data confirm that mtDNA do not recombine and have lower silent site 350 diversity than do nuclear genes, which supports the prediction that the mitochondrial genome has a lower N_e than does the nuclear genome. We also show a skew in the site-frequency spectrum toward rare alleles that likely has two sources: 1) the accumulation of new mutations on what appears to be a mtDNA haplotype that has swept to high frequency in the recent past, and 2) the ancestral polymorphisms contained on migrant or remnant haplotypes (RAL-639 and RAL-335) 355 that are now rare in this population. Despite the apparent reduction in N_e for mtDNA, our findings indicate that selection is equally effective at purging deleterious polymorphisms from the mitochondrial and nuclear genomes of *D. melanogaster* and *H. sapiens*.

Given its uniparental and haploid transmission, the expectation under neutrality is that the mtDNA has one-quarter the population size of the autosomes. This reduced value of *N* (and 360 subsequently N_e) matches that expected for the Y (or W) chromosome, and, like the Y, the mtDNA has little to no recombination. However, very much unlike the Y chromosomes that have been sequenced (e.g. Charlesworth and Charlesworth 2000; Carvalho *et al.* 2009; Carvalho and Clark 2013; Bellott *et al.* 2014), animal mtDNA genomes do not show an accumulation of

transposable elements, and the gene content of the mitochondrial genome is remarkably stable,
365 with few gene losses and even fewer pseudogenes (Boore 1999; Ballard and Rand 2005). Furthermore, d_N/d_S is two to fifteen times lower for mitochondrial genes than for nuclear genes in mammals (Popadin *et al.* 2012), and average values of d_N/d_S for mitochondrial genes are well under 0.1 and are, on average, only 13% that of nuclear genes in *Drosophila* (Bazin *et al.* 2006; Montooth *et al.* 2009). This pattern of amino acid conservation is particularly striking given that
370 the mutation rate in the *D. melanogaster* mtDNA is an order of magnitude greater than the per-site mutation rate in the nuclear genome (Haag-Liautard *et al.* 2007; Haag-Liautard *et al.* 2008). While heteromorphic Y chromosomes do show signatures of less effective purifying selection, such as proliferation of satellite repeats and reduced codon bias (Bachtrog 2013; Singh *et al.* 2014), the single copy, X-degenerate genes that have remained on the human Y chromosome
375 experience effective purifying selection (Rozen *et al.* 2009; Bellott *et al.* 2014), as do the protein sequences of *Drosophila* Y-linked genes (Singh *et al.* 2014). Thus, despite early loss of many genes when heteromorphic Y chromosomes and mtDNA formed, both these non-recombining chromosomes have maintained a stable set of genes that experience effective purifying selection despite their reduced N_e.

380 Many researchers have cited the early work on *NI* in *Drosophila* and mammals in support of the idea that mtDNA accumulate deleterious mutations (e.g., Meiklejohn *et al.* 2007; Green *et al.* 2008; Neiman and Taylor 2009; Akashi *et al.* 2012). In fact, this idea has become so engrained that it is regularly cited in reviews of mitochondrial gene evolution (e.g. Ballard and Whitlock 2004; Lynch 2007, p. 338). What is surprising about this conversion of a small set of
385 intriguing initial studies into dogma is that the early studies themselves were quite circumspect about the implications of their results. For instance, Nachman (1998), in noting that very few

nuclear loci were available for comparison, stated "It is also unclear whether the patterns reported here are unique to mitochondrial DNA." Data from the few nuclear genes that had been sequenced raised "the possibility that the patterns reported here for mtDNA may also be found at
390 some nuclear loci" (Nachman 1998). Even the relatively more recent studies that did have access to additional nuclear datasets were only able to calculate *NI* for 36 nuclear loci (Weinreich and Rand 2000), and the *NI* values that were available often did not deviate significantly from neutrality (Nachman 1998; Weinreich and Rand 2000). Those that did reject neutrality tended to do so weakly, perhaps due to the small number of polymorphisms in mitochondrial samples even
395 when the number of individuals sampled is high (e.g. ND3, Nachman *et al.* 1996). Nevertheless, there are mitochondrial genes that do strongly reject neutrality and some of these had *NI* values that greatly exceeded *NI* for the sampled nuclear loci. Based on these and similar comparisons, many authors have reached the conclusion that mtDNA evolves in a manner distinct from the nuclear genome. Our results using the whole genomes of flies and humans suggest that they are
400 not evolving differently.

Reductions in N_e—due either to reductions in census population size or to the increased effect of linked selected variants in regions of low recombination—are expected to result in a reduction in the efficacy of selection. Indeed, comparisons of McDonald-Kreitman test results across a range of species with different values of N_e has revealed this expected relationship (e.g.
405 Li *et al.* 2008; Wright and Andolfatto 2008; Gossmann *et al.* 2010), as have comparisons of *NI* across regions of the *D. melanogaster* genome with different recombination rates (Presgraves 2005; Langley *et al.* 2012). Therefore, all things being equal, mitochondrial loci would be expected to harbor an excess of replacement polymorphisms relative to nuclear loci due to reduced N_e. Our results suggest that all things are not equal between these two cellular

410 compartments, and that there may be features of the mitochondrion that make it less likely to accumulate deleterious mutations. One such feature is the "bottleneck" that occurs in the number of mtDNAs that are passed from mother to offspring—this event makes it possible for selection to act within hosts, possibly increasing the power of selection to remove deleterious mutations (Bergstrom and Pritchard 1998; Rand 2011) and reducing variability in mitochondrial N_e among
415 taxa, relative to nuclear genomes. The additional layers of selection imposed by mitochondrial inheritance, combined with stronger negative selective effects of amino acid changing mutations in mitochondrial genes, may therefore allow the mtDNA to escape the accumulation of deleterious mutation, resulting in equal values of *NI* between nucleus and mitochondria. If the selective effects of mutations in mitochondrial genes are beyond the "horizons" where all
420 mutations will behave similarly regardless of N_e (Nachman 1998; Eyre-Walker and Keightley 2007), then we expect patterns of mitochondrial polymorphism and divergence to be largely independent of N_e.

Our results come with several caveats. First, we have only studied two organisms—it may be that a more comprehensive review of *NI* in mtDNA and nuclear loci across many species
425 will reveal a difference in the average efficacy of selection. The early meta-analyses of *NI* contained loci from a wide range of animals (Nachman 1998; Weinreich and Rand 2000), possibly suggesting that using only *Drosophila* and humans results in a view that is too limited. Nevertheless, these are two model organisms for evolutionary biology that span a large range of mtDNA:nuclear substitution rates, and studies of these species have set the bar for much of
430 modern population genetics. Second, it is clear from our analysis of the *D. melanogaster* mtDNA that it is not at equilibrium, and may be recovering from a partial cytoplasmic sweep that may be associated with *Wolbachia* (Richardson et al. 2012). Much of the theory used to predict *NI*

values from N_e and s assumes mutation-selection-drift balance (see, e.g. Nachman 1998), and deviations from this equilibrium can result in more complex relationships between N_e, s, and *NI*
435 (Messer and Petrov 2013). While non-equilibrium histories may mean that mtDNA *NI* values are not at equilibrium, it is equally likely that nuclear genes from *D. melanogaster* are not at mutation-selection-drift equilibrium (Hahn 2008; Langley *et al.* 2012). Whether or not the mtDNA is at equilibrium, and whether or not the *NI* values calculated from this snapshot of two species represent equilibrium values, our results still imply that there is no difference between
440 nuclear and mitochondrial measures of the efficacy of selection.

Despite the mitochondrial genome experiencing a distinct population genetic environment relative to the nuclear genome, our whole-genome analyses uncovered no evidence for an excess accumulation of slightly deleterious mutations in mitochondrial genomes. In fact, the only strong evidence for a reduced efficacy of selection in mtDNA, relative to nuclear
445 genomes, comes from comparative studies of nuclear and mitochondrial tRNAs (Lynch 1996; Lynch 1997). As discussed above, in the absence of a pattern in *NI*, there are essentially no other patterns of molecular evolution in mtDNA indicative of deleterious mutation accumulation. This pattern is in stark contrast to the patterns found in analogous nuclear regions with reduced N_e and low recombination, like the Y chromosome. Determining whether mtDNA accumulate
450 deleterious polymorphisms and substitutions more readily than nuclear DNA in a larger sample of species—and what type of loci may be affected—will be a particularly fruitful avenue for future studies.

455

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Supporting Information

File S1 FASTA file with the 38 assembled and aligned genomes used in this study

Table S1 mtDNAs assembled in this study along with the average coverage

650 **Table S2** Correlations between linkage disequilibrium (LD) and distance (bp) between pairs of SNPs in the *D. melanogaster* mtDNA

Table S3 Counts of polymorphic (*P*) and divergent (*D*) nonsynonymous (*N*) and synonymous (*S*) sites along with summary statistics of the McDonald-Kreitman table using 36 of the 38 mitochondrial haplotypes in our sample that were independently sequenced and assembled by Richardson et al. (2012).

655 **Table S4** Effects of sampling on *NI*

Table S5 Accession numbers for mtDNAs used in Table S4

Table 1 Synonymous and nonsynonymous variation in *D. melanogaster* mitochondrial genes and OXPHOS complexes

	Synonymous sites[b]					Nonsynonymous sites				
Gene/Complex[a]	n (bp)	*S*	π	Θ_W	*D*	n (bp)	*S*	π	Θ_W	*D*
COI	347	16	1.57	3.81	-1.91	1183	0	0	0	undef
COII	142	5	0.31	1.19	-1.90	539	1	0.05	0.24	-1.13
COIII	169.33	9	0.72	2.14	-1.96	613.67	1	0.05	0.24	-1.13
NDI	194	3	0.16	0.71	-1.72	739	3	0.16	0.71	-1.72
NDII	198	5	0.41	1.19	-1.69	822	6	0.34	1.43	-2.07
NDIII	68	1	0.05	0.24	-1.12	280	1	0.05	0.24	-1.12
NDIV	273.33	7	0.51	1.67	-1.95	1061.67	7	0.38	1.67	-2.17
NDIVL	56	1	0.05	0.24	-1.13	229	2	0.11	0.48	-1.49
NDV	351	10	0.68	2.38	-2.16	1365	4	0.21	0.95	-1.88
NDVI	103.33	4	0.26	0.95	-1.75	415.67	2	0.11	0.48	-1.49
Complex I	1243.66	31	2.12	7.38	-2.48	4912.34	25	1.35	5.95	-2.64
Complex III	239	5	0.55	1.19	-1.38	892	2	0.16	0.48	-1.29
Complex IV	658.33	30	2.60	7.14	-2.21	2335.67	2	0.11	0.48	-1.49
Complex V	174.67	2	0.16	0.71	-1.72	650.33	6	0.32	1.43	-2.10
Total	2315.66	68	5.43	16.42	-2.44	8790.34	35	1.93	8.33	-2.70

[a] Complex I, ND, 7 loci; Complex III, Cyt-b, 1 locus; Complex IV, CO, 3 loci; Complex V, ATPase, 2 overlapping loci

[b] For synonymous and nonsynonymous sites we calculated the number of segregating sites (*S*), heterozygosity (π), Watterson's Θ_W, and Tajima's *D*. The heterogeneity test for differences between synonymous and nonsynonymous *D* was never significant ($P > 0.35$ for all comparisons).

Table 2 Counts of polymorphic (*P*) and divergent (*D*) nonsynonymous (*N*) and synonymous (*S*) sites along with summary statistics of the McDonald-Kreitman table for *D. melanogaster* mitochondrial genes

Gene	P_N	P_S	D_N	D_S	NI[a]	Z^* [b]	DoS[c]
ATPase6	5	2	11	35	7.955	-0.778	-0.475
ATPase8	1	0	2	8	6	-0.778	undef
COI	0	16	8	101	0.667	0.176	undef
COII	1	5	6	39	1.3	-0.280	-0.033
COIII	1	9	8.5	47.5	0.621	-0.009	0.052
CytB	2	5	17.5	67.5	1.543	-0.267	-0.080
NDI	3	3	11	45	4.091	-0.584	-0.304
NDII	6	5	25	41	1.968	-0.275	-0.167
NDIII	1	1	5	22	4.4	-0.584	-0.315
NDIV	7	7	24	63	2.625	-0.408	-0.224
NDIVL	2	1	1	7	14	-0.778	-0.542
NDV	4	10	55.833	107.167	0.768	0.063	0.057
NDVI	2	4	21.5	22.5	0.523	0.203	0.155

[a] For *ATPase8* and *COI*, a count of 1 was added to each cell when calculating $NI = \frac{D_S P_N}{D_N P_S}$. For no gene was the neutral expectation rejected via a Fisher's exact test of the MK table at the table-wise Bonferroni corrected *P*=0.004.

[b] $Z^* = Log_{10}\left(\frac{(D_N+1)(P_S+1)}{(D_S+1)(P_N+1)}\right)$, as in (Presgraves 2005).

[c] $DoS = \frac{D_N}{D_N+D_S} - \frac{P_N}{P_N+P_S}$ was not calculated for loci with a count of zero (Stoletzki and Eyre-Walker 2011).

Table 3 Summary statistics of the McDonald-Kreitman table for mitochondrial OXPHOS complexes and nuclear genes

Species	Genome	Gene set[a]	*NI* [b]	NI_{TG}[c]	*Z* [b]	*DoS* [b]
D. melanogaster	mtDNA	Complex I	1.73	1.59 (0.94, 3.17)	-0.238	-0.129
	mtDNA	Complex IV	0.56	0.55 (0, 1.30)	0.255	0.045
	mtDNA	**Complex V**	**9.92**	**9.64 (undef)**	**-0.997**	**-0.518**
	mtDNA	All coding	1.59	1.67 (1.03, 2.86)	-0.201	-0.095
	nuclear	All coding	1.13	1.35 (1.31, 1.38)	-0.053	-0.023
H. sapiens	mtDNA	Complex I	1.19	1.20 (0.56, 2.40)	-0.074	-0.027
	mtDNA	Complex IV	1.98	2.00 (0.86, 3.61)	-0.296	-0.060
	mtDNA	Complex V	1.62	1.66 (0.79, 2.42)	-0.209	-0.097
	mtDNA	All coding	1.46	1.48 (0.90, 2.24)	-0.165	-0.059
	nuclear	All coding	1.51	1.57 (1.51, 1.63)	-0.180	-0.101

[a] Complex I, ND, seven loci; Complex IV, CO, three loci; Complex V, ATPase, two overlapping loci. Complex II is nuclear encoded and Complex III has only a single mitochondrial locus, *CytB.*

[b] *NI*, $Z = Log\left(\frac{D_N P_S}{D_S P_N}\right)$, and *DoS* were calculated using counts of P_N, P_S, D_N and D_S summed across genes within gene sets. **Bold** indicates *P* = 0.007 for Fisher's exact test of the MK table, which is significant after sample- but not table-wise Bonferroni correction.

[c] $NI_{TG} = \frac{\sum D_{Si} P_{Ni}/(P_{Si}+D_{Si})}{\sum P_{Si} D_{Ni}/(P_{Si}+D_{Si})}$ with confidence intervals from 5000 bootstrap samples (Stoletzki and Eyre-Walker 2011). Only the *D. melanogaster* nuclear dataset had significant heterogeneity as assessed by Woolf's test of homogeneity.

Table 4 The sensitivity of *NI* to sampling

Sample	1 African 1 European 1 Japanese [a]		1 African 1 European 1 Japanese [b]		1 African 1 European 1 Japanese [b]		19 African-American 20 European-American[c]		30 African-American 30 European-American[c]	
	NI [d]	*Z**	*NI*	*Z**	*NI*	*Z**	*NI*	*Z**	*NI*	*Z**
ATPase	2.27	-0.37	2.78	-0.44	2.84	-0.45	1.62	-0.22	1.65	-0.22
COI	**12.50**	**-1.08**	6.89	-0.88	**10.33**	**-1.03**	**3.61**	**-0.56**	**3.68**	**-0.56**
COII	3.33	-0.52	5.80	-0.82	3.28	-0.52	0.86	-0.13	0.64	-0.01
COIII	2.36	-0.53	1.38	-0.14	1.60	-0.39	1.38	-0.23	0.95	-0.08
Cyt-b	**3.78**	**-0.57**	3.64	-0.55	3.64	-0.55	2.29	-0.36	**3.23***	**-0.50**
NDI	1.58	-0.28	2.51	-0.43	2.63	-0.45	1.49	-0.20	1.49	-0.19
NDII	**5.86**	**-0.76**	**5.86**	**-0.76**	**7.73**	**-0.86**	3.59	-0.55	**2.95**	**-0.46**
NDIII	2.83	-0.52	6.80	-0.77	2.83	-0.52	1.33	-0.28	1.52	-0.25
NDIV	0.93	-0.09	0.52	0.05	0.52	0.05	**0.13**	**0.60**	**0.11**	**0.70**
NDIVL	2.20	-0.34	2.63	-0.42	2.63	-0.42	5.00	-0.62	4.00	-0.54
NDV	2.00	-0.32	2.38	-0.39	2.23	-0.37	1.20	-0.09	1.44	-0.16
NDVI	**30.71***	**-1.22**	5.50	-0.74	1.79	-0.25	1.30	-0.20	1.17	-0.16
All coding	**2.88***	**-0.46**	**2.50***	**-0.40**	**2.41***	**-0.39**	**1.46**	**-0.17**	**1.55**	**-0.19**
NI_{TG} (CI)[e]	**2.85** (1.83,4.99)		**2.60** (1.65,3.91)		**2.56** (1.59,3.81)		1.48 (0.92,2.25)		1.59 (0.93,2.36)	

[a] MK table counts from Nachman et al. (1996)

[b] MK table counts as above, but substituting two different, randomly chosen Japanese samples

[c] MK table counts from African-American and European-American sequences sampled from (Just *et al.* 2008) and (Rubino *et al.* 2012) with the chimpanzee mitochondrial reference genome as an outgroup (Horai *et al.* 1995).

[d] A count of 1 was added to each cell when calculating NI for an locus with a zero count in any cell. **Bold** indicates $P \leq 0.05$; * indicates significant a sample-wise Bonferroni-corrected *P*-value of less than 0.004 for Fisher's exact test of the MK table.

[e] Calculated as in Table 3. No sample rejected Woolf's test of homogeneity ($P > 0.19$ for all samples).

Bold indicates that the confidence intervals do not overlap the neutral expectation of 1.

Figure 1

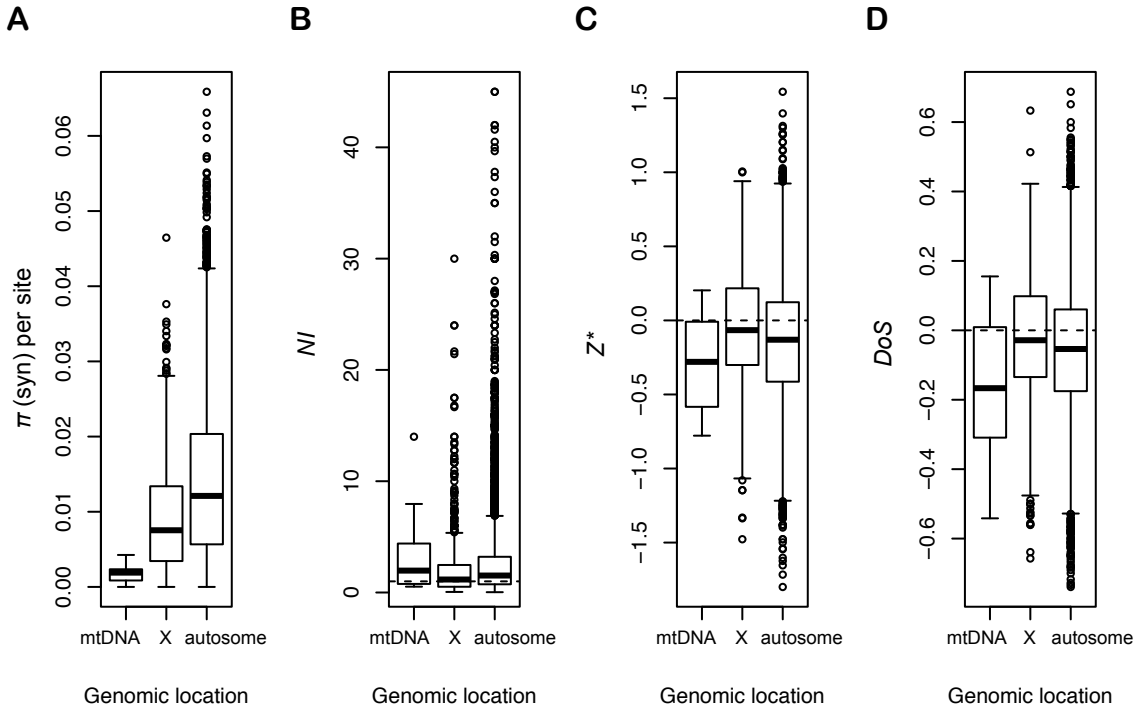

Figure 1 Effect of genomic location on silent-site heterozygosity and on summary statistics of polymorphism and divergence in *D. melanogaster.* (A) Genomic location has a significant effect on silent-site heterozygosity ($P_{MWU} < 0.001$ for all pairwise contrasts), consistent with predicted differences in N_e between these chromosomes. The ratio of median mitochondrial to autosomal silent site heterozygosity was 0.157, less than predicted for neutral sites if mitochondrial N_e is one quarter that of the autosomes. mtDNA, X-chromosome, and autosome data sets contained 12, 1255, and 8073 genes, respectively. (B-D) Distributions of neutrality indices *NI*, *Z** and *DoS* are similar between mitochondrial and nuclear genes despite differences in N_e. Dashed lines represent the neutral expectations for these statistics. Three nuclear loci for which *NI* exceeded 50 were excluded from (B) to improve visualization. See main text for description of statistics, number of genes in each dataset and statistical results.

Figure 2

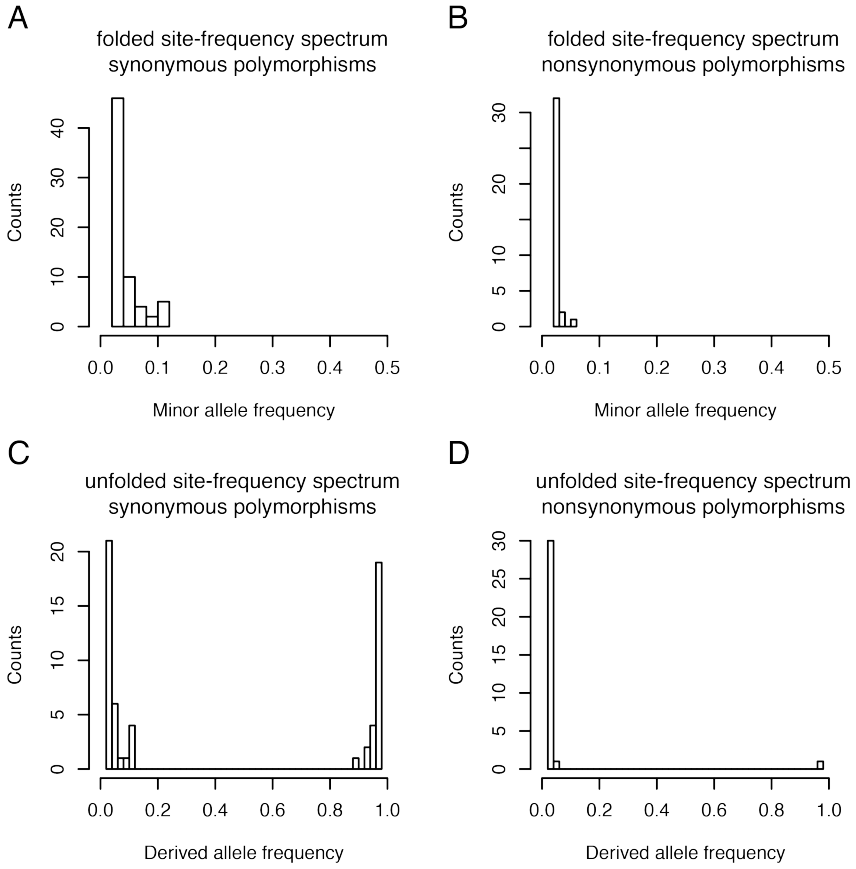

Figure 2 Site-frequency spectra of synonymous and nonsynonymous polymorphisms in the *D. melanogaster* mtDNA. (A, B) Folded site-frequency spectra for synonymous and nonsynonymous segregating sites across the mitochondrial coding region reveal that mitochondrial polymorphisms are skewed to low frequencies and are more so for nonsynonymous sites. (C, D) Unfolded site-frequency spectra reveal that derived, synonymous polymorphisms are almost equally likely to be at low frequency (56% of 59 sites at frequencies less than 0.11) or nearly fixed (44% of 59 sites at frequencies greater than 0.89), while derived, nonsynonymous polymorphisms are nearly always present as singletons (94% of 32 sites). There are essentially no mitochondrial polymorphisms at intermediate frequencies. Sites were omitted from the unfolded site frequency spectra if neither allelic state was shared with *D. yakuba*. The number of sites included in each distribution is 67 (A), 35 (B), 59 (C), and 32 (D).

Figure 3

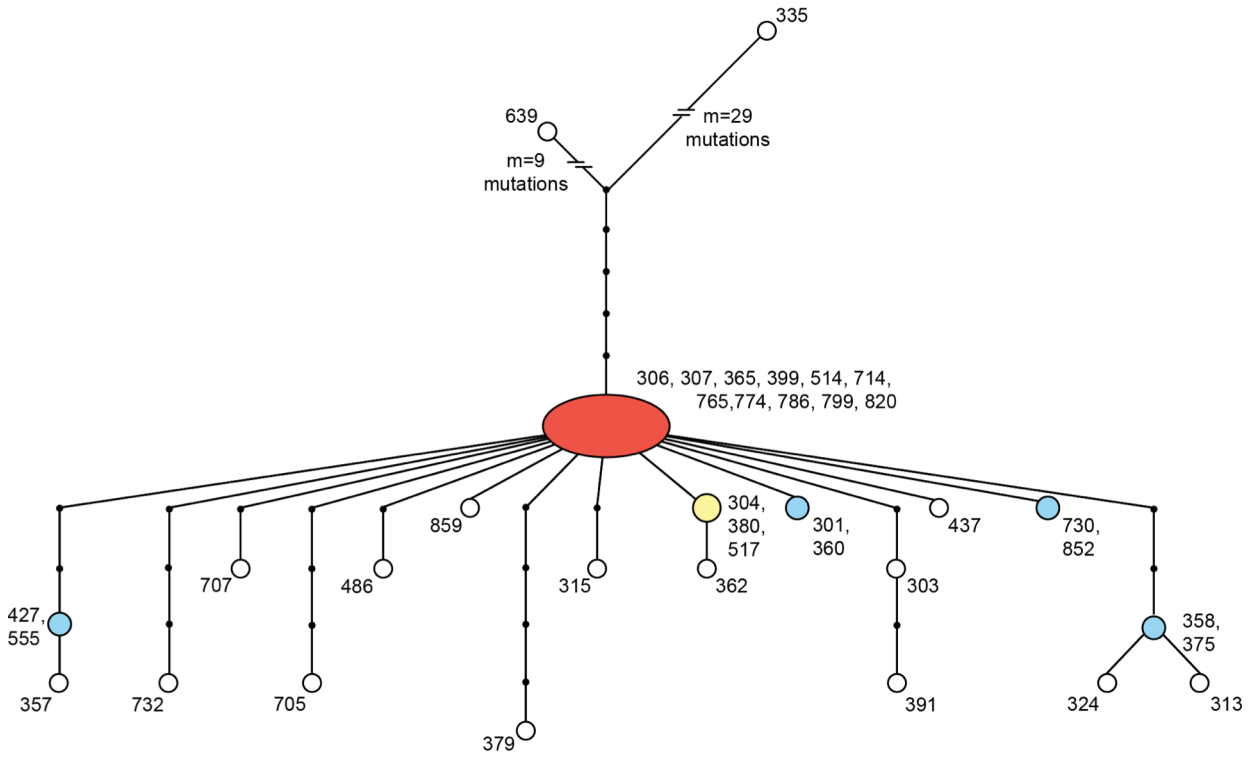

Figure 3 Haplotype network for 38 *D. melanogaster* mtDNAs sampled from Raleigh, NC. The network, inferred from 80 coding region SNPs with no missing information, reveals that nearly 30% of individuals sampled (11/38) share the same common haplotype (red) and an additional 65% of individuals carry a haplotype only a few mutations away from this haplotype. This common set of mitochondrial haplotypes is highly diverged from the two other mtDNAs sampled in the population; lines RAL-639 and RAL-335 differ from the common haplotype at 14 and 34 SNPs, respectively. At least one of these two haplotypes carries the ancestral state (shared with *D. yakuba*) at 38% of these SNPs. Numbers represent the Raleigh (DGRP) line carrying the haplotype. Red, yellow, blue and white nodes were present in 11, 3, 2 and 1 lines, respectively.

Figure 4

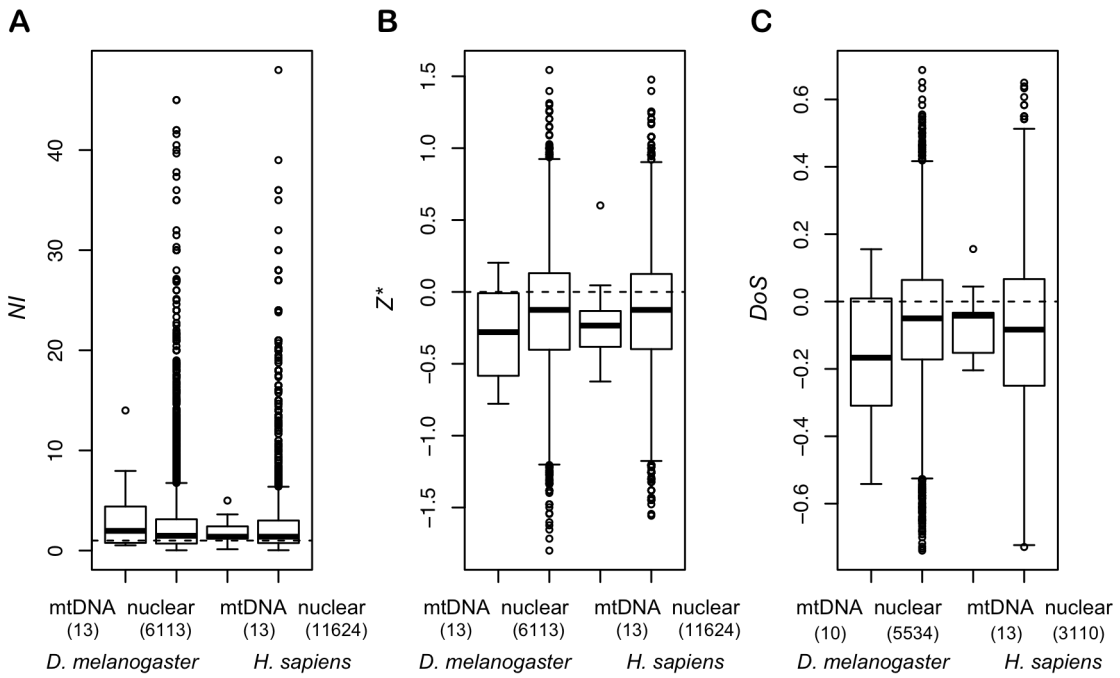

Figure 4 Distributions of polymorphism and divergence summary statistics for mitochondrial and nuclear genes in flies and in humans. (A) Distributions of *NI* are similar between genomes within species and across species. Values of *NI* greater than 50 were removed to improve visualization (3 nuclear genes for flies and 2 nuclear genes for humans). Distributions of (B) *Z** and (C) *DoS* for mitochondrial genes are also similar to those for nuclear genes in both flies and humans (see text for statistical contrasts). Positive values of these summary statistics of the MK contingency table are consistent with positive selection and negative values with negative selection. Dashed lines represent the neutral expectation for each statistic, and the numbers of genes in each set are indicated in parentheses.