CHAPTER

2

Base Substitution in Fish
Mitochondrial DNA:
Patterns and Rates

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I. Introduction

Many of the authors in this volume use mitochondrial DNA (mtDNA) sequences because they are easily accessible, have high rates of evolution, and generally follow a clonal pattern of inheritance well suited to phylogenetic reconstruction (Wilson et al., 1985). This chapter is about the natural history of these sequences. Just as morphological systematists strive to analyze characters for which the pattern of development and effects of the environment are well known, so molecular systematists should begin by understanding the biology underlying the characters they use for inferring phylogenies. By understanding how changes accumulate in sequences, accurate models of substitution can be developed for use in phylogenetic inference.

Molecular sequences are deceptively simple in structure. There are just four bases common in DNA. These bases appear to be freely interchangeable, but in fact, mutation interconverts some nucleotides more frequently than others. Selection and drift then act on this spectrum of mutations in such a way as to prevent most substitutions from becoming fixed in the population. Neither mutation nor selection is homogeneous along a sequence of nucleotides; close examination reveals important differences in the pattern of mutation and selective constraint among nucleotide sites. Additional differences can be observed in comparisons among species.

Probably more is known about evolutionary patterns in animal mitochondrial genomes than for any other DNA sequence. Although some aspects of the substitutional pattern (e.g., the high proportion of transitions) are unique to animal mtDNA, this molecule is still an excellent model system to illustrate the analytic method needed to reconstruct phylogenies from DNA sequence data. This chapter focuses on patterns of mtDNA evolution in cichlid fishes. Examples are drawn from continuing studies of the gene encoding NADH dehydrogenase subunit 2 (ND2) in East African cichlids (Kocher et al., 1995). This data set is particularly useful because it includes a large number of closely related molecules which provide insights into the pattern of substitution usually obscured in comparisons among more highly diverged sequences.

II. Simple Models of Substitution

A. Mutational Models

At the core of most phylogenetic reconstruction algorithms is a simplified mutational model of the sub-
though additional substitutions continue to occur. The true evolutionary rate is hidden by the occurrence of multiple substitutions at a site.

Appropriate statistical corrections can be applied to transform the observed differences into a measure of the total number of changes that have occurred (total divergence, or evolutionary distance). These corrections can be derived for any of the mutational models, but are accurate only in the early stages of differentiation, before saturation has been closely approached. Furthermore, these corrections are accurate only if all of the nucleotide sites are evolving according to the same substitutional model.

C. Selectional Filter

Although mutational models have been widely used to describe the process of substitution, they ignore the influence of selection, which may be the dominant force regulating change in real sequences. It is easy to show, by comparison of nucleotide substitution rates at silent and amino acid replacement sites, that selection filters out more than 90% of all mutations which occur in mtDNA. Any concordance between the predictions of mutational models and the evolution of real sequences is therefore fortuitous.

Most simple models assume that substitutions occur randomly among sites following the Poisson distribution. Numerous demonstrations of the inadequacy of this model have been published (Fitch and Markowitz, 1970; Uzzell and Corbin, 1971; Kocher and Wilson, 1991). Substitutions do not occur with equal probability at each site. Instead, selection resists substitution at some sites, while allowing mutations at other sites to become fixed. A better model of this process uses a gamma distribution (Bliss and Fisher, 1953; Tamura and Nei, 1993), or a covarion model (Fitch and Markowitz, 1970; Miyamoto and Fitch, 1995), to allow rates of substitution to vary among nucleotide sites. The gamma distribution models have been mathematically formulated so that it is straightforward to correct distances for multiple hits (Tamura and Nei, 1993), but this is not yet possible for the covarion model. Few studies have attempted to estimate either the gamma parameter or the size and exchange rate of the covarion. It is important to remember that estimates of these parameters must be made from close relatives, as they provide the best information to quantify the process of substitution, free from the effects of multiple substitution.

For protein-coding sequences, it is possible to classify sites a priori according to the known selective constraints of the coding function. At the very least, it is recognized that first, second, and third positions of co-
III. Evolution of Real Sequences

To evaluate which theoretical models provide the most appropriate basis for phylogenetic reconstruction, the evolution of real sequences must be quantified. Here we examine a set of 56 mitochondrially encoded NADH subunit 2 (ND2) sequences (34 codons) obtained from 45 species of cichlid fish, mostly from East Africa. The most divergent comparisons involve New World species which presumably diverged from the African lineages more than 60 million years (MY) ago. The most closely related sequences are intraspecific polymorphisms differing by just a few nucleotides. These sequences not already reported in Kocher et al. (1995) are deposited in GenBank.

Ideally, we would plot the divergence of molecules with respect to geologic times of divergence. For these fishes, however, few reliable divergence times are available. Instead we will use the proportion of third position sites which have experienced a transversion as a measure of divergence. Transversions occur relatively rarely and in a nearly Poisson fashion (Irwin et al., 1991). These divergences are corrected for multiple substitution using a two-state model \(d = -0.5 \ln (1-Q)\), where \(Q\) is the observed proportion of transversions.

A. Changes in the Third Position of Codons

Many substitutions at the third positions of codons are synonymous (i.e., do not change the amino acid sequence of the encoded protein) and thus escape selection on protein structure. These sites therefore provide the most direct view of the mutational process. Although these sites are often thought to evolve according to a purely mutational model, some selective constraint does exist (Perna, 1996; Xia et al., 1996). While it would be inappropriate to equate substitutions at third positions with mutation, these sites approximate the underlying mutational spectrum more closely than the first or second positions.

The dominant feature of mtDNA evolution is the high rate of transition substitutions relative to transversions. At third positions the ratio of transition-transversion differences is at least 5:1 initially (Fig. 2), consistent with a strong transition bias in the underlying mutation process.

As transitions begin to occur repeatedly at the same sites, the ratio of transitions:transversions observed in pairwise comparisons drops. At a 10% transition difference, the ratio is only 2.5:1, and in the deepest comparisons it drops to 1:1. At a 10% transversion difference, the actual number of transition substitutions that have occurred is at least twice as great as the observed number of differences. The transition-transversion ratio is thus one way to quantify the degree of multiple substitution that has occurred since the common ancestor of two sequences.

Base composition influences the maximum observed difference. Figure 3 shows the accumulation of the two kinds of transitions possible: those involving the purines (A and G) and those involving the pyrimidines (C and T). It is interesting to note that the initial rate of transitions is the same for the two types of nucleotides. The purines, however, show saturation at a lower level of divergence than the pyrimidines. This pattern arises because the frequencies of A and G are much more unequal than the frequencies of C and T. At third positions the proportions are A,C,C,T: 0.32, 0.05, 0.38, 0.26. The maximum divergence of two sequences is calculated as 1 — probability of chance identity. For the purines described earlier, where only two states are possible (e.g., A or G), this is calculated as

\[
d_{max} = 1 - \left( \frac{f_a}{f_a + f_g} \right)^2 - \left( \frac{f_c}{f_c + f_t} \right)^2. \tag{1}
\]

The very unequal frequencies of A and G allow a maximum difference of just 23% instead of the 50% that would be expected given equal frequencies of the two nucleotides. For C and T, the maximum difference is higher, about 48% (Kocher et al., 1995). These differences explain why the purine transitions reach saturation before the pyrimidine transitions.

These mitochondrial sequences approach saturation rapidly. Evidence of multiple substitutions is quite apparent at only 2% transversion difference. The mammalian fossil record suggests that this corresponds to about 2 MY of divergence (Irwin et al., 1991). The fossil record of cichlids is more difficult to interpret, but a similar rate does correlate well with the geologic history of East Africa (Kocher et al., 1995). The fact that saturation effects begin to arise after just 2 MY of divergence underscores the importance of corrections for multiple substitution when constructing phylogenies of more distantly related taxa.
B. Changes in First and Second Positions and Amino Acid Substitution

At the first and second position of codons, selection dominates the substitution process. This is apparent from the rate of transition substitution, which is 6- and 15-fold slower at first and second positions, respectively, than the rate at third positions (Fig. 4). Because there is no reason to suspect a slower mutation rate at these sites, the difference must arise because selection prevents fixation of most mutations. Selection also constrains the maximum amount of difference that is observed between two sequences. Second positions plateau at approximately 3% transition difference, while first positions plateau at about 8%. The comparable value at third positions is 25%.

Selective constraint has a strong effect on base composition, which differs among the three codon positions. First positions are relatively rich in GC because of the high leucine and alanine content of the ND2 protein. Second positions show a high proportion of T and C (37.9 and 30.4%, respectively), probably because hydrophobic amino acids required for this membrane-spanning protein are encoded by either C or T at the second position (Naylor et al., 1995).

Probably the most important characteristic of selective constraint is that it varies from site to site along the molecule according to the structural function of the
FIGURE 3 Differences in the accumulation of transition differences involving purines (●) and pyrimidines (○). Because the frequencies of A and G are very unequal, saturation for sequence difference occurs earlier and at a lower value, than for C and T.

FIGURE 4 Accumulation of sequence difference at the first and second codon positions. The rate of accumulation of transition substitutions is 6- and 15-fold slower, respectively, than at the third positions.
encoded protein. Some positions in the protein can accept substitution relatively easily, whereas selection acts to prevent substitution at others. In ND2, this pattern of constraint can be visualized by plotting the proportion of variable sites along the molecule (Fig. 5). For many mitochondrial encoded proteins, the membrane-spanning helices of the protein experience high levels of substitution, whereas the turns between these helices appear relatively conserved (e.g., Irwin et al., 1991). The structure of ND2 is less well known, but it appears that regions of the protein outside the membrane may be constrained by selection for appropriate contacts with other proteins.

If selection did not alter substitutional probabilities among sites we might expect to model the occurrence of multiple hits using a Poisson distribution. In fact, real molecules rarely fit a Poisson. Table I shows the number of changes per site estimated by parsimony over a tree for 10 closely related cichlids. The 72 inferred substitutions are distributed among just 53 of the 348 positions in the sequence. These data do not fit a model in which substitutions occur randomly along the sequence. Rather, the significant deviation from Poisson expectation demonstrates that the substitutions are clustered at a relatively small number of sites.

It appears that selection is preventing substitutions at a large fraction of sites. Because the magnitude of selective constraint is likely to be a continuously distributed variable, it is most appropriate to model this variation as a gamma distribution. The gamma distribution uses an additional parameter to quantify the variance in rate among sites. Most real sequences can be modeled rather well with this distribution.

Fitch and Markowitz (1970) coined the term covarion to describe the set of "concomitantly variable codon positions." In their view, only a small proportion of the codons for a protein can experience an amino acid substitution at any given instant (the covarion). Substitution is thought to slowly alter the selective constraints so that the sites making up the covarion change over time (Fitch, 1971). The data in Table I can be made to fit a Poisson model, if it is assumed that the covarion con-

TABLE I. Estimated Distribution of the Number of Amino Acid Substitutions per Site Estimated over a Parsimony Tree of 10 East African Cichlid Taxa* *

| Number of changes per site over tree | 0 | 1 | 2+
|--------------------------------------|---|---|---
| Assuming 348 variable sites          |   |   |   |
| Observed                             | 205 | 37 | 16 |
| Poisson expectation                  | 202.96 | 58.54 | 6.48 |
| Assuming 100 variable sites         |   |   |   |
| Observed                             | 47 | 37 | 16 |
| $\chi^2 = 0.1713, p > 0.9$           | 48.68 | 33.05 | 16.28 |

*The 10 species and their assumed relationships are (Hemichromis sp., Lethrinops aurita, Pseudotropheus zebra), Cynotilapia jollyi, Cynotilapia nigrofasciata, Cynotilapia freshwater, Paratilapia microlepis), Lethrinops aurita), and Panagurichromis boultoni).
sits of only 100 of the amino acid sites. Selection may be acting to prevent substitutions at the other 48 sites in the molecule.

Miyamoto and Fitch (1995) demonstrate that the co-varion model is more biologically correct than models based on the gamma distribution. It remains to be seen whether the difference between the co-varion and the gamma models is important to phylogenetic analysis and whether the co-varion model can be mathematically formulated for convenient use.

IV. Implications for Phylogenetic Reconstruction

A. Choosing a Model

There are two important decisions to be made when attempting to recover phylogenetic information from an analysis of molecular sequences. First, a substitutional model must be chosen which accurately models the evolution of the molecule under study, including whether rates vary among sites. Second, a particular tree-building algorithm must be selected. Although much has been written about the accuracy of various tree-construction algorithms, the choice of a substitutional model probably has a far greater impact on the phylogenetic conclusions.

Since the pattern of evolution varies among genes (e.g., nuclear versus mitochondrial), it is important to adjust the substitution model for each analysis. Some tree-building algorithms allow this modeling more easily than others because the assumptions are more explicit or the model more easily adjusted. In general, it is easier to adjust the evolutionary model using methods which first calculate a distance statistic. Probably the best is the gamma distance developed by Tamura and Nei (1993), which incorporates transition/transversion bias, compositional inequalities, and variance in rate among sites. Once accurate distances are calculated, almost all tree algorithms will yield the correct topology (Huelsenbeck and Hillis, 1993). The neighbor-joining algorithm (Saitou and Nei, 1987), for example, can construct trees from distance matrices calculated according to many different models. There are several advantages to focusing on the development of a distance matrix prior to initiating tree construction. First, calculation of the distance matrix focuses attention on the model of sequence evolution to be used. Second, it is usually more efficient to estimate topologies from a distance matrix than by searching for minimum evolution trees among a universe of possible topologies.

Third, it is easier to calculate standard errors for each divergence than to evaluate the support for nodes using a bootstrap approach. This is particularly true for sequences that are near saturation.

B. Power Analysis for mtDNA Phylogenetics

Because of the heterogeneous nature of evolution along a DNA sequence, sites differ in the amount of information they carry about a particular phylogenetic relationship. It is important to determine which sites and classes of change give the greatest signal/noise ratio for testing a particular phylogenetic hypothesis. Characters must be chosen to match the time scale of the divergences being studied. An analysis using third position transitions to evaluate basal (60–80 MYB) relationships in the Cichlidae would be fundamentally flawed, since these substitutions are completely saturated after about 10 MY of divergence (Fig. 2). Neither would it be appropriate to focus exclusively on third position transversions to recover relationships among close relatives because of the large amount of information that would be lost by not including transitions.

We wish to know which characters from the ND2 sequence give the greatest power for resolving relationships among cichlids at various divergences. First, we must specify the model for the evolution of bases in the sequence. Because of the importance of both base composition and variance in rate among sites, the Tamura–Nei gamma-distance model is the most appropriate for these data. This model describes how sequences diverge through time by calculating the expected proportion of transitional differences for purines (P1) and pyrimidines (P2) and the expected proportion of transversional differences (Q). The mean values of these expected differences are used, assuming that rates vary among sites according to a gamma distribution [Tamura and Nei (1993) Eqs. (12–14)].

\[
\bar{P}_1 = \frac{2\gamma_{8c} \gamma_{8s}}{\gamma_{8s}} \left[ \gamma_{8s} + \frac{a}{a + 2\gamma_{8s} \gamma_{8c} \gamma_{8s} B_1} \right],
\]

\[
\bar{P}_2 = \frac{2\gamma_{8c} \gamma_{8s}}{\gamma_{8s}} \left[ \gamma_{8s} + \frac{a}{a + 2\gamma_{8s} \gamma_{8c} \gamma_{8s} B_1} \right],
\]

\[
\bar{Q} = 2\gamma_{8s} \gamma_{8s} \left[ 1 - \left( \frac{a}{a + 2\gamma_{8s} B_1} \right) \right].
\]
where $g_A$, $g_C$, $g_T$, and $g_G$ are the nucleotide frequencies, $a$ is the gamma parameter, $\alpha_1$ and $\alpha_2$ are the rates for transitional changes for purines or pyrimidines, and $\beta$ is the rate for transversional change. These equations give the time-dependent differences between two sequences. We use the equations for $P$ and $Q$ rather than $s$ and $v$ as they clearly represent data regarding the saturation of sequence differences resulting from multiple substitutions.

We consider the power of the first, second, or third position transversions or transversions to identify a 1 MY period of shared ancestry between sequences B and C, after their divergence from sequence A (see Fig. 6), at various times in the past. This is done by first calculating the number of sequence differences accumulating in that 1 MY interval, at various times in the past. In order to calculate the number of differences, $\Delta P$ or $\Delta Q$ which accumulate within some time interval we need to calculate the derivatives of the previously described equations.

\[
\frac{dP}{dt} = \frac{4g_A g_C}{g_A} \left( \frac{a}{a + 2(g_A \alpha_1 + g_C \beta)} \right)^{t-1} - g_A \beta \left( \frac{a}{a + 2\beta t} \right)^{t-1} \tag{5}
\]

\[
\frac{dQ}{dt} = \frac{4g_A g_C}{g_A} \left( \frac{a}{a + 2(g_A \alpha_2 + g_C \beta)} \right)^{t-1} - g_A \beta \left( \frac{a}{a + 2\beta t} \right)^{t-1} \tag{6}
\]

\[
\frac{dQ}{dt} = 4g_A g_C \beta \left( \frac{a}{a + 2\beta t} \right)^{t-1} \tag{7}
\]

Based on these derivatives, we can calculate the number of transitional or transversional differences accumulating during a time interval, $\Delta t$, from

\[
\Delta P = \left( \frac{dP}{dt} + \frac{dP}{dt} \right) \Delta t \tag{8}
\]

\[
\Delta Q = \frac{dQ}{dt} \Delta t. \tag{9}
\]

Typical parameters for vertebrate evolution can be obtained from the cichlid ND2 sequences as follows. Gene frequencies are determined from the sequence data. If we assume

\[
\bar{\alpha}_1 = \bar{\alpha}_2 = \bar{\alpha}
\]

we can solve for $\alpha$ and $\beta$ using Eq. (5) plus Eq. (6) and Eq. (7) to get the following equations:

\[
\alpha = \frac{dP}{dt} (t = 0) \quad \text{and} \quad \beta = \frac{dQ}{dt} (t = 0)
\]

Using Figs. 2 through 4, we can estimate $dP/dt$ and $dQ/dt$ at time $t = 0$. The parameters determined from the ND2 data are given in Table II. Gamma values are taken from an analysis of mammalian cytochrome b genes (Irwin et al., 1991). Figure 7 plots the number of sequence differences accumulating in the 1 MY interval of interest at various times in the past. We have calculated the average value of $\Delta P$ or $\Delta Q$ over the 1 MY time interval.

Clearly, third position transitions contribute the greatest number of changes in the first few million years—more than all the other characters combined. With time and the effects of saturation, the accumulation of third position transition differences decreases. After only 5 MY, the combination of changes at first and second positions with third position transversions
contributes a greater number of differences in a 1 MY interval than do third position transitions. This plot represents only the signal, however. There will also be variability in the differences between the sequences resulting from the variance contributed by the differences estimates from the long terminal branches of the tree. This variability will contribute noise which, if large enough, will obscure the signal we hope to observe. The noise is determined from the standard deviation which is simply related to the variance. The binomial variance and standard deviations for \( P \) and \( Q \) are given by

\[
\text{var}(P) = \frac{P(1-P)}{n} \quad \sigma(P) = \sqrt{nP(1-P)} \\
\text{var}(Q) = \frac{Q(1-Q)}{n} \quad \sigma(Q) = \sqrt{nQ(1-Q)}
\]

Figure 8 plots both the number of changes contributed in a 1 MY interval at various times in the past and the standard deviation associated with differences accumulated on the terminal branches. The crossing points of the curves give an indication of when the phylogenetic signal uniting taxa B and C will be hidden by the variance associated with the long terminal branches.

These crossing points have been calculated for various periods of common ancestry (1, 2, 5, and 10 MY) for data sets of various sizes (102 codons representing typical cytochrome b fragment data, 348 codons representing the eichlid ND2 data set, 1000 codons representing approximately three mitochondrial genes, and 3754 codons representing the complete coding regions for human mitochondrial DNA) as a function of the terminal branch length. Figure 9 summarizes the calculations in a format useful for planning phylogenetic studies. To use the figure, first determine the approximate time of divergence for the taxa under study (either through fossil evidence or by estimating third position transition difference from a pilot study). Next, identify the curve associated with the desired degree of resolution (1, 2, 5, or 10 MY between divergences). Finally, read the number of codons which must be sequenced from the y-axis.

**FIGURE 7** The number of sequence differences accumulating in a 1 MY interval at various times in the past and observed at time zero. Third position transitions contribute the greatest number of differences over the recent past, but the sum of transitions and transversions at first and second positions soon dominates.
FIGURE 5  Power analysis for mitochondrial sequence data. The situation considered is shown in Fig. 6. The number of codons required to detect 1, 2, 5, or 10 MY periods of common ancestry, at various times in the past. The base composition and rates of evolution of the cichlid ND2 gene (Table II) are assumed. The curves represent the number of codons required so that 1 SD of the estimate of terminal branch length is equal to the mean number of differences arising along the internal branch of the tree. Data are shown for transitions (2P) and transversions (3Q) in first (1), second (2), and third (3) positions. The x axis assumes a transversion divergence of 1% per million years. A spreadsheet is available from the authors for calculating crossing values for taxa with radically different base compositions or rate matrices.
Several findings can be gleaned from these graphs. First, it is quite surprising that, even with the complete ND2 sequence, none of the characters have power to resolve a 1 MY difference beyond about 2.5 MY (only transitions at third positions are effective over this period). More slowly accumulating differences (e.g., changes at first and second positions), although not saturated (Fig. 4), are not effective in resolving the relationship because of the large variance associated with the small number of substitutions.

Second, the greatest statistical power, even for deeply divergent lineages, is found in the third position transversion data. This is because third position transversions accumulate relatively rapidly and do so in a nearly Poisson fashion. The slower rates, and greater variation in rate among sites for other characters, sharply reduce their power.

The third surprising finding is that mtDNA data have the potential to resolve rather deep divergences. Complete mitochondrial coding sequences should be able to resolve a 10% difference in divergence times, even 75 MY in the past. However, 100 codons give only coarse resolution for very recent events.

We expect that this figure will be accurate for most vertebrate mtDNAs. Our preliminary examination of other taxa suggests that base composition is an important determinant of the power for resolving short time periods of shared ancestry. However, it is clear that a variation in rates among sites is the single most important factor affecting the performance of a given sequence in phylogenetic analysis. Differences in the assumed gamma parameter can have a dramatic effect on the accuracy of distance estimation.

V. Conclusions

A. Choice of mtDNA Sequence Characters

It is well known that third position transitions, although useful for recent divergences, saturate rapidly. It is somewhat surprising that transversions at the third position outperform changes (both transitions and transversions) at the first and second codon positions. The advantage seems to arise because third position transversions approximate a Poisson distribution, whereas strong selection at first and second positions creates a large variance in substitution probability among sites.

B. Power Analysis

Poor resolution of branching order deep in the tree is a common result in phylogenetic analyses. Investigators frequently attribute this result to rapid radiation of the taxa in question. The alternative, that data are simply insufficient to resolve even moderately spaced speciation events, is rarely considered objectively. The graphs in Fig. 9 are a good tool for gauging the power of a particular data set to resolve closely spaced bifurcations. The steep slopes of the curves suggest that many published studies may have failed to gather sufficient data to detect even a 10% difference in divergence times.

C. Adequacy of Mutational Models

Selection dominates the substitution process at almost all sites in the mitochondrial genome. It is therefore surprising that the models of sequence evolution most often used in phylogenetic analysis still focus on the mutation process. Use of mutation-based models is probably not appropriate for anything but third position transitions (Xia et al., 1996). Approaches that explicitly model the selection filter should be pursued, as they may allow modeling of sequence divergence further back in time.

D. Reality of Rate Variation

Suggestions have been made that the rate of mitochondrial DNA evolution varies among lineages (e.g., Martin et al., 1992; Berningham et al., 1997). Attention has begun to shift toward identifying physiological correlates which might account for this rate variation (Martin and Palumbi, 1993; Rand, 1994). While rate variation may well exist, the search for the causes of such variation may be premature. The rate of substitution is the result of a delicate interplay of forces, including mutation, selective constraints arising at both molecular and organismal levels, and population-level events. We should be careful not to jump from poorly supported correlations to hypotheses of causation. It is hoped that this chapter will promote the development of a consistent set of evolutionary rate estimates, incorporating corrections for both unequal base composition and patterns of selective constraint. When used in relative-rate tests, or in well-substantiated absolute calibrations of evolution rate, these estimates may lead to new hypotheses on both the generality of mitochondrial clocks and the forces which regulate their ticking. A further benefit is that the refinement of substitution models will improve our ability to reconstruct phylogenetic relationships among species.

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References


