

# Near Neutrality, Rate Heterogeneity, and Linkage Govern Mitochondrial Genome Evolution in Atlantic Cod (*Gadus morhua*) and Other Gadine Fish

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The mitochondrial DNA (mtDNA) genome figures prominently in evolutionary investigations of vertebrate animals due to a suite of characteristics that include absence of Darwinian selection, high mutation rate, and inheritance as a single linkage group. Given complete linkage and selective neutrality, mtDNA gene trees are expected to correspond to intraspecific phylogenies, and mtDNA diversity will reflect population size. The validity of these assumptions is, however, rarely tested on a genome-wide scale. Here, we analyze rates and patterns of molecular evolution among 32 whole mitochondrial genomes of Atlantic Cod (*Gadus morhua*) as compared with its sister taxon, the walleye pollock (*Gadus [Theragra] chalcogrammus*), and genomes of seven other gadine codfish. We evaluate selection within *G. morhua*, between sister species, and among species and intraspecific measures of linkage disequilibrium and recombination within *G. morhua*. Strong rate heterogeneity occurs among sites and genes at all levels of hierarchical comparison, consistent with variation in mutation rates across the genome. Neutrality indices (dN/dS) are significantly greater than unity among *G. morhua* genomes and between sister species, which suggests that polymorphisms within species are slightly deleterious, as expected under the nearly neutral theory of molecular evolution. Among species of gadines, dN/dS ratios are heterogeneous among genes, consistent with purifying selection and variation in functional constraint among genes rather than positive selection. The dN/dS ratio for ND4L is anomalously high across all hierarchical levels. There is no evidence for recombination within *G. morhua*. These patterns contrast strongly with those reported for humans: genome-wide patterns in other vertebrates should be investigated to elucidate the complex patterns of mtDNA molecular evolution.

## Introduction

No other molecule features more prominently in vertebrate molecular systematics and evolution than mitochondrial DNA (mtDNA). From the restriction maps of the 1970s to the present-day currency of whole mitochondrial genome sequences, this genome has dominated the stage of the entire field of study now known as phylogeography, the interpretation of the geographic distribution of genetic lineages within species (Avice 2004). The vast mitogenomic database compiled for genealogical investigation into the history of our own species (Torroni et al. 2006) stands as a continuing testament to the richness of information content stored within ~16,500 bp.

The contemporary and historical significance of the mtDNA molecule to vertebrate evolutionists rests on a well-known suite of characteristics and assumptions, including high mutation rate, maternal inheritance, and an effective population size one-quarter that of nuclear genes (Avice 2004 and references therein). The two most important aspects of this mantra are the presumed freedom from positive Darwinian (adaptive) natural selection and lack of recombination of the mitochondrial genome. Together, these two properties mean that mtDNA gene genealogies should serve as maternal intraspecific phylogenies and that intrapopulation diversity of the mtDNA molecule should reflect population size. These endowments in turn underlie the evolutionary utility of mtDNA.

The importance of the protein products of mtDNA genes in cellular respiration mean that it is reasonable to hypothesize that some of the variation in the molecule could

be adaptive. Nonetheless, of the numerous single-gene studies of mtDNA to challenge its neutrality in the past two decades, very few have been able to reject neutrality in favor of positive Darwinian selection, other than important studies of the invertebrate *Drosophila* (Rand et al. 1994; García-Martínez et al. 1998). Indeed, the emerging picture from early studies in vertebrates is one in which different levels of variability are attributable to functional constraints and/or slightly deleterious polymorphisms, a pattern consistent with near neutrality (e.g., Templeton 1996; Nachman et al. 1996).

Nonetheless, a comprehensive study by Bazin et al. (2006) of a broad range of vertebrate and invertebrate animals demonstrated a lack of relationship between population size and mtDNA diversity, which they attributed to recurrent bouts of adaptive evolution, due to "genetic draft," whereby the increased efficiency of positive natural selection in larger populations offsets their reduced drift (Gillespie 2000). Now, as large libraries of human whole-mtDNA genome sequences accumulate, genome-wide analyses have begun to paint a more complex picture of natural selection. For example, Mishmar et al. (2003) reported functionally significant amino acid changes in the ATP6, CYTB, and COXI for consistency with subsequent usage genes, correlated with climatic zone. Elson et al. (2004) found a pattern more consistent with negative selection, rather than a model of climatic adaptation. Moilanen et al. (2003) observed different levels of functional constraint in different haplogroups and also reported a pattern of nucleotide variability that did not correlate with position in the genome. Eyre-Walker (2006) also noted that human populations provide an exception to the phenomenon described by Bazin et al. (2006), and Mulligan et al. (2006) described a positive correlation between mtDNA diversity and allozyme diversity in eutherian mammals, which usage is non-restrictive typically, have small population sizes.

Key words: mitogenomics, rate heterogeneity, nonsynonymous-to-synonymous ratio, recombination, *Gadus morhua*, gadine fishes, slightly deleterious polymorphisms.

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Recombination breaks down the correlation in genealogical history between different regions of a genome. Its phylogenetic signature is excess homoplasy relative to recurrent mutation alone (Maynard Smith and Smith 1998), and its consequence may be incorrect inference of evolutionary history (Schierup and Hein 2000). The issue of recombination of the human mitochondrial genome was raised by Awadalla et al. (1999) but disputed by Ingman et al. (2000). Initial evidence for recombination in the human mitochondrial genome has subsequently been attributed variously to sequencing errors (Macauley et al. 1999), bouts of adaptive evolution (McVean 2001), or mutational hot spots (Galtier et al. 2006). Nonetheless, the necessary enzymes for recombination are present in the mitochondria, and a few paternal mitochondria do penetrate the egg during fertilization (Thyagarajan et al. 1996). Recombination is thus possible, at least in humans. Recent broad surveys of animal mitochondrial genomes have concluded that recombination is widespread (Piganeau et al. 2004; Tsaousis et al. 2005).

Whole-mtDNA genome studies can address issues of selection and recombination with heretofore unachievable power as compared with single-gene studies. These issues have not been considered on an intraspecific scale in vertebrates other than humans. We have recently employed mitogenomic approaches to resolve phylogenetic relationships among and within species in the important codfish subfamily Gadinae. Among gadines, we clarified the taxonomy and biogeographic origins of 10 taxa of codfish and pollock (Coulson et al. 2006). Within the species *Gadus morhua* (Atlantic cod), phylogeographic analysis of multiple complete mitochondrial genomes indicates population expansion during the previous glacial period 59 Kya, rather than as part of a postglacial emergence from marine refugia 12 Kya (Carr and Marshall 2008a). Here, we report on rates and patterns of mtDNA sequence variation in Atlantic cod and its close relatives and perform specific tests designed to detect recombination and selection. Such analyses are of interest in their own right and also have implications for the use of mtDNA to estimate population structure and phylogeny. With inclusion of data from the walleye pollock, the sister taxon to *G. morhua*, we can examine patterns of DNA sequence variation at three hierarchical levels: within species, between sister taxa, and among closely related species and genera.

## Materials and Methods

### Sequences

We compiled a total of 52 mitogenomic sequences. We obtained 32 Atlantic cod sequences (*G. morhua*) from Carr and Marshall (2008; GenBank accession numbers EU877710–EU877741). We obtained 10 walleye pollock sequences (*Theragra chalcogramma*; see below) from Yanagimoto et al. (2004; GenBank accession numbers AB094061, AB182300–AB182308) and one from Coulson et al. (2006; GenBank accession number DQ356946) as well as two Norwegian pollock sequences (*Theragra finnmarchica*) from Ursvik et al. (2007; GenBank accession numbers AM489718 and AM489719)

for a total of 13 sequences. Coulson et al. (2006) demonstrated a sister taxon relationship between *G. morhua* and walleye pollock and referred the latter to *Gadus chalcogrammus* Pallas, 1,811 as originally described (Coulson et al. 2006). Ursvik et al. (2007) demonstrated that the Norwegian pollock sequences clustered within walleye pollock Latin binomial creates confusion here (cf. Carr and Marshall 2008b). Accordingly, we include walleye and Norwegian pollock sequences in *G. chalcogrammus* and describe its comparison with *G. morhua* as a sister-species comparison. Finally, we obtained sequences from seven other gadine species (Coulson et al. 2006): *Gadus macrocephalus* (Pacific cod, including Greenland cod *G. macrocephalus ogac*; GenBank accession number DQ356941), *Arctogadus glacialis* (polar cod; GenBank accession number DQ356935), *Boreogadus saida* (Arctic cod; GenBank accession number DQ356936), *Melanogrammus aeglefinus* (haddock; GenBank accession number DQ356942), *Merlangius merlangus* (whiting; GenBank accession number DQ356943), *Pollachius virens* (saithe; GenBank accession number DQ356945), and *Microgadus proximus* (Pacific tomcod; GenBank accession number DQ356944). There are, thus, three levels of hierarchical comparison: intraspecific variation within *G. morhua*, variation between the sister species *G. morhua* and *G. chalcogrammus*, and interspecies variation among nine species within the subfamily Gadinae. (*G. macrocephalus* is the sister taxon to *G. morhua* and *G. chalcogrammus* [Coulson et al. 2006]; we include its comparison with the latter two among the interspecies comparisons.)

All 52 sequences were aligned, edited, and compared with the software Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI). The region compared spans a total of 15,584 bp, and includes all 13 protein-coding genes and 21 of the 22 tRNA genes. The tRNA<sup>Glu</sup> gene and the Control Region were omitted.

### Sequence Diversity

Measures of sequence diversity were calculated and correlated with position in the genome with the sliding-window option of the software DNAsp 4.10 (Rozas et al. 2003). Within the *G. morhua* sample and among representatives of the nine gadines, we calculated the nucleotide diversity (the average number of nucleotide differences per site between two sequences or  $\pi$ , Nei 1987) as a measure of sequence diversity. To quantify divergence between the *G. morhua* and the *G. chalcogrammus*, we calculated the net number of nucleotide substitutions per site among all individuals from the two species ( $d_A$ , Nei 1987). We used a window size of 500 bp and a step size of 100 bp.

### Tests of Neutrality

#### McDonald–Kreitman Tests and Neutrality Indices

We performed McDonald–Kreitman style tests of neutrality (McDonald and Kreitman 1991) and calculated neutrality indices (Rand and Kann 1996) for sequences within *G. morhua* and between *G. morhua* and *G. chalcogrammus*, as follows. All tests were performed for the entire coding portion of the genome and separately for each gene.

First, within *G. morhua*, we mapped each of the nucleotide substitutions among the 32 individuals onto their phylogeny (fig. 3 in Carr and Marshall 2008a) and then divided them into four categories: synonymous shared, synonymous private, nonsynonymous shared, and nonsynonymous private. The shared and private designations refer to the occurrence of a substitution at an internal or a tip branch, respectively. The significance of the  $2 \times 2$  table of the ratio (nonsynonymous private to nonsynonymous haplogroup-associated mutations)/(synonymous private to synonymous haplogroup-associated mutations) was assessed with Fisher's exact test. An intraspecies neutrality index was calculated as the ratio of nonsynonymous-private to nonsynonymous-shared substitutions, divided by the ratio of synonymous-private to synonymous-shared substitutions, as described by Elson et al. (2004).

Neutrality indices between *G. morhua* and *G. chalcogrammus* were calculated as the ratio of nonsynonymous polymorphisms to fixed differences, divided by the ratio of synonymous polymorphisms to fixed differences for the 32 *G. morhua* individuals relative to the 13 *G. chalcogrammus* individuals. Ratios were evaluated with Fisher's exact test as before.

#### *Distribution of Synonymous and Nonsynonymous Polymorphisms among Genes within G. morhua*

We also compared the distribution of observed substitutions in each protein-coding gene with the expected distribution in *G. morhua*, for both synonymous and nonsynonymous site categories. Expected distributions were generated from the total observed polymorphisms in each category multiplied by the proportional size of the gene. The significance of the difference between the two distributions was evaluated by a chi-square test of independence. Under neutrality, the observed and expected distributions should not differ within the nonsynonymous category. To control for rate variation among sites (i.e., if the observed-to-expected distribution does differ for synonymous sites), we also calculated the expected distribution of nonsynonymous sites among genes from the total number of nonsynonymous polymorphisms, multiplied by the proportion of observed synonymous polymorphisms found in that gene. This expected distribution was then compared with the observed distribution of nonsynonymous sites among genes.

#### *dN/dS Ratios and Site-Specific Positive Selection among Gadines*

We calculated dN/dS ratios among the set of 10 gadines and tested their significance with Z-tests (Nei and Kumar 2000) for each of the 13 protein-coding genes, as implemented by MEGA version 4 (Tamura et al. 2007). We also tested for site-specific positive selection with Bayesian posterior probabilities under the Ny98 fitness regime ( $0 < \omega_1 < 1$ ,  $\omega_2 = 1$ , and  $\omega_3 > 1$ ; Nielsen and Yang 1998) as described and implemented by MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003).

#### Tests of Recombination *Linkage Disequilibrium*

Following Ingman et al. (2000), we chose a standard measure of linkage disequilibrium ( $|D'|$ ; Lewontin 1964) that allows allele frequency to be taken into account. We also calculated the Hill and Robertson (1968) measure of linkage disequilibrium ( $r^2$ ), as was done by Awadalla et al. (1999) for humans. Each measure was calculated for all pairs of parsimony-informative sites with DnaSp 4.0 (Rozas et al. 2003). Measures of linkage disequilibrium were regressed against physical distance between pairs of sites: Decay in linkage disequilibrium with distance would indicate recombination.

#### *Pairwise Homoplasmy Test of Recombination*

To avoid the confounding effects of recurrent mutation and population expansion on the inference of recombination, we also employed a relatively new and robust statistical estimator of recombination called the pairwise homoplasmy test ( $\Phi_W$ ; Bruen et al. 2006). This is a coalescent-based estimator of the genealogical correlation or compatibility among sites, which is negatively correlated to recombination. The estimator was calculated and its significance estimated via a permutation test, with Splitstree 4.10 (Huson and Bryant 2006).

## Results

### Rates and Patterns of DNA Sequence Variation in the Mitochondrial Genomes of Codfish and Relatives

Rates and patterns of DNA sequence diversity were compared among 32 *G. morhua* individuals, 13 *G. chalcogrammus* individuals, and 1 representative each of the gadines *G. macrocephalus ogac*, *A. glacialis*, *B. saida*, *M. aeglefinus*, *M. merlangus*, *P. virens*, and *M. proximus*. The phylogenetic relationships among these species are shown in figure 1. Within *G. morhua*, 293 nucleotide sites were found to be variable; 196 of these are singleton sites. Between *G. morhua* and *G. chalcogrammus*, there are on average 618 nt differences. Among pairs of gadine species, there are from 599 (*G. macrocephalus ogac* and *Arctogadus*) to 1,539 (*Microgadus* and *Melanogrammus*) pairwise nucleotide differences.

The distributions of sequence diversity across the genome for the three hierarchical levels of within *G. morhua* ( $\pi$ ), between sister taxa (*G. morhua* and *G. chalcogrammus*) ( $d_A$ ), and among gadine species ( $\pi$ ) are presented in figure 2. Notably, at all hierarchical levels of comparison, genes from the NADH dehydrogenase (ND) complex show the most elevated rates of sequence diversity. This is most pronounced at the lowest level of comparison, among *G. morhua* individuals, where the ND2 gene is the most variable. Between sister species of *Gadus*, the ND4 gene is the most variable, and the cytochrome oxidase (COX), cytochrome *b* (CYTB), and ATPase (ATP) genes have begun to accumulate more diversity. Among gadines, the ND1 and ND2 and especially ND5 and ND6 genes are the most variable, but the remaining protein-coding genes

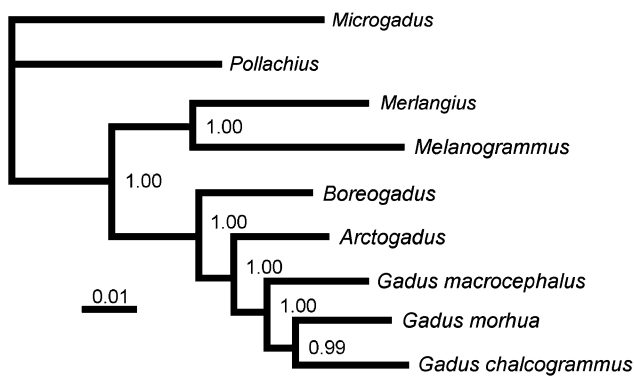


FIG. 1.—The phylogeny of the gadine taxa used in this study. Adapted from figure 1 of Coulson et al. (2006), with numbers at nodes representing Bayesian posterior probabilities.

have accumulated substantial diversity. Troughs in the distribution associated with tRNA and rRNA genes indicate reduced diversity within these genes.

#### Tests of Neutrality

##### McDonald–Kreitman Tests and Neutrality Indices

Results of the McDonald–Kreitman style tests and neutrality indices within and between species are presented in table 1. Within *G. morhua*, the ratio (nonsynonymous private to nonsynonymous haplogroup-associated mutations)/(synonymous private to synonymous haplogroup-associated mutations) provides an intraspecies neutrality index and test of neutrality, whereas the conventional McDonald–Kreitman test and interspecies neutrality index were derived from the ratio of nonsynonymous to synonymous within *G. morhua* polymorphisms versus fixed substitutions between *G. morhua* and *G. chalcogrammus*. Significance of the comparisons was evaluated with Fisher's exact tests; a neutrality index greater than 1 is consistent with purifying selection, equal to 1 with neutrality, and less than 1 with positive Darwinian selection.

Within species, in no gene except ND5 are there any haplogroup-associated nonsynonymous mutations, whereas all ND genes and the COX1 gene exhibit private nonsynonymous mutations. This pattern is strongly indicative of purifying selection: indeed, neutrality indices cannot even be calculated for other loci, where the number of nonsynonymous mutations in the denominator is zero. When mutation events are pooled among genes, the neutrality index of 5.39 is significantly greater than unity. The neutrality index is also greater than unity for the ND5 gene but not significantly so by Fisher's exact test. The distribution of synonymous and nonsynonymous mutations is consistent with the distribution of diversity documented in the previous section, with lower numbers of both types of change in non-ND genes.

At the interspecific level of comparison, the pattern holds although a greater number of statistically significant results are observed for individual genes because of the greater numbers of mutation events. In particular, the neutrality indices are significantly greater than 1 for the ND2 and ND4 genes and at a higher level of significance for the

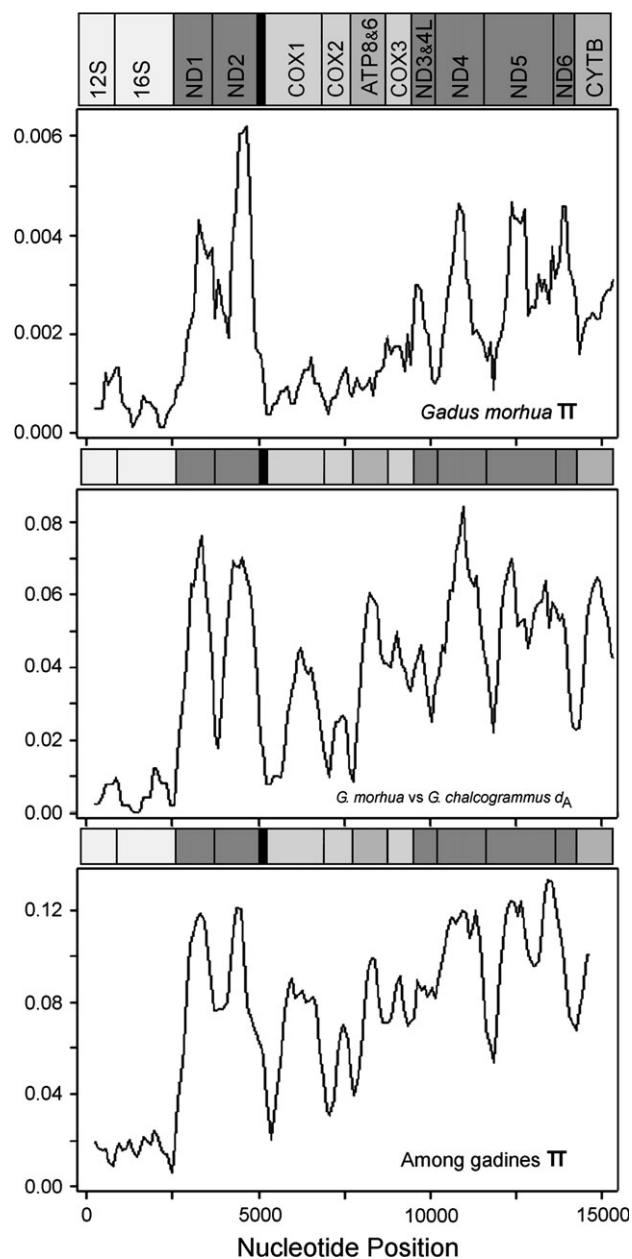


FIG. 2.—Sequence diversity of the mitochondrial genome by nucleotide position—within *Gadus morhua* ( $\pi$ ), between *G. morhua* and *Gadus chalcogrammus* ( $d_A$ ), and among gadine species ( $\pi$ ). The diversity measure was calculated for a sliding window of 500 bp with a step size of 100 bp.

ND5 gene and the pooled data. For the COX1 and CYTB genes, the neutrality index could not be computed as there were no nonsynonymous fixed differences between species; however, Fisher's exact test indicates that there are significantly more nonsynonymous mutations polymorphic within species than nonsynonymous substitutions fixed between species. Notably, in the ND4L and ATP6 genes, the numbers of fixed nonsynonymous changes exceed the numbers of nonsynonymous polymorphisms. For the former, the pattern is matched by the synonymous changes, but for the latter, the neutrality index of zero is not inconsistent with positive Darwinian selection.

**Table 1**  
**McDonald–Kreitman (MK) Style Tests of Neutrality within *Gadus morhua* and between *G. morhua* and *Gadus chalcogrammus***

Gene	Intraspecies MK-Like Test			Interspecies MK Test		
	Within <i>G. morhua</i>			<i>G. morhua</i> versus <i>G. chalcogrammus</i>		
	Haplogroup N/S	Private N/S	NI	Fixed N/S	Polymorphic N/S	NI
ND1	0/8	1/25	—	3/46	3/39	1.18
ND2	0/10	6/29	—	1/52	7/48	7.58 ( $P = 0.06$ )
COX1	0/4	1/11	—	0/38	3/30	— ( $P = 0.09$ )
COX2	0/1	0/6	—	0/13	0/9	—
ATP8	0/0	0/0	—	0/0	0/2	—
ATP6	0/3	0/11	—	1/36	0/22	0
COX3	0/2	0/5	—	0/26	1/17	—
ND3	0/4	1/4	—	1/15	2/11	2.73
ND4L	0/0	1/0	—	2/5	1/1	2.50
ND4	0/12	3/28	—	1/77	6/54	8.56 ( $P = 0.04$ )
ND5	2/18	11/29	3.41	2/80	18/68	10.59 ( $P = 0.0002$ )
ND6	0/6	3/15	—	0/16	4/28	—
CYTB	0/7	0/25	—	0/53	3/44	— ( $P = 0.10$ )
All	2/75	27/188	5.39 ( $P = 0.01$ )	11/457	48/373	5.35 ( $P = 4.6 \times 10^{-8}$ )

NOTE.—N and S refer to nonsynonymous and synonymous sites, respectively. NI is the neutrality index; a dash indicates an undefined value, where the denominator is zero.

#### *Distribution of Synonymous and Nonsynonymous Substitutions among Genes in G. morhua*

Among individual *G. morhua*, the observed distribution of synonymous as well as nonsynonymous polymorphisms among genes differs significantly from the expected distribution, as calculated from the total observed substitutions in each class and the proportional size of the gene in nucleotides (fig. 3A and B;  $P = 0.00488$  for the synonymous class and  $P = 6.95 \times 10^{-6}$  for the nonsynonymous class). When the nonsynonymous distribution is adjusted to reflect the observed distribution of synonymous variants, the observed nonsynonymous distribution still differs from the expected, but with only marginal significance (fig. 3C;  $P = 0.0510$ ). The discrepancy appears to be attributable to an excess of nonsynonymous polymorphisms in the ND5 gene and a deficiency in the CYTB gene.

The heterogeneous distribution of synonymous site variation in figure 3A is consistent with the pattern of rate variation across sites in figure 1A, but the excessive polymorphism of the ND genes in general (except ND4L) and CYTB is more pronounced, whereas the COX genes show a reduction in polymorphism.

#### *Patterns of Synonymous and Nonsynonymous Rate Variation among Gadines*

As a conservative measure of selection, Z-tests were conducted for each protein-coding gene between sister species and among all species. In all cases,  $dN < dS$  is highly significant ( $P = 0$ ), which is consistent with strong purifying selection of mitochondrial genes at these two levels of hierarchical comparison. The  $dN/dS$  ratios differ among genes (fig. 4), which could potentially be attributable to positive selection or to variation in levels of functional constraint among genes. Specifically, the ND4L gene is characterized by a  $dN/dS$  ratio well above average at both levels of comparison, and among species, the COX1 and COX2

gene ratios are lower than average. However, site-specific positive selection inferred from Bayesian posterior probabilities indicates no sites under positive selection, for any gene among species.

The ND4L result is exceptional not only because of the elevated  $dN/dS$  ratio but also because it results from both an anomalously lower  $dS$  than average (second lowest among species; lowest between sister taxa) and a higher  $dN$  (highest at both levels of comparison).

#### Tests of Recombination

Neither of the measures of linkage disequilibrium,  $r^2$  or  $|D'|$ , are correlated with physical distance between sites within *G. morhua* mtDNA sequences (fig. 5). The absence of decay of linkage disequilibrium with distance is a strong indication of complete linkage among sites. The pairwise homoplasmy test statistic also provided no evidence of recombination ( $\Phi = 0.14$ ;  $P = 0.540$ ). Hence, the assumption of complete linkage of the mtDNA genome appears justified in *G. morhua*.

#### Discussion

To investigate rates and patterns of mitochondrial genome evolution among *G. morhua* and relatives, we conducted several tests designed to detect departure from strict neutrality and the signature of recombination and described the pattern of rate variation among sites and genes at different hierarchical levels. We find no indication of a role for recombination in generating mitochondrial diversity. We document strong heterogeneity of rate among sites and genes. We demonstrate a strong role for purifying selection, consistent with the nearly neutral model of molecular evolution in shaping the pattern of diversity observed among genes and among hierarchical levels of comparison.

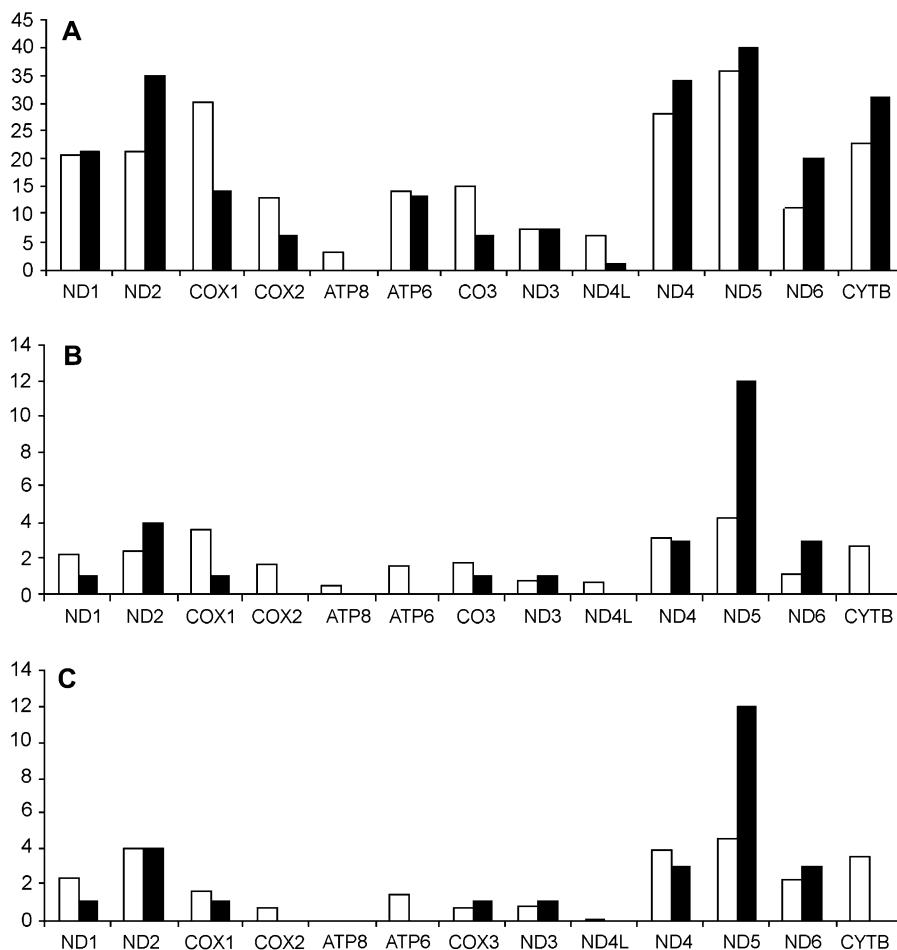


FIG. 3.—Expected (white) and observed (black) numbers of synonymous (A) and nonsynonymous polymorphisms (B) within *Gadus morhua*. Expected distribution of nonsynonymous polymorphisms adjusted according to the observed distribution of synonymous polymorphisms (C).

### Strong Rate Heterogeneity Exhibited by the Mitochondrial Genomes of Gadines

The mitochondrial genomes of gadines exhibit striking patterns both of polymorphism and substitution rate heterogeneity among sites, as evidenced by sliding-window estimates of sequence diversity within *G. morhua*, between *G. morhua* and *G. chalcogrammus*, and among nine species of gadines (fig. 2). Most notably, rates are elevated in the ND genes and reduced in the ribosomal genes, a pattern that is first evident within species and maintained among species. Somewhat unexpectedly, this pattern cannot be attributed simply to differences in selection pressure among genes as the pattern of rate heterogeneity among genes is observed for both synonymous and nonsynonymous changes. The observed distributions of both types of polymorphism within *G. morhua* differ significantly from the expected distributions. When adjusted according to the observed distribution of synonymous polymorphisms, the observed distribution of nonsynonymous changes is only marginally different from expected (fig. 2). Hence, rate heterogeneity appears to be due to the rate of change at synonymous sites.

The possible explanations for synonymous rate heterogeneity are selection pressure via differential tRNA avail-

ability or variation in the mutation rate itself among different genes or across the genome. Because serine and leucine are the two amino acids in the mitochondrial genetic code represented by six codons, and for which only two tRNA species are used, synonymous substitution rate might be expected to decline with leucine + serine content if selective pressure favored a particular tRNA. We observe instead a significant positive correlation between leucine + serine content and synonymous substitution rate in each of the 13 protein-coding genes both within *G. morhua* and among gadines ( $R^2 = 0.34$ ,  $P = 0.040$ ;  $R^2 = 0.39$ ,  $P = 0.02$ , respectively), a trend not observed for other combinations of amino acids.

The more probable explanation for the observed rate heterogeneity is variation in mutation rate. The two inter-related factors involved are base composition bias and properties of the L-strand during mtDNA replication. Because mtDNA replicates in a strand-asymmetric manner starting at the origin of H-strand replication (OriH), the L-strand remains single-stranded until the L-strand origin of replication (OriL) is reached (Tanaka and Ozawa 1994; Nedbal and Flynn 1998). The two regions of the genome that will persist in this single-stranded fashion are the genes immediately downstream of the OriH (CYTB, ND6, ND5, and ND4 in that order) and those immediately upstream of

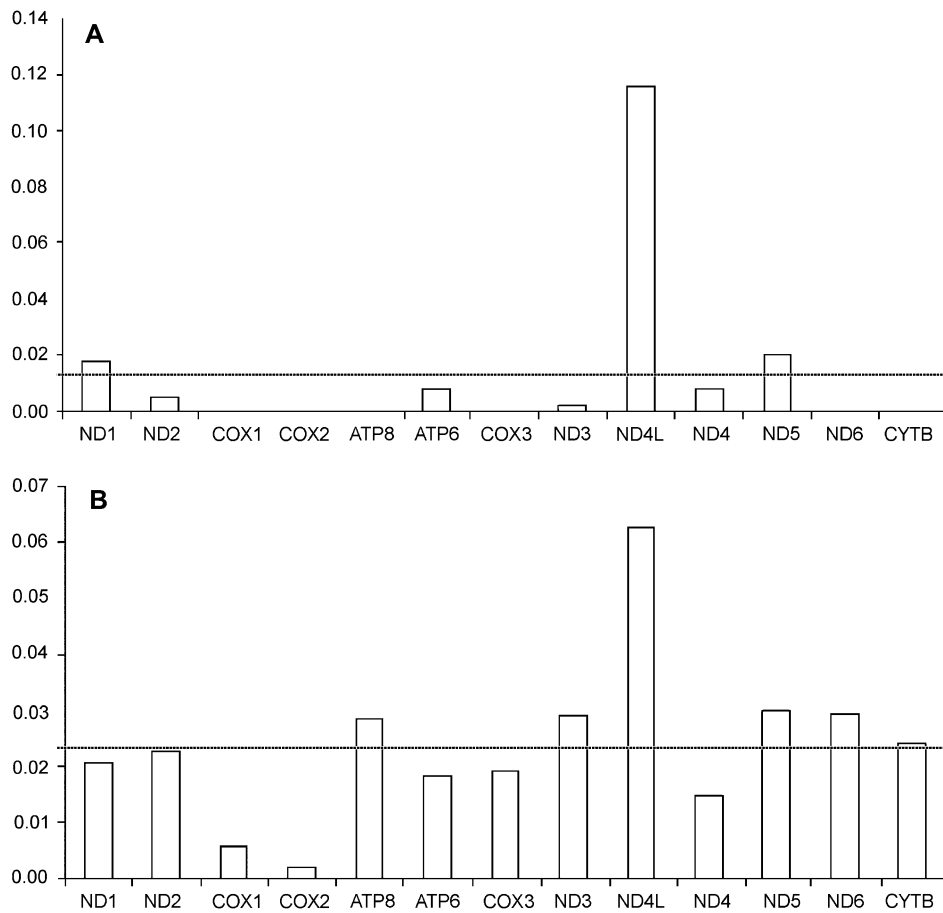


FIG. 4.—Nonsynonymous/synonymous rate ratios per gene among gadines (A) and between *Gadus morhua* and *Gadus chalcogrammus* (B). The average ratio is indicated by the dotted line.

the OriL (ND2 and ND1). These are the genes where we observe the greatest variability. The base composition bias between the two strands (L-strand rich in C + A and H-strand in G + T) has been attributed to this strand-asymmetric mode of replication (Tanaka and Ozawa 1994). Because the L-strand is subject to mutation during its single-stranded phase and G → A and T → C mutations occur more frequently than the reverse, an accumulation of A and C on the L-strand is predicted (Tanaka and Ozawa 1994). In support of such a mutation rate effect, the data show a significantly positive correlation between G + T content at third positions of the L-strand and rate of synonymous substitution among genes both within *G. morhua* and among gadines ( $R^2 = 0.48$ ,  $P = 0.0088$ ;  $R^2 = 0.47$ ,  $P = 0.0095$ , respectively).

The pattern of heterogeneity across the mitochondrial genome observed here differs from previous whole-genome intraspecific studies in humans, where either the particular pattern differs (Moilanen et al. 2003) or only the nonsynonymous distribution is heterogeneous (Elson et al. 2004). It also differs from both inter- and intraspecific patterns in *Drosophila*, where the synonymous distribution is more homogeneous, particularly within species (Ballard 2000). Comparisons at the familial and ordinal levels in mammals show significant rate heterogeneity and site-specific hypermutability, which occur at least partially as

the consequence of neutral mutational effects (Galtier et al. 2006). Hence, our results may be the norm rather than the exception. Such patterns will influence the choice of regions of the mtDNA used to investigate phylogeography because the more variable regions of the genome harbor excess homoplasmy relative to the more slowly evolving regions (Galtier et al. 2006), a pattern we also observed (observed distribution of homoplasies fits expected based on number of synonymous changes). Hence, even within species, it may be wiser to choose more slowly evolving regions for analysis, with an increase in the sequence length examined accordingly.

#### No Signature of Recombination in the Mitochondrial Genomes of *G. morhua*

A presumed absence of recombination is one of the main features of mtDNA that makes it a phylogeographically useful molecule: if recombination occurred, different parts of the molecule would have different genealogical histories. The net effect of this would be to increase homoplasmy and decrease the accuracy and confidence of phylogenetic inference made from the recombinant molecule.

The uniparental inheritance of mtDNA means recombination should be rare or absent. The evidence for

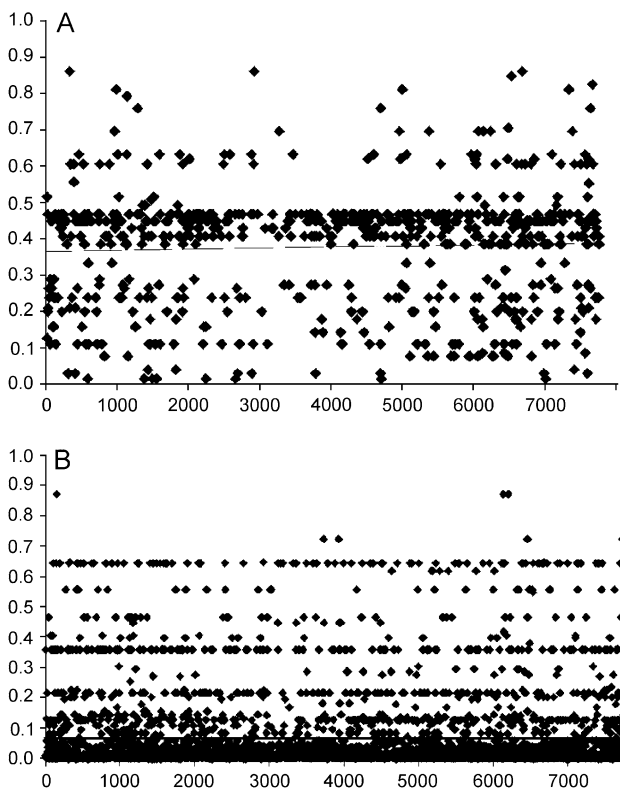


FIG. 5.—Measures of linkage disequilibrium versus distance in nucleotides between sites. (A)  $|D'|$ ,  $R^2 = 0.0023$ , values of  $|D'| = 0$  were omitted. (B)  $r^2$ ,  $R^2 = 6 \times 10^{-6}$ .

recombination of the mtDNA molecule in human populations has been thoroughly criticized on several grounds (Macauley et al. 1999; Galtier et al. 2006); however, there remains one documented case of paternal leakage and subsequent recombination in humans (Schwartz and Vissing 2002; Kraysberg et al. 2004). A broad survey of 267 published animal mtDNA data sets by Piganeau et al. (2004) provided indirect evidence of recombination in more than 14% of cases, including *M. merlangus* (represented by three mtDNA genomes) studied here. Thus, it would seem prudent to evaluate the evidence for recombination on a case-by-case basis in any newly collected data set, prior to phylogenetic or phylogeographic analysis.

Here we had the opportunity to perform tests of recombination in a 32-genome intraspecific data set of a teleost fish species, *G. morhua*. Due to inherent biases in different methods for detecting low levels of recombination, we performed several different tests. First, we evaluated two measures of linkage disequilibrium,  $r^2$  and  $|D'|$ . The former measure was the one initially used in the human studies, whereas the latter allows all informative sites to be considered and takes allele frequency into account (Ingman et al. 2000). Due to the sensitivity of these tests to rate heterogeneity (Piganeau et al. 2004) and mutational hot spots (McVean et al. 2002) and the sensitivity of certain coalescent-based estimators (e.g., the composite likelihood index; McVean et al. 2002) to demographic factors such as population growth (Tajima's  $D$  [Tajima 1989] is significantly negative in *G. morhua*), we also calculated the pairwise homoplasy index, a simple but robust estimator of compat-

ibility among sites (Bruen et al. 2006). In none of these tests was any evidence of recombination detected. Hence, it is unnecessary to consider the effects of recombination on the evolution of mtDNA in *G. morhua*.

#### Mitochondrial Polymorphisms within *G. morhua* Are Slightly Deleterious

The pattern of molecular evolution within *G. morhua* and between *G. morhua* and *G. chalcogrammus* evident from the McDonald–Kreitman style tests is strongly indicative of slightly deleterious mutations that occur as polymorphisms in younger lineages and which are progressively eliminated at the older internal branches within *G. morhua* or between sister species. This is consistent with the nearly neutral theory of molecular evolution proposed by Ohta (1973), in which most mutations are neutral, but a small fraction is nearly neutral or slightly deleterious. Although many of our observations are not significant on a per-gene basis, they all show the same trend toward a lower dN/dS ratio as hierarchical divergence increases, and the neutrality index for the whole genome is significantly greater than unity both within species and between sister species. Our results therefore support, on a genome-wide scale, the majority of single-gene-mtDNA studies that have investigated the role of natural selection in shaping mtDNA diversity and which reported consistency either with neutrality or with near neutrality. This is not the same as the genetic draft effect proposed by Bazin et al. (2006).

If polymorphisms within populations have a tendency to be slightly deleterious, there are important implications for inference of population size and coalescence times, both of which may be overestimated, especially in small populations. This is not to say that the overall pattern of phylogeographic inference would be compromised, as also pointed out by Zink (2005). Incorrect estimation of population size in turn impacts the correct assignment of endangered populations and species in conservation efforts. Furthermore, if the maintenance of slightly deleterious polymorphisms is higher in small populations, this has practical implications for the health of small populations. Popadin et al. (2007) reported an accumulation of slightly deleterious polymorphisms in mitochondrial genes of large mammals, with small population sizes, relative to small mammals, with larger population sizes, and point to the contribution of this effect toward the decline and extinction of large mammals. It remains to be seen if such a trend holds in fishes. It is clear that the relationships between polymorphism, divergence, and population size are complex and in need of investigation in a broader range of vertebrates than has been considered to date.

A preponderance of slightly deleterious polymorphisms was first reported in humans in an analysis of the mitochondrial genomes of humans, chimpanzees, and gorillas (Hasegawa et al. 1998). Elson et al. (2004) presented neutrality indices for variation within and between haplogroups of modern humans that were similar in general pattern to ours, but with some notable differences. First, they were able to calculate the neutrality index for all genes,



which we were unable to do within *G. morhua* because of the absence of nonsynonymous variants at the haplogroup level of comparison in most genes. Given the similar total depths of the cod and human mtDNA coalescents (162 Kya in *G. morhua*; Carr and Marshall 2008a), this is likely due to the much greater sampling effort by Elson et al. (2004). Despite this, they reported significant results for only four genes, COXI, COXIII, ND4, and ND6. Second, they reported (nonsignificant) neutrality indices of less than unity in two genes, ND2 and ND3. Finally, despite a neutrality index greater than unity, they interpreted the results of the ATP6 gene as evidence for positive selection. They attributed the discrepancy to the large number of substitutions contributed by the African lineages, which obscured the signal between Asian and European lineages (Elson et al. 2004). In our data, there are no genes in which the nonsynonymous-to-synonymous ratio is higher at haplogroup-associated sites; that is, no cases where the neutrality index suggests positive selection within *G. morhua*. Between sister species, the only exception to the general pattern is the ATP6 gene, which has a neutrality index of zero, which though not inconsistent with positive selection does not approach statistical significance.

The Pattern of Variation among Mitochondrial Genes in Gadines Is Consistent with Purifying Selection

The patterns of molecular evolution within *G. morhua* and between sister species are therefore consistent with nearly neutral theory, with an excess of slightly deleterious nonsynonymous polymorphisms among the closest hierarchical comparisons. The patterns of molecular evolution documented among gadines are an extension of this, with no evidence for gene (*Z*-tests) or site-specific (Bayesian posterior probabilities) positive selection. Furthermore, both between *G. morhua* and among nine species of gadines, nonsynonymous-to-synonymous rate ratios are relatively consistent among genes, with the notable exception of the very high ratio of the ND4L gene. Additionally, several genes (notably COX1 and COX2) fall well below the average ratio. In the absence of evidence for positive selection and in light of the neutrality indices described previously, it is easiest to interpret these results in terms of varying levels of functional constraint in the different genes.

What accounts for the unusual rate ratio of the ND4L gene? The high ratio is associated both with an abnormally high nonsynonymous rate and an abnormally low synonymous rate. Upon closer inspection, there is no particular pattern either to the type of amino acid change or to the lineage in which it occurs. Most changes are singletons, and no particular amino acid change is prevalent or lineage affected (Table 2). The ND4L gene encodes a small (98 amino acid) polypeptide, 1 of 46 subunits of the oxidative phosphorylation enzyme complex I, and catalyzes the reduction of ubiquinone to ubiquinol. The protein consists of one putative functional domain (amino acids 3–98) of the oxidored<sub>q2</sub> superfamily and resides in the membrane domain of complex I (along with the other mitochondrially encoded subunits; Mishmar et al. 2006). Variation in ND4L is biomedically important: one amino acid position in is associ-

**Table 2**  
**Amino Acid Substitutions in the Mitochondrial ND4L Gene among Gadines**

Taxon	Amino Acid Position								
	7	16	48	56	59	62	82	83	92
<i>Gadus morhua</i>	T	M	S	C	A	L	A	R	A
<i>Gadus chalcogrammus</i>	S	L	*	*	*	*	*	*	*
<i>Gadus macrocephalus</i>	*	*	*	*	*	*	V	G	G
<i>Arctogadus</i>	*	*	A	*	*	*	*	E	*
<i>Boreogadus</i>	*	L	*	*	*	*	*	*	*
<i>Merlangius</i>	*	*	*	*	V	*	*	*	*
<i>Melanogrammus</i>	*	*	*	Y	*	M	?	?	?
<i>Pollachius</i>	*	*	*	*	*	*	*	*	*
<i>Microgadus</i>	*	L	*	*	*	*	*	*	*

NOTE.—The single letter code is used, and \* indicates identity to *G. morhua*.

ated with Leber hereditary optic neuropathy (LHON) (Val65Ala; OMIM entry 516004) and another with colorectal cancer (Cys32Arg; OMIM entry 516004). Neither of these positions is variable among gadines: interestingly, position 65 is fixed for alanine, which suggests that this substitution is not the sole cause of LHON in humans. Although the dN/dS ratio of this gene among gadines is strikingly high, especially when compared with humans, in whom a very low ratio was reported (see Ingman and Gyllensten 2007), none of the statistical tests suggest positive selection. Hence, the most parsimonious explanation for the pattern observed remains a reduced mutation rate, in conjunction with reduced functional constraints in this gene.

Previously, we observed a priori evidence of selection on the ND5 gene of *G. morhua* and *G. chalcogrammus*, in the form of two amino acid substitutions at the carboxy terminus of the protein at which *G. chalcogrammus* is identical to non-*Gadus* gadines and differs from other *Gadus* species (Ser454Pro and Thr490Ala, in *chalcogrammus* vs. *morhua*, respectively; Coulson et al. 2006). Neither of these substitutions is considered “radical” (Grantham 1974), and in the present *G. morhua* sequences, the latter turns out to be polymorphic. Site-specific tests of positive selection are not as effective in the mitochondrial genome as in nuclear genes due to the lack of recombination. Nonetheless at this point, there is no evidence to support a role for positive selection at this gene in gadines. The convergent amino acid substitution in *G. chalcogrammus* and other gadines could have resulted from recurrent mutations, coupled with reduced functional constraints. It is worth noting that the N-terminus of the ND5 protein is highly conserved in haplogroup J of humans and is the location of many of the disease-causing substitutions (Moilanen et al. 2003). Mishmar et al. (2006) also report the influence of adaptive selection on Cys330 of the ND5 protein in primates.

Our results are especially interesting in light of the findings in the human studies, in which different genes are thought to be under positive selection in different phylogeographic lineages of human, thus suggesting the involvement of a role of climate. By way of contrast, we find no clear evidence for positive selection in any gene at three levels of hierarchical comparison, encompassing species in habitats as climatically diverse as the Pacific, Atlantic, Arctic, and Mediterranean waters. However, Ingman

and Gyllensten (2007) reexamined the results of the human studies by comparing rates of change in the functional domains within mitochondrial genes and concluded that each of the results previously reported in humans could be explained without invoking positive selection, by considering only genetic drift and functional constraints. Thus, the case for positive selection may not be particularly strong in humans either.

### Concluding Remarks

Within *G. morhua* and among gadines, then, we find that patterns of molecular evolution are best explained by mutation rate heterogeneity, slightly deleterious polymorphisms within species, and purifying selection with different levels of functional constraint among species. The finding of mutation rate heterogeneity is notable, and it will be of interest to investigate this in other taxonomic groups. Within *G. morhua*, there is no need to invoke recombination to explain the pattern of variation. This is not to say that positive selection and recombination are not important in other taxa. Meiklejohn et al. (2007) address the possible explanations for the genetic draft effect detailed by Bazin et al. (2006) and suggest the particularly intriguing possibility of selective sweeps in nematodes and insects caused indirectly by the maternally transmitted intracellular parasite *Wolbachia*, which would account for the high dN/dS ratios observed among haplotypes of *Drosophila simulans* (Ballard 2000). Complex factors contribute to mtDNA evolution, and these differ in different groups of organisms, therefore requiring examination on a case-by-case basis. The results here clearly demonstrate the value of whole-genome versus single-gene studies where mtDNA is used in a phylogeographic context. Novel, high-throughput biotechnology, such as multispecies microarray iterative sequencing platforms (the ArkChip: Carr et al. 2008), permits routine collection of whole-mtDNA genome data sets in a variety of fish, mammal, and bird species. Assessment of the effects of recombination and selection can now be brought out of the realm of humans and model organisms and into the broader world of the numerous “nonmodel” vertebrate species of interest to evolutionary biologists and conservation geneticists.

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