

CYTONUCLEAR COADAPTATION IN *DROSOPHILA*: DISRUPTION OF CYTOCHROME C OXIDASE ACTIVITY IN BACKCROSS GENOTYPES

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Abstract.—The cytochrome *c* oxidase enzyme (COX) is comprised of 10 nuclear-encoded subunits and three mitochondrial-encoded subunits in close physical association in the inner mitochondrial membrane. COX passes electrons from cytochrome *c* to molecular oxygen and pumps protons into the inner mitochondrial space for ATP production. Selection on nuclear-mitochondrial interactions within species should lead to coadaptation of the proteins comprising this important enzyme. Under this model, there should be relatively little disruption of COX activity when mitochondrial genomes are crossed among strains within species. A more pronounced disruption of activity is expected when the mitochondrial genome is expressed in the nuclear background of a different species. We test these hypotheses in *Drosophila* using hybridization and backcrossing among lines of *D. simulans* and *D. mauritiana*. Disrupted cytonuclear genotypes were constructed using backcrosses between two lines of *D. simulans* (*siI* and *siII*) that introduced each divergent mitochondrial DNA (mtDNA) into each nuclear background due to maternal inheritance of mtDNA. Similar crosses were used to introduce each *D. simulans* mtDNA into the *D. mauritiana* nuclear background. Reconstituted cytonuclear control genotypes were constructed by backcrossing the initial F₁ females to males of the maternal genotype. COX enzyme activities were compared among these disrupted and reconstituted backcross genotypes within and between species. The disruption effect on COX activity was restricted to males of interspecific genotypes. These data support the coadaptation hypothesis and are consistent with predictions that the evolution of modifiers of male mitochondrial dysfunction is hindered by the maternal inheritance of mtDNA. New sequence data for nuclear encoded subunits of COX identified amino acids that may play a role in the disruption effect.

Key words.—Coadapted gene complex, coevolution, mitochondrial DNA, oxidative phosphorylation.

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It is now clear that mitochondria are descendants of free-living bacteria that became incorporated into the cytoplasm of ancestors of present-day eukaryotic cells (Margulis 1970; Gray et al. 1999). Whole-genome sequence analysis of bacterial and endosymbiont genomes has clarified the role of gene transfer from protomitochondrion to host nuclear genome in the origin of eukaryotes (Andersson et al. 1998; Gray 1999; Rotte et al. 2000). As a consequence of this fusion of genomes, mitochondrial function in animals requires the coordinated expression of hundreds of nuclear-encoded genes and 37 mitochondrial genes. Grivell et al. (1999) estimated that about 12% of the yeast nuclear proteome functions in the mitochondria, and a recent study identified a large class of novel nuclear genes with mitochondrial functions (Steinmetz et al. 2002). While gene transfer from mitochondrial DNA (mtDNA) to nucleus may have ceased in animals (Boore 1999), fitness or performance traits are likely to depend critically on nuclear-mitochondrial interactions.

A number of studies have tested for nuclear-mitochondrial interactions using either fitness assays (e.g., Clark and Lyckegaard 1988; Hutter and Rand 1995; Rand et al. 2001), enzyme activities (e.g., Kenyon and Moraes 1997; Burton et al. 1999; Rawson and Burton 2002), or sequence comparisons (e.g., Schmidt et al. 2001). These interactions provide an appealing context for the experimental analysis of coadaptation for several reasons. First, nuclear and mitochondrial genomes have a long evolutionary history of association and their interactions are essential for eukaryotic function. Second, analyses of mtDNA sequence divergence have estab-

lished that deleterious mutations are a primary force in mtDNA evolution (Lynch and Blanchard 1998; Nachman 1998; Rand and Kann 1998; Rand 2001). Because coadapted gene complexes can evolve from compensatory mutations that follow deleterious mutations in interacting loci, the genetics of nuclear-mitochondrial interactions likely predispose them for coadaptation. Third, the maternal inheritance of mtDNA allows for easy manipulation of nuclear-mitochondrial genotypes so that the crucial empirical tests of coadaptation can be conducted. The power of these tests lies in clear predictions of the manipulations: fitness should be reduced when a given mtDNA is transplanted onto a foreign nuclear genetic background.

Support for nuclear-mitochondrial coadaptation has been provided from both cell culture and whole-animal studies. Mitochondrial activity is reduced in somatic cell models where mouse nuclear chromosomes and rat mtDNA reside in the same cell (Dey et al. 2000; McKenzie and Trounce 2000; Yamaoka et al. 2000). In primate cells where the mitochondria from one species are introduced into the nuclear background of a different species, these “xenomitochondrial cybrids” allow a detailed analysis of biochemical function. Several studies suggest a threshold effect of nuclear-mitochondrial disruption. Oxidative respiration was restored in human mitochondria-less cells repopulated with mitochondria from either chimpanzees or gorillas, but not when orangutan mitochondria were introduced (Kenyon and Moraes 1997). ATP synthesis also shows a threshold effect as a function of the proportion of mutant mtDNAs within heteroplasmic cells (D’Aurelio et al. 2001). Further analyses of human-orangutan cybrids indicate that nuclear-nuclear interactions may play a role in the genetic basis of mitochondrial dysfunction (Barrientos et al. 1998). While cell culture methods

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provide a powerful means of manipulating nuclear-mitochondrial interactions, it is not clear how these results relate to fitness differences among genotypes that might be observed in nature.

In whole-animal systems, cytonuclear genotypes can be manipulated through hybridization and backcrossing. The maternal inheritance of mitochondria in animals allows mtDNA to be crossed onto two different nuclear backgrounds, so that disrupted and native cytonuclear genotypes can be compared experimentally. Fitness assays have shown that nuclear-mitochondrial interactions can be detected among geographic lines of *Drosophila melanogaster*, but clear evidence for coadaptation (i.e., native cytonuclear genotypes having higher fitness than disrupted genotypes) is not evident within species (Clark 1985; Fos et al. 1990; Kilpatrick and Rand 1995). A population cage study using *D. pseudoobscura* and *D. persimilis* supported the coadaptation hypothesis on one nuclear background, but not the other (*D. pseudoobscura* mtDNA showed higher fitness on the *D. pseudoobscura* nuclear background but was neutral on the *D. persimilis* background; Hutter and Rand 1995).

Whole-animal studies of cytochrome *c* oxidase (COX) activity are an appealing system with which to study nuclear-mitochondrial coadaptation because the enzyme is comprised of both nuclear and mitochondrial subunits in physical contact in the inner mitochondrial membrane (Saraste 1999). In a series of studies with intertidal copepods (*Tigriopus californicus*), Burton and coworkers have demonstrated that nuclear-nuclear and nuclear-mitochondrial interactions are partly responsible for variation in COX activity, and that backcrossing of isolated intraspecific lines can disrupt COX activity in some cases (Burton et al. 1999; Edmands and Burton 1999). The characterization of cytochrome *c* (*cyt c*) from this species (Rawson et al. 2000) has allowed the identification of effects on viability of *cyt c*-cytoplasm interactions, suggestive of coadaptation among specific proteins in the oxidative phosphorylation pathway (Willett and Burton 2001; Rawson and Burton 2002). An attractive feature of *Tigriopus* is the extensive divergence among conspecific populations at genes encoding COX subunits in mtDNA and in *cyt c*, providing phenotypically distinct genotypes for experimental analysis within species (Burton and Lee 1994; Rawson et al. 2000). While the *Tigriopus* studies provide compelling evidence for coadaptation among some pairs of populations, it is clear that the biochemistry, cell biology, and cytonuclear genetic architecture of COX activity are complex (Edmands and Burton 1998). Additional analysis of nuclear and mitochondrial components of COX activity are needed in systems that are also amenable to genetic manipulation.

Drosophila provides an attractive model with which to dissect cytonuclear coadaptation. Many genetic tools are available and complete mtDNA sequences have been published for divergent lineages (Ballard 2000a), many of which can produce fertile F₁ females for backcross analyses. To date, no studies of the evolutionary genetics of COX activity have been conducted in *Drosophila*. As a first step in dissecting the genetic architecture of COX in *Drosophila*, we present a backcross-based approach to studying the coadaptation of COX nuclear and mitochondrial factors in the *D. simulans* clade. We used two divergent lines of *D. simulans* carrying

distinct mtDNAs (*siI* and *siII*) and the *maI* line of *D. mauritiana* as a nuclear outgroup (Harr et al. 1998; Kliman et al. 2000). Mating incompatibilities prevent reciprocal crosses between *D. simulans* and *D. mauritiana*, but the two *D. simulans* mtDNAs can each be introgressed into intraspecific (*siI*, *siII*) or interspecific (*maI*) nuclear backgrounds. Compatible backcrosses between these lines created disrupted or reconstituted cytonuclear genotypes for comparison of COX activity.

The mtDNA phylogeny shows that the *D. mauritiana* (*maI*) haplotype is virtually identical to the *D. simulans* *siIII* haplotype, which is allopatric from the other two *D. simulans* mtDNAs (*siI* and *siII*; Solignac et al. 1986; Ballard 2000a,c). This implies a history of introgression between female *D. simulans* *siIII* and *D. mauritiana* (Ballard 2000c). Although the *maI* line of *D. mauritiana* may carry some *D. simulans* nuclear alleles, its use as an interspecific nuclear background provides a conservative test of the coadaptation hypothesis. Because primate cell culture studies suggest that cytonuclear disruption is only apparent with hybrid pairs more distant than human-gorilla (Kenyon and Moraes 1997), evidence for cytonuclear disruption within the *D. simulans* clade would provide motivation for further studies of the genetic architecture COX activity in the *Drosophila* system. Here we show that backcross genotypes between *D. simulans* mtDNA haplotypes and males of a *D. mauritiana* *maI* line have reduced COX activity relative to the reconstituted controls. This disruption effect is stronger in males than females, as has been predicted by some models of mitochondrial dysfunction (Frank and Hurst 1996). We report new sequence data for eight of the nuclear-encoded subunits of COX in an effort to identify amino acid changes related to the disruption of COX activity.

MATERIALS AND METHODS

Fly Lines and Maintenance

Three fly lines were used in these experiments: *D. simulans* Kenya (mitochondrial type *siII*), *D. simulans* Seychelles (*siI*), and *D. mauritiana* G122 (*maI*). The nuclear phylogeny of these three lines clearly places *D. mauritiana* as an outgroup to the *D. simulans* lines (Harr et al. 1998; Kliman et al. 2000; Ting et al. 2000). The mtDNA phylogeny of these three lines, however, places *siII* and *D. mauritiana* *maI* as sister taxa, with *siI* as an outgroup (Ballard 2000a). The *maI* mtDNA is virtually identical to a third haplotype of *D. simulans* (*siIII*) presumably stemming from introgression of *siIII* mtDNA into the nuclear background of *D. mauritiana* (Ballard 2000a,c). Despite these discordant phylogenies and introgression, an average allele in the *siI* or *siII* nuclear genomes has had a longer history of association with their respective mtDNAs than has an average allele in the nuclear genome of *D. mauritiana*. Because there is no reciprocal monophyly for nuclear alleles between the two mtDNA lines within *D. mauritiana* (*maI* and *maII*; Hey and Kliman 1993; Kliman et al. 2000), *maI* nuclear alleles should be effective at disrupting cytonuclear function in combination with either *siI* or *siII* mtDNA. As mentioned above, *maI* provides a conservative test of the coadaptation hypothesis. Below we refer to crosses involving *maI* males as interspecific crosses.

Flies were maintained at 25°C. Seven-day-old adults were frozen in liquid nitrogen and stored at -80°C for use in the COX assay. All fly stocks were treated with 0.25 mg/ml tetracycline added to dry food (Carolina Biological, Burlington, NC) for two generations to remove *Wolbachia* bacteria. All hybridization and backcrossing was initiated after tetracycline treatment was completed.

Backcrossing Procedure

Approximately 10 virgin females were placed in a vial with 10–15 males in the following combinations: *D. simulans* Seychelles female × *D. simulans* Kenya male (denoted the SK cross), Kenya female × Seychelles male (KS cross), *D. simulans* Seychelles female × *D. mauritiana* G122 male (SM cross), and *D. simulans* Kenya female × *D. mauritiana* G122 male (KM cross). Because the cross *D. mauritiana* females × *D. simulans* males is not fertile, reciprocal crosses could only be established within *D. simulans* (i.e., the *maI* mtDNA was not incorporated in the crossing design). Virgins were collected from these hybrid matings, and 10 virgin females of each F₁ genotype (SK, KS, SM, KM) were put in two sets of new vials and backcrossed to males from either the paternal line or the maternal line. Because mitochondria are inherited from the maternal line, all hybrids will have 100% of the maternal mtDNA, but only 50% maternal nuclear DNA. Backcrossing to males from the paternal line will gradually increase the proportion of nuclear alleles from the paternal line, and thus will eventually re-establish the paternal chromosomal constitution, but with the maternal mtDNA (referred to as a “disruption” cross). When backcrossing is done using males from the maternal line, this will restore the nuclear alleles from the maternal line, and thus will reconstitute the original maternal stock (the “reconstitution” cross). For example, KM virgin females (from a *D. simulans* Kenya female × *D. mauritiana* male) were backcrossed to *D. mauritiana* males producing the disrupted KM × M cyto genotype. Siblings of these same KM virgin females were backcrossed to Kenya males producing the reconstituted KM × K cyto genotype. Thus, disrupted cytonuclear genotypes serves as experimentals, and reconstituted cytonuclear genotypes serve as controls. This backcross procedure was carried out for each viable F₁ hybrid cross. Listed as disruption/reconstitution pairs, the following experimental genotypes were used: SK × K/SK × S; KS × S/KS × K; KM × M/KM × K; SM × M/SM × S.

Backcrossing continued through five generations (F₁, plus B₁–B₅). Flies were frozen after each generation and analyses were done on generations B₁–B₅ with sample sizes 220, 162, 148, 118, 118, respectively, across the eight genotypes. Assuming neutrality and free recombination during the backcrossing, the proportion of nuclear alleles in a given generation should be $1 - (1/2)^n$, where $n = 1$ for F₁, 2 for B₁, 3 for B₂, and so on. As described below, disruption effects do increase with backcross generation, but the main analyses were done on data pooled across backcross all generations. The weighted average of the proportion of nuclear alleles paired with a given mtDNA was 0.88, based on these assumptions and the sample sizes for backcross generations B₁–B₅ stated above.

COX Assay

Procedures for microplate assays of COX were modified from previously published methods in (Clark and Keith 1989; Edmands and Burton 1998, 1999). Cyt *c* from horse heart (Sigma Chemical, type VI) was used as the substrate for all COX assays. The effect of species origin of cyt *c* on COX activity was examined by performing the assay on a sample of *D. melanogaster* flies using horse, chicken, or yeast cyt *c* as a substrate. The correlation between the activities of the lines using horse versus chicken cyt *c* was significant with an R^2 of 85% (see Fig. 1 and Results below). Cyt *c* was prepared by diluting 100 mg of protein in 10 ml of water, reducing this solution with 5–10 mg of ascorbic acid, and dialyzing at 4°C overnight, in the dark, with 500 ml of phosphate buffer (1 M Na₂H₂PO₄, pH 7.1; Spectra/Por 7 dialysis tubing with a molecular weight cut off 3500 was used; Spectra Laboratories, Rancho Dominguez, CA). The reduced cyt *c* was then diluted to 25 μM using an extinction coefficient at OD₅₅₀ of 29.5. The reduction of the cyt *c* was tested before each use by comparing the OD at 550 nm and 565 nm; if the ratio was under 8, the substrate was not used.

Three flies of the same genotype and sex were pooled and ground in 100 μl of cold homogenization buffer (0.05% Tween-80 in phosphate buffer). The homogenates were then diluted with an additional 500 μl of homogenization buffer, to a total concentration of one fly per 200 μl. The samples were then spun down at 2000 g for 5 min at 4°C and 40 μl of each sample was aliquotted in triplicate to two replicate 96-well plates. A third plate, for protein concentration determination, was aliquotted in triplicate with 10 μl per well of sample. Plates were frozen at -80°C until use.

To run the COX assay, a plate was thawed and allowed to equilibrate to room temperature. A volume of 150 μl of reduced cyt *c* was added to each well, and the plate was assayed in a plate reader, reading every 10 sec at 550 nm for 4 min and 30 sec in a Molecular Devices (Global Medical Instrumentation, Albertville, MN) Vmax microplate reader. As COX oxidizes cyt *c*, the optical density of the solution declines at 550 nm. The plate reader calculates the maximum slope of the plot of optical density, which is taken as V_{\max} for the reaction. The V_{\max} data from the Molecular Devices Softmax software was then transferred to an Excel spreadsheet for analysis. Protein concentration for each homogenate was determined using the BCA method (Pierce, Rockford, IL).

Data Analysis

A total of 776 COX activity assays were determined among the eight cytonuclear genotypes. Sample sizes for the disrupted/reconstituted genotype pairs were as follows: SK × K/SK × S (98/110); KS × S/KS × K (107/100); KM × M/KM × K (88/109); SM × M/SM × S (73/81), split approximately evenly between males and females. Analyses were done on flies from backcross generations B₁–B₅, as described above. Within a genotype, sex, and generation, sample sizes ranged from six to 15 assays. Samples for SM × M were not available in backcross generation B₅, but the results presented below are the same when all B₅ data are omitted.

Homogenates from all genotypes and both sexes were

TABLE 1. Microsatellite primers used for analysis of backcross genotypes. Chromosome band refers to the chromosome band location of the primer. Primers were obtained from the Web sites of the laboratories listed in the Source column: Aquadro (<http://www.mbg.cornell.edu/aquadro/aquadrolab.html>), Schlötterer (<http://i122server.vu-wien.ac.at/c.s.laboratory.html>), and Goldstein (<http://fruitfly4.aecom.yu.edu/links.html>).

Locus	Chromosome band	Forward	Reverse	Source
DMZW3K25	3A	ATTGTCATTTTATTGCTGCCG	TAACGAAGAGAGTTGCGGAGA	Aquadro
DS00146	4A4-B2	GAGTCAACGAGCCAGCAAAGT	AACAATACAGAGCAGCACACG	Schlötterer
DELTEX	6A1-2	TACGCAATAAGTTGGCGTA	AATCAGGATAATGCCTAATACTAGT	Aquadro
Drosev1	10A1-2	ATACAAGATACATCCGTGCGC	CCCAACTGAAAAGCAACTCC	Aquadro
Drogpdha	25F5-26A	CATTGGAAAAGTGAGCGGAT	CGGACAACAACAAATCGTTG	Schlötterer
Bib	30F	TCGCAAGGATCAGCGGTGAC	TTGGGCCTCAGCGGCAGAAT	Aquadro
ac006302	34C4-D2	TGTTTTCCATGCCAGCTAGT	GCCCGGAAAATTCTTGTTTA	Goldstein
ac004759	38E1-E9	ACAGACGGAAAGCCAAAATG	CACTCCGCCTCGTTTCTTAC	Goldstein
Master	50C	CAGCAGCAGATCCAAGTTCA	GTTTGCATTGTAGGGCGAGT	Aquadro

blocked onto individual microplates in triplicate. Space limitations on each plate required backcross generation to be confounded with block (i.e., plate) effects. To standardize COX activity across the dataset, all raw activity scores generated by the Softmax microplate software (maximum slope of the plot of optical density) were corrected for protein concentration based on the BCA assay of the same homogenates (see above). These protein-corrected activities were then subjected to ANOVAs to test the hypothesis that disruption/reconstitution, intra/interspecific, male/female, and backcross generation had main and interaction effects. These ANOVAs revealed that the disrupted-interspecific-male samples showed significantly reduced COX activity when pooled across backcross generation. To test the hypothesis that this effect could be due to spurious plate effects, residuals were generated from the two-way ANOVA: protein-corrected activity = plate + well + (plate × well) + error, where well is an individual well in a microplate. The disrupted-interspecific-male effect was observed again. We report the simpler protein-corrected result as they do not differ qualitatively from the corrections based on residual analyses of plate effects. While COX activities in the disrupted-interspecific-male samples decreased with backcross generation, these trends were not statistically significant. Below we present results for both the pooled data and for the individual backcross generations. The JMP statistical package (Statistical Discovery Software, <http://www.jmp.com>) for Macintosh computers was used for all analyses.

Microsatellite Analysis

To check that the backcrossing introduced the paternal alleles into the experimental genotypes, microsatellites were scored for several loci in samples of backcross flies. The parental lines were scored for diagnostic microsatellites, and then a sample of four female flies (eight alleles) for each genotype was scored in the fourth backcross generation. Flies were prepared using a Tris/NaCl/Proteinase K buffer following the procedures described in Rand et al. (1994). Microsatellites were amplified in 25-ml reactions using 2 µl of fly homogenate, 2.5 µl of Promega (Madison, WI) Taq buffer, 2.0 µl of 10 mM dNTPs, 2.5 mM MgCl₂, 0.3 µl of each primer (from a 10-pM/µl stock), and 0.1 µl of Promega Taq polymerase. Amplification was for 35 cycles of 95°C for 30 sec, 54°C for 1 min, 72°C for 1 min, followed by 10 min at 72°C.

The primers used in this analysis are listed in Table 1. The reverse primers were fluorescently labeled. Amplified microsatellites were run on an ABI 377 automated sequencer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's specifications. Fragment sizing was done using ABI GenScan software.

Sequence Analysis

The nucleotide sequences of the mtDNA-Encoded subunits of COX were obtained from the GenBank files reported in Ballard (2000a). Due to selective sweeps within *D. simulans* (Ballard 2000b), there is very little variation within the *siI* or *siII* haplotypes, hence the published sequences should be nearly identical to the lines of *siI* and *siII* used in our COX assays. There is relatively little mtDNA variation within *D. mauritiana* *maI* (Solignac et al. 1986). While there may be some synonymous variation between the reported sequence of *maI* and the G122 line used in our study, we assume that there are no amino acid differences.

Sequence data from eight of the nine nuclear encoded subunits of COX that have been identified in *D. melanogaster* (Szuplewski and Terracol 2001) were determined for each of the three lines used in this study (*siI*, *siII*, and *maI*). Genomic DNA was prepared from an individual female of each line using a Tris/NaCl/Proteinase K buffer following the procedures described in Rand et al. (1994). Subunits IV, Va, Vb, VIb, VIc, VIIa, VIIc, and VIII were amplified from genomic DNA using the primers listed in Table 2. Primers were designed in noncoding DNA flanking the coding region as reported in the complete *Drosophila* genome (Rel. 2, Berkeley *Drosophila* Genome Project available on FlyBase: www.fruitfly.org). The Vb sequence was derived from primers that overlap the 5' and 3' ends of the coding sequence, so differences in these regions will be missed. Putative nuclear subunits were based on BLAST searches reported in Szuplewski and Terracol (2001). Amplified products were purified in QIAquick PCR purification columns (Qiagen, Valencia, CA) and sequenced on an ABI 377 automated sequencer. All sequences were determined in both directions. All primer pairs amplified the desired sequence from *D. melanogaster*, and because no ambiguous amplification products were produced, we assumed that the homologous sequence was also amplified from *D. simulans* and *D. mauritiana* lines used in this study. Sequences were aligned in ClustalW and

TABLE 2. Polymerase chain reaction primers used to amplify nuclear-encoded cytochrome *c* oxidase subunits in *Drosophila* species.

Gene	Primer (5' → 3')	Primer name
COX 4	CTACCTTGCAGACTCTGTGAG	ext4F
	CTTAATCACACACAATCGCGG	ext4R
COX 5A	CGTGTATATTGTGTTCTGTG	ext5AR
	GTGAGTCGAGCTGTTACTTA	ext5AF
COX 5B	ATGGCATCGATCTGTGGACGCA	ext5BF
	TAAACAGCTGCCTTCTCCACCAGC	ext5BR
COX 6B	ATGTCCAAAAACAAAAGCAG	6BF
	TTAGATGCGCGCCGGGAATG	6BR
COX 6C	GCGTTTAATGGCAGCTGCCT	ext6CF
	TCAATACAGAAGCGCAACTC	ext6CR
COX 7A	GTTCGGAATACTCCAGAAAG	ext7AF
	GTCGTACAATGGTATTGTC	ext7AR
COX 7C	GGGCCGAGCAGTGTGATTG	ext7CF
	CATTGTGTTGCTGTGCCT	ext7CR
COX 8	CTCTCTACTTTACAAGCGTA	ext8R
	TTTACGCCAGCGTATTTTGC	ext8F

showed no stop codons or alignment ambiguities. The numbers of synonymous and nonsynonymous differences among the three lines were determined in MEGA (Kumar et al. 2001), and d_N/d_S ratios were calculated in PAML (Yang 2002) using the (Nei and Gojobori 1986) method. The primers used to amplify and sequence these genes are listed in Table 2. The sequences have been deposited in GenBank (accession numbers AY307958–AY307981).

RESULTS

Microsatellite Analysis

Table 3 shows estimates of allele frequencies in the backcross genotypes analyzed for COX activity. The data listed are the frequencies of the backcross male allele, and the expected allele frequency for this backcross 4 generation is 0.94, assuming neutrality and free recombination. The samples are generally very close to this expectation, showing clear effects of the backcross process. One reconstitution genotype (KM × K) appears to have retained some of the *D. mauritiana* alleles (frequency of the paternal K alleles = 0.79).

COX Activities

Figure 1A shows that the use of different cyt *c* substrates produces very similar COX activities across a panel of *D. melanogaster* homogenates. Males and females from five different wild lines of *D. melanogaster* were scored for COX activity using horse, chicken, and yeast cyt *c* as substrate. The horse and chicken data are significantly correlated ($R^2 = 0.85$, $P < 0.01$). The horse and yeast data are not, due to one outlier ($R^2 = 0.35$, $P > 0.05$), although a trend is clear. These data indicate that the COX assay we used is a reliable indicator of genotype.

Figure 1B shows the COX activity for the parental lines used in the backcrosses. For females, *D. mauritiana* has significantly higher COX activity than *D. simulans* ($P < 0.01$), but the two *D. simulans* lines do not differ significantly. For males, *D. mauritiana* shows the highest activity, *siII* (from Kenya) is intermediate, and *siI* (from Seychelles) is lowest ($P < 0.05$ for each comparison, Tukey-Kramer HSD tests adjusted for multiple tests). The primary result from these analyses is that the *D. mauritiana* line does not have lower COX activity than either *D. simulans* line, which might com-

TABLE 3. Microsatellite allele frequencies in backcross genotypes. The genotypes listed at the top refer to female × male × backcross male, and the R or D in parentheses refers to reconstituted or disrupted with respect to cytonuclear composition (see text for details). The DMZW, Bib, and ac004759 loci were only informative for the K and M lines and were not used for scoring the genotypes involving K and S. The frequencies reported are for the backcross male allele (e.g., K allele in KS × K flies). The data are based on a sample of four females (eight alleles) from the fourth backcross generation (the two entries of 0.83 are from samples showing five of six paternal alleles).

Locus	Intraspecific				Interspecific			
	KS × K (R)	KS × S (D)	SK × S (R)	SK × K (D)	KM × K (R)	KM × M (D)	SM × S (R)	SM × M (D)
DS00146	1.00	1.00	1.00	0.88			0.83	0.88
DELTEX	1.00	1.00	1.00	1.00			1.00	1.00
DROSEV1	1.00	1.00	1.00	0.63	0.75	1.00	0.88	1.00
DROGPDHA	0.88	0.88	0.88	1.00			1.00	1.00
ac006302	0.88	1.00	1.00	0.88			1.00	1.00
Master	1.00	1.00	1.00	0.63			1.00	
DMZW						1.00		
Bib					0.83	1.00		
ac004759					0.75	1.00		
Average	0.96	0.98	0.98	0.83	0.79	1.00	0.95	0.98

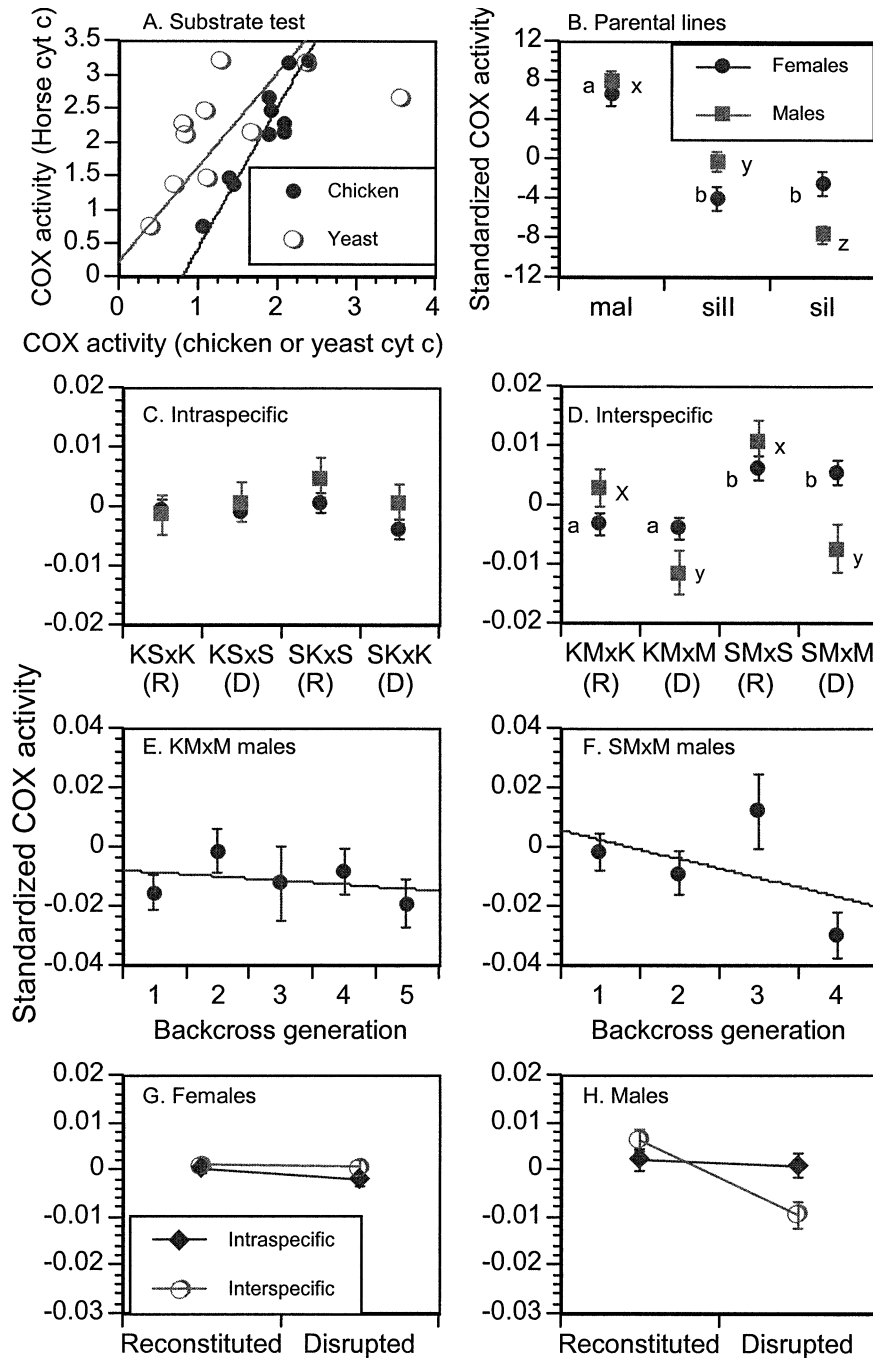


FIG. 1. Cytochrome *c* oxidase (COX) activities in different *Drosophila* samples. (A) COX activities for 10 *D. melanogaster* homogenates using three different cytochrome *c* substrates (horse, chicken, and yeast). The horse-chicken correlation is significant ($R^2 = 0.85$, $P < 0.01$), indicating that the heterologous source of cytochrome *c* provides a reliable assay. (B) COX activities for the parental strains used in this study (*mal*, *D. mauritiana*; *sil*, *D. simulans* from Kenya; *sil*, *D. simulans* from Seychelles). Different lowercase letters indicate significant differences ($P < 0.05$ a, b, or c for females; x, y, or z for males; Tukey-Kramer HSD tests corrected for multiple comparisons). (C) COX activities for intraspecific disruption (D) and reconstitution (R) backcross genotypes within *D. simulans* (KS × S refers to the disrupted genotype of K mitochondrial DNA [*D. simulans sil*] from Kenya) in an S [*D. simulans* Seychelles] nuclear background; see Materials and Methods for details). None of the samples are significantly different. (D) COX activities for interspecific disruption (D) and reconstitution (R) backcross genotypes between *D. simulans* and *D. mauritiana* (MK × M refers to the disrupted genotype of K [*sil*] mitochondrial DNA in an M [*D. mauritiana*] nuclear background). (E) COX activities in backcross generations of the KM × M disrupted males. (F) COX activities in backcross generations of the SM × M disrupted males. The B₄ generation is significantly lower than the others ($P < 0.05$). (G) Pooled data for females (no effects are significant). (H) Pooled data for males (the interaction between disruption/reconstituted and intra-/interspecific is significant; ANOVA, $P < 0.004$; see Table 4 for details). Standardization of COX activity was done separately for the data shown in panels A, B, and C–H, as reflected in the different scales on the y-axes (see Materials and Methods for details).

plicate the detection of a disrupted (i.e., lower) COX activity in backcross genotypes.

Figure 1C and 1D show COX activities for pooled data across the five backcross generations (B_1 – B_5). Protein-corrected, standardized COX activities are shown for the disrupted (D) and reconstituted (R) genotypes within *D. simulans* (Fig. 1C) and between *D. simulans* and *D. mauritiana* (Fig. 1D). S and K refer to the *D. simulans* Seychelles (*siI*) and Kenya (*siII*) lines, respectively, and M refers to *D. mauritiana maI*. The genotype notation on the x-axis is (F_1 : female \times male) \times backcross male (see Materials and Methods). Thus, $KS \times K$ is a reconstitution (R) cytonuclear genotype and $KS \times S$ is a disruption (D) genotype in the Kenya/*siII* cytoplasm. There is no significant difference between any of the four genotypes for either sex within *D. simulans* (Tukey-Kramer HSD tests). ANOVAs of COX activity in these samples cannot detect any main effects or interaction terms where sex and being (or not being) a disrupted genotype are modeled.

Figure 1D shows the COX activities for reconstituted and disrupted cytonuclear genotypes between *D. simulans* and *D. mauritiana*. As before, S and K refer to *siI* and *siII* lines of *D. simulans*, and M refers to *D. mauritiana*. Following the genotype notation of (F_1 : female \times male) \times backcross male, $KM \times K$ is a reconstituted interspecific cytonuclear genotype and $KM \times M$ is a disrupted genotype in the Kenya (*siII*) cytoplasm. In both cytoplasmic comparisons, disrupted males have significantly lower COX activity than their respective reconstituted controls (Fig. 1D; lowercase letters indicate significant differences between genotypes, $P < 0.05$, Tukey Kramer HSD tests correcting for multiple comparisons). The two reconstituted genotypes ($KM \times K$ and $SM \times S$) do not differ from each other, nor do the two disrupted genotypes ($KM \times M$ and $SM \times M$). In females, there is no difference in COX activity between disrupted and reconstituted genotypes, although the genotypes carrying the S mtDNAs show significantly higher COX activity than those carrying the K mtDNA (the parental S and K lines also differed in this direction; see Fig. 1B). Figure 1E and 1F show the COX activities for the disrupted interspecific male genotypes in each generation of backcrossing. A negative trend is observed for both genotypes, but the slopes are not significant. However, generation 4 of the $SM \times M$ genotype is significantly lower than the three earlier generations ($P < 0.05$, Tukey Kramer HSD tests).

The disruption effect on males in the interspecific comparison (Fig. 1D) is not simply a consequence of these backcrossed flies carrying *D. mauritiana* nuclear alleles coding for low COX activity. Protein concentrations were not significantly different between disrupted and reconstituted males, females of these same genotypes show no disruption effects, and *D. mauritiana* shows the highest relative COX activity of the parental lines (Fig. 1B). Together these data reveal a male-specific nuclear-cytoplasmic (mtDNA) interaction effect on COX enzyme activity.

This sex-specific disruption effect is summarized in Figures 1G and 1H, which present the data shown in Figures 1C and 1D with individual genotypes pooled into broader categories (male/female, disrupted/reconstituted, intra-/interspecific). Each panel was analyzed as a two way ANOVA

TABLE 4. Analyses of variance (ANOVA) for cytochrome *c* oxidase activity in females and males. Replicates and genotypes were pooled into intra- and interspecific categories and into disrupted and reconstituted cytonuclear categories as described in Materials and Methods. The significance of the disruption effect is greater in males. For males the pooling is justified by lack of significance among pooled genotypes. Females show a significant difference between the genotypes carrying the K and S mitochondrial DNAs (see Fig. 1D), but the same results are obtained when this ANOVA design is applied to genotypes within a mitochondrial DNA type (either K or S) where the categories used here do not require pooling of differentiated samples.

Effect	df	F-ratio	Prob > F
Females			
Intra-/interspecific	1	1.52	0.21
Disrupted/reconstituted	1	1.26	0.26
Interaction	1	0.52	0.46
Model	3	1.15	0.33
Males			
Intra-/interspecific	1	1.51	0.2195
Disrupted/reconstituted	1	11.67	0.0007
Interaction	1	8.52	0.0038
Model	3	6.42	0.0003

within each sex (Table 4). In females neither the main effects (disrupted/reconstituted or intra-/interspecific) nor the interaction term are significant. In males, there is a highly significant interaction term. With the exception of the interspecific female samples, pooling these samples is justified by Tukey-Kramer HSD tests that show no significant differences between pooled samples (Figs. 1C, D). When the female data are separated by mtDNA type (e.g., similar two-way ANOVAs run within just the S or K cytoplasm so that significantly different samples are not pooled), neither main effect or interaction term is significant.

Divergence of COX Subunits

Table 5 reports the patterns of divergence among the lines used in this study for the three mitochondrial subunits and eight of the nine nuclear subunits of COX (but note that additional isoforms of some subunits were reported for *Drosophila* COX by Szuplewski and Terracol 2001). The d_N/d_S ratios are well below one for all genes, indicating that purifying selection is operating on COX subunits. The nuclear-encoded proteins tend to have higher d_N/d_S ratios than the mitochondrial proteins (COX 4 and 5A have d_N/d_S ratios around 0.3, while COX2 equals 0.08; see summary at the bottom of Table 5). This probably reflects the high rate of synonymous divergence in the mitochondrial genes. Because the nuclear genes are rather short, the error associated with these d_N/d_S ratios will be high, but is not likely to affect the general conclusion that both nuclear and mitochondrial subunits of COX are subject to purifying selection. The mitochondrial gene for COX 3 and the nuclear genes for COX 6B, 6C, and 7C show no amino acid substitutions among the lines. Summing across all proteins, the data are consistent with previous patterns of divergence for these lines. The mtDNA data place *siI* as the outgroup, whereas nuclear data place *maI* as the outgroup (Harr et al. 1998; Kliman et al.

TABLE 5. Nucleotide divergence at cytochrome *c* oxidase (COX) subunit genes in the *Drosophila* lines used in this study. COX 1–3 are encoded in mitochondrial DNA; all others are nuclear encoded. *siI* and *siII* refer to *D. simulans* from Seychelles and Kenya, respectively; *maI* refers to *D. mauritiana*. The numbers of synonymous and nonsynonymous differences (No. syn, No. nsyn, respectively) are listed, followed by the d_N/d_S ratio determined from Nei and Gojobori (1986) corrected distances from PAML analyses (see Materials and Methods). Specific amino acid changes are shown in parentheses, with the two amino acids separated by their position in the respective proteins.

Gene (no. codons)	<i>siI</i> vs. <i>siII</i>			<i>siI</i> vs. <i>maI</i>			<i>siII</i> vs. <i>maI</i>		
	No. syn (amino acid substitutions)	No. nsyn (amino acid substitutions)	d_N/d_S	No. syn (amino acid substitutions)	No. nsyn (amino acid substitutions)	d_N/d_S	No. syn (amino acid substitutions)	No. nsyn (amino acid substitutions)	d_N/d_S
COX 1 (511)	54	2 (I450V, F468Y)	0.01	38	2 (I450V, F468Y)	0.015	43	0	0
COX 2 (229)	15 (S115N, T129S, T130I, V165I)	4	0.085	16	1 (S115N)	0.032	11 (S129T, I130T, I165V)	3	0.071
COX 3 (263)	22	0	0	15	0	0	17	0	0
COX 4 (182)	1	0	0	2	2 (A88T, M163I)	0.313	3 (A88T, M163I)	2	0.208
COX 5A (149)	1	1 (S22T)	0.321	3	0	0	4	1 (S22T)	0.075
COX 5B (121)	1	1 (L46F)	0.270	3	1 (L46F)	0.088	2	0	nd
COX 6B (93)	0	0	nd	0	0	nd	0	0	nd
COX 6C (77)	1	0	0	1	0	0	2	0	0
COX 7A (106)	2	1 (I28F)	0.156	6	2 (I28F, N30D)	0.101	4 (N30D)	1	0.076
COX 7C (51)	0	0	nd	0	0	nd	0	0	nd
COX 8 (68)	0	0	nd	0	0	nd	0	1 (I34V)	nd
Total or mean d_N/d_S									
Mitochondrial DNA	91	7	0.0317	69	4	0.0157	71	3	0.0237
Nuclear	6	3	0.1494	15	5	0.1004	15	4	0.0898
Both	97	10	0.1053	84	9	0.0686	86	7	0.0614

2000; Ting et al. 2000). These patterns hold for both synonymous and nonsynonymous sites.

The most promising candidate amino acid changes are those unique to *maI* nuclear subunits because the activity disruption effect involves *D. simulans* mtDNAs in the *maI* nuclear background. Three such candidates are listed in Table 5: two in COX 4 (A88T and M163I) and one in COX 7A (N30D). Note that the *maI* mtDNA was not involved in the backcross design, so substitutions in COX 1–3 of this mtDNA are not relevant to the disruption effect. A structural model for COX has been proposed based on the bovine heart protein (Tsukihara et al. 1995), but *Drosophila* appears to have one fewer nuclear subunit (Szuplewski and Terracol 2001). From the bovine model position 88 in COX 4 lies in transmembrane helix 1 and is spatially adjacent to helix 7 in this subunit (Tsukihara et al. 1995). The bovine COX 4 protein has only 150 amino acids, so it is difficult to interpret the functional significance of the substitution at position 163 in the *Drosophila* protein. Likewise, the amino acid sequence of the putative *Drosophila* 7A subunit is longer than that of mammalian subunits, and approximately the first 40 amino acids show no homology in protein BLAST searches of SWISS-PROT using the fly subunit as a query sequence. This makes it difficult to interpret the functional significance of the COX 7A N30D substitution in *D. mauritiana*. Although these three amino acid residues may affect COX function, we note that substitutions in COX assembly proteins may also contribute to the observed disruption effects.

DISCUSSION

Interacting genes that share a line of descent should co-evolve to maintain coordinated functions. Deleterious mutations in one gene may create the context for adaptive, compensatory mutations in interacting genes or sequence motifs (Stephan and Kirby 1993; Shaw et al. 2002). It follows that genes from distinct lineages with limited gene flow should diverge in their coordinated functions, and that the degree of dysfunction should be correlated with the amount of divergence between lineages. Here we have tested these predictions of coadaptation using activity assays of COX (complex IV of the electron transport chain), an enzyme jointly encoded by nuclear and mitochondrial genes (for reviews see Saraste 1999; Burton et al. 1999).

We used experimental and control backcrosses to create disrupted and reconstituted cytonuclear genotypes within the *D. simulans* clade. The maternal inheritance of mtDNA allows one to move this genome onto distinct nuclear chromosomal backgrounds through hybridization and backcrossing. The *D. simulans* clade is convenient for these analyses because it exhibits considerable mtDNA divergence among lines that can be crossed to produce fertile F₁ females. Our data show that the COX activities of cytonuclear genotypes carrying disrupted pairs of nuclear and mtDNAs were consistently lower than for control genotypes carrying native nuclear and mitochondrial genomes. This effect was greater in the inter-specific crosses, where *D. mauritiana* nuclear chromosomes were backcrossed onto either of two *D. simulans* mitochon-

drial genomes. Our data support the two basic predictions of mitochondrial-nuclear coadaptation outlined above and thus contribute to the growing body of evidence that suggests these genomes are involved in a coevolutionary process (Kenyon and Moraes 1997; Edmands and Burton 1999; Schmidt et al. 2001).

What might be the genetic and biochemical bases of the disruption phenotype we have described? One possibility is that COX enzymes comprised of divergent nuclear and mitochondrial subunits are less efficient at transferring electrons from cyt. *c* to oxygen. This might result from functionally mismatched amino acid residues being placed in close proximity in the holoenzyme, resulting in disrupted biophysical properties of electron transport. Support for such a residue-contact hypothesis comes from studies of primates, in which the evolutionary rates in COX residues encoded by mtDNA that are in contact with residues encoded by nuclear genes are elevated over noncontact mtDNA residues (Schmidt et al. 2001). These authors suggest that the elevated mutation rate in mtDNA allows for greater opportunity to maintain a coadapted state among interacting subunits of COX.

A related hypothesis concerning the disruption of COX activity is improper assembly of the COX enzyme due to disrupted interactions between mitochondrial subunits and nuclear assembly or localization proteins that properly position the subunits in the holoenzyme (Smeitink et al. 2001). The distinction between these hypotheses lies in the focus on contact residues that affect enzymatic activity versus those residues that affect enzyme assembly. However, both hypotheses invoke the unmasking of deleterious mutations when disrupted cytonuclear genotypes are created among divergent lineages that presumably harbor compensatory mutations among interacting nuclear and mitochondrial proteins.

Sequence surveys and neutrality tests of animal mtDNA indicate that deleterious mutations are a common aspect of mitochondrial gene evolution (Lynch 1997; Nachman 1998; Rand and Kann 1998; Rand 2001). Such deleterious mutations may establish the conditions for compensatory mutations in nuclear subunits that could lead to coadaptation. Our sequence data for nuclear and mtDNA subunits of COX from the experimental *Drosophila* lines indicate that purifying selection is operating on both nuclear and mitochondrial subunits. These sequences reveal three amino acid changes in nuclear encoded subunit of COX that are unique to *D. mauritiana* and are candidate residues for studies of the activity disruption effects we observe. A structural model of mammalian COX has been proposed (Tsukihara et al. 1995), but analyses of the *Drosophila* genome have identified only nine of the 10 nuclear-encoded subunits of COX found in mammals (Szuplewski and Terracol 2001). This potential difference in quaternary structure, plus considerable primary sequence divergence between bovine and *Drosophila* nuclear COX subunits makes it difficult to determine the functional significance of the observed amino acid differences among *Drosophila* lines from our sequence data (Table 5).

Coadaptation of COX need not involve COX subunits alone. Evidence for functional coadaptation between COX and cyt *c* has been provided in the intertidal copepod, *T. californicus* (Rawson and Burton 2002). The striking differentiation among tide-pool populations in this species estab-

lishes conditions that could promote the evolution of coadapted gene complexes. Different populations show distinct variants of COX and cyt *c* proteins, and COX activity is significantly higher when assayed with cyt *c* variants from the same population (Rawson and Burton 2002).

Alternatively, the COX disruption effects we have observed in backcrossed *Drosophila* may have little to do with the function of individual holoenzymes. COX assays in whole-fly homogenates must depend upon density of COX enzymes in the inner mitochondrial membrane, the total surface area of the inner membrane, and the density of mitochondria per unit protein. This COX-density hypothesis implies a role for nuclear-mitochondrial subunit interactions in the proper biogenesis of the inner mitochondrial membrane. Indeed, some mitochondrial diseases related to specific enzyme functions have been attributed to assembly proteins, such as SURF (Coenen et al. 2001; Smeitink et al. 2001). Some human cases of nuclear-encoded deficiencies of COX activity show no mutations in any of the subunits of the enzyme (Shoubridge 2001). These cases are best explained by defects in COX assembly proteins or other cofactors, which are numerous. Because more than 12% of the yeast proteome has some role in mitochondrial function (Grivell et al. 1999; Steinmetz et al. 2002), there are many other such nuclear-encoded genes that may affect COX activity that do not affect the key catalytic residues within a protein. The distinction between this COX density hypothesis and the residue-contact hypotheses above is the focus on the cellular and organellar titer, rather than the structure and biophysical properties, of the COX enzyme. Each of these hypotheses is testable in *Drosophila* through further genetic and biochemical dissection of COX activity.

Mitochondrial Dysfunction in Males

The significant COX disruption effects we observed were restricted to males (Fig. 1D). One possible explanation for this pattern is a Haldane's rule effect on total protein or general fly viability that might affect males more than females. However, all activities were corrected for total protein, and a one-way ANOVA with disruption as the main effect and protein concentration as the dependent variable show no decline in total protein in disrupted males (data not shown).

An alternative explanation for the male disruption effect invokes sex-specific differences in the evolution of modifiers of mitochondrial function. A male cannot transmit any combination of mtDNA, X chromosome, or Y chromosome to an individual offspring. In females, mtDNA and X chromosome are cotransmitted at higher rates than mtDNA and autosomes (see Frank and Hurst 1996; Rand et al. 2001). This means that modifiers that suppress deleterious interactions among these chromosomes that may not be cotransmitted cannot easily become established in populations. This indicates that the various mtDNAs, sex chromosomes, and autosomes that combine in males may be more likely to be deleterious than those cytonuclear genotypes that can be assembled in females. As suggested by Frank and Hurst (1996), a mitochondrial mutation that is only weakly deleterious in females but strongly deleterious in males can still persist in a population, perhaps accounting for more severe disease pheno-

types among males than females for some mitochondrial diseases.

This effect might be especially strong in males due to mtDNA–Y chromosome interactions (Gemmell and Sin 2002). Because mtDNAs and Y chromosome can never be cotransmitted (barring paternal leakage) and both have no (or low) recombination, this chromosomal pair may be predisposed to deleterious associations when divergent lineages are crossed. This argument of increased cytonuclear dysfunction in males parallels genetic arguments concerning the evolution of reproductive isolation among interacting genetic factors (e.g., Orr and Turelli 2001). It is plausible that the interactions between these genetic components are partly or fully responsible for the sex-specific nature of COX dysfunction observed here. Detailed genetic analyses of mitochondrial, sex chromosome, and autosome interactions are needed to dissect the nature of variation in the enzymes of oxidative phosphorylation.

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