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RATES OF MOLECULAR EVOLUTION: Phylogenetic Issues and Applications

David P. Mindell and Christine E. Thacker
Department of Biology and Museum of Zoology, University of Michigan, Ann Arbor, Michigan 48109-1079

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ABSTRACT

The proper relationship between systematics practice and our understanding of evolution has been long debated. Systematists seek to avoid assumptions about evolutionary process in their methods, yet a growing body of evidence indicates that patterns in rates of evolution can be used to reduce effects of homoplasy. We review variable evolutionary rates for molecular characters in the context of constraints on mutation and fixation. Some constraints, like the genetic code for protein-coding genes, are consistent in the direction of their effects, whereas others, like population size and cladogenesis frequency, are historically variable within and among lineages.

We review methods for assessing rate variability, and we estimate comparative absolute rates of change for five sets of mitochondrial DNAs in 12 vertebrates for application in phylogenetic analyses. Unequal weights for subsets of mitochondrial DNAs improved congruence with the most highly corroborated tree in many but not all cases. The largest data set (12,120 bases) yielded the same tree under all four weighting alternatives. This is consistent with the notion, echoing the law of large numbers, that as data sets increase in size, homoplasy will tend to cancel itself out. Even if this notion has validity, however, evolutionary biology will remain vital in systematics if we want to: match sets of taxa with characters likely to be historically informative (when data sets are not sufficiently large); avoid comparing characters with different histories due to reticulations, horizontal transfer, or lineage sorting; avoid assuming random distribution of homoplasy; be alerted to the possibility of long-branch attraction problems; and understand the cause of the hierarchy of taxa in nature as inheritance of genetic material and descent with modification.

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INTRODUCTION

An appealing aspect of cladistics is its claim of relative freedom from assumptions about evolutionary processes (29, 86, 120). If we presume the existence of 1) a natural, divergent hierarchy of organisms based on common descent and 2) identifiable shared-derived characters (homologies) for taxa within the hierarchy, then discovery of monophyletic groups appears to be a straightforward task. Preference for a parsimony criterion in this discovery process derives from the general scientific practice of minimizing ad hoc assumptions (assumptions of homoplasy in the case of phylogenetic analyses) and the notion that parsimony correctly determines which phylogenetic hypothesis is best supported by the character evidence (32, 85). One need not invoke any specifics of evolutionary process to estimate genealogy. This enhances objectivity of phylogenetic analyses by separating them from preconceived notions of evolutionary process.

However, homologies can be difficult to identify. This may be attributed in part to the existence of a finite number of character states (four in the case of DNAs) and rates of change sufficient to yield independent expressions of the same state. Putative homologies, like putative relationships among taxa, are products of phylogenetic analysis (85); attempts to improve them must, therefore, involve refinements in phylogenetic analyses. Such refinement has long been sought in the use of conservative characters. By giving greater weight in phylogenetic analyses to characters changing less frequently, confounding effects of homoplastic similarity can be reduced.

Thus, improved understanding of rates of molecular evolution has the potential to improve phylogenetic analyses. However, for some this may appear to conflict with cladistic parsimony's appeal of making minimal assumptions about evolutionary process; and so several questions arise. Does character weighting based on relative rates of change conflict with a "total evidence" approach to character analysis (61, 111)? Can parsimony analyses recover genealogy without recourse to knowledge and theory regarding biological evolution? If not, what knowledge of evolution is needed? How is that knowledge gained, and how might it be applied? These questions focus on the relevance of biology for phylogenetic inference.

ASSESSING EVOLUTIONARY RATES

Organismal Rates

Absolute rates of molecular evolution have been estimated using fossil or biogeographic events together with pairwise distance measures, to provide a minimum age for the divergence of pairs of taxa. Such estimates must presume 1) that the rate is constant over time and across taxa for species involved in the

pairwise distance comparisons and 2) that the fossils or biogeographic dates accurately reflect lineage divergence times. Unfortunately, these are not safe presumptions. Use of discrete character branch lengths, rather than distances, can obviate the first but not the second assumption.

A brief look at avian divergence time and rate estimates illustrates the difficulties. Sibley & Ahlquist's (106) calibration for DNA-DNA hybridization distances was based on assuming that a sister relationship existed between the ostrich and rhea lineages, and that the Atlantic Ocean created a dispersal barrier to their most recent common ancestor about 80 million years ago (mya). However, the sister relationship of ostriches and rheas as well as the Gondwana origin of ratites (including ostriches and rheas) has been contradicted by paleontological evidence (55) and mitochondrial DNA (mtDNA) analyses (18). Shields & Wilson (104) estimated the mtDNA divergence rate in geese at 1.8% to 2.3% per my, based on distances from RFLP data. This estimate is similar to absolute rate estimates of 2.0% per my for mtDNAs in mammals (122), suggesting similar rates in birds and mammals. However, the calculation for birds suffers from being based on a single divergence time estimate and has led to estimates of effective population sizes inconsistent with observed sizes (5, 90). This calculation also conflicts with evidence indicating slower rates of mitochondrial sequence evolution in birds relative to mammals (59, 76). Thus, the assumptions inherent in calibrating require that they be interpreted cautiously (52).

Relative rate tests for lineages are based on the expectation of equal amounts of character change in sister taxa relative to an outgroup, if rates of evolution in the sister taxa are equal. This follows from the fact that sister taxa have had equal amounts of time for accumulation of change relative to the outgroup, and it makes the test independent of fossil or biogeographic event dating. Relative rate tests based on distances (66, 98, 125) must assume equal amounts of homoplasy across taxa. Relative rate tests using branch lengths from phylogenetic trees to estimate amounts of unambiguous character state change and a binomial test to assess departure from the expected 50% of all change found in each of two ingroup taxa (75), together have the potential for recovering differential homoplasy amounts across taxa. The advantages of discrete character relative rate tests, compared to relative rate tests based on genetic distances, are that 1) different kinds of character change may readily be used in seeking to emphasize those with lower levels of homoplasy; 2) no assumptions of equal amounts of homoplasy across taxa are made, as homoplasious similarity may be recognized and tallied on phylogenetic tree branch lengths; and 3) internal branches shared by ingroup taxa may be excluded from analyses, thereby preventing one form of nonindependence (Figure 1). Alternative pairwise comparisons involving the same species, however, are not independent.

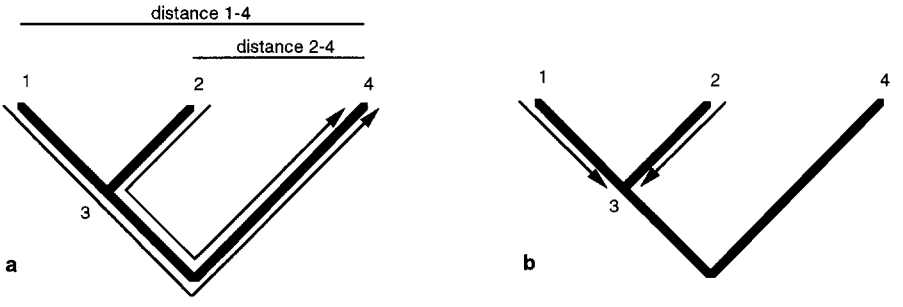


Figure 1 Comparison of distance versus parsimony-based relative rate tests (RRTs). *1a.* Distance-based RRTs have non-independence problems in counting changes twice that accumulate historically between 4 and 3. *1b.* Parsimony-based RRTs compare only autapomorphies for *1* and *2*. Further, assumptions of equal amounts of homoplasy across taxa are not made as in *1a* because homoplasious similarity may be recognized and tallied on phylogenetic tree branch lengths.

Evolutionary rate estimates may be biased in favor of finding faster rates in clades with more taxa, as overwritten character state changes can be discovered only by adding taxa to a clade (36, 45). Although less widely recognized, addition of taxa may also result in less of the actual change being detected due to convergent events (see 78, 97). Both potential biases stem from analyses of data sets with high levels of homoplasy. Focus on relatively conserved types of sequence change and data sets with low levels of homoplasy works to minimize these potential biases. Relative rate tests may lead to false conclusions if the two ingroup taxa are so recently diverged that actual differences in rate have not yet appeared, or if the designated outgroup is actually more closely related to one or the other of the ingroup taxa.

Though we focus on parsimony-based relative rate tests, least-squares and maximum-likelihood methods have also been developed and are designed for identifying sequences that differ in rate compared with an average rate for all sequences (35). Muse & Gaut (83) implement a likelihood approach in comparing relative rates of change across taxa for all characters as well as for transitions (TIs), transversions (TVs), synonymous and nonsynonymous nucleotide substitutions in their program CODRATES. Their evolutionary model uses the codon, rather than the nucleotide, as the unit of evolution, and it seeks to account for dependencies among nucleotides and multiple hits within a codon. F-ratio tests (96) have also been used to investigate rate variation across taxa by comparing length of sister branches from distance trees (103, 110), though these tests assume both additivity and independence (34).

Character Rates

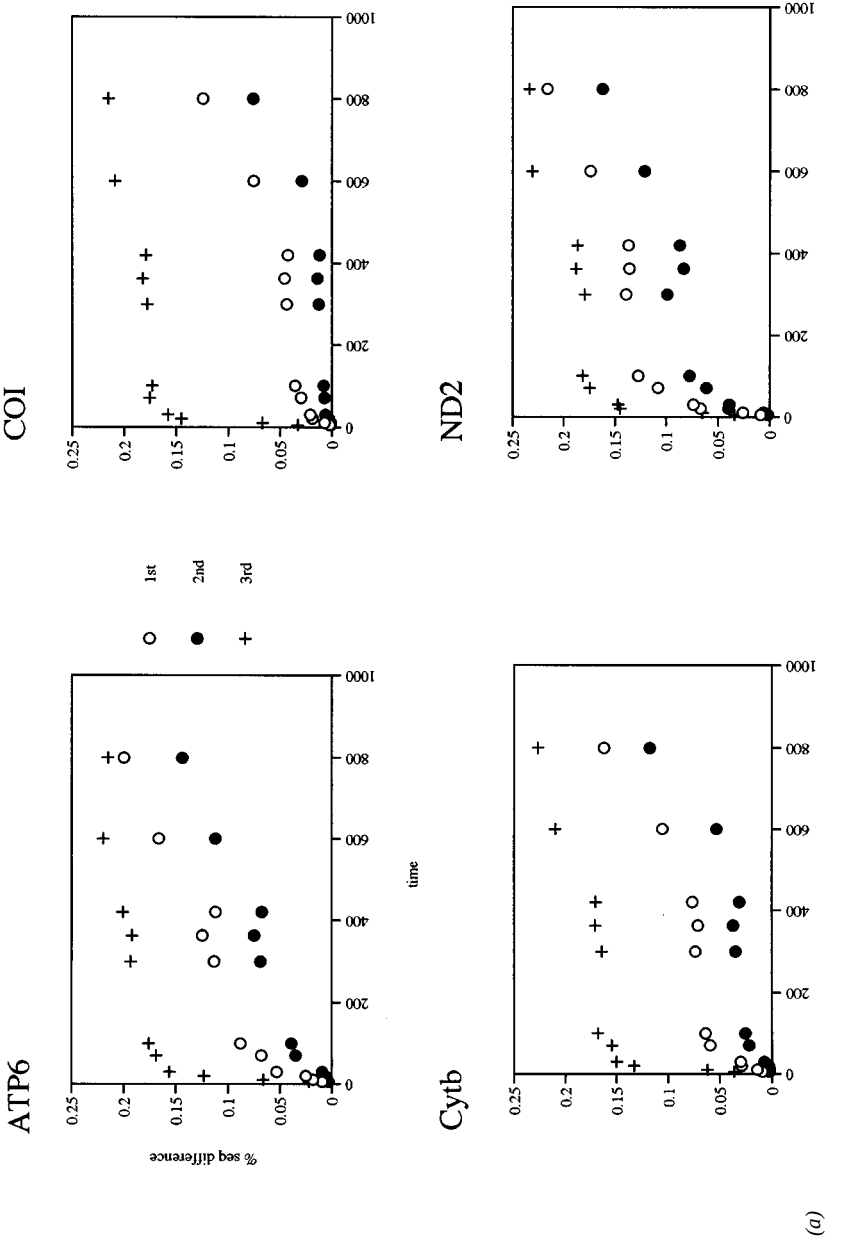
Number of nucleotide substitution types found in pairwise comparisons of taxa may be plotted against estimated time since divergence of those taxa to estimate absolute rates of character change. Absolute rate estimates can be compared for various kinds of characters to determine their degree of saturation with change over time (Figure 2). Alternatively, the number of substitutions of different kinds can be plotted against each other to provide an estimate of relative rates of change (73, 116). If one kind of substitution decreases in frequency relative to another, or relative to absolute time, this suggests multiple changes at single nucleotide positions and some degree of saturation with change.

However, such interpretation of rate estimates entails assumptions and must be considered cautiously. Homoplasy can be recognized only in the context of a phylogeny and will be underestimated in pairwise comparisons (38). Comparisons averaging rates of change across sites will tend to underestimate biases in rates of change for specific nucleotide substitutions (116a). Application of pairwise comparisons also assumes a degree of rate constancy over time and across taxa. Increased sampling of taxa and of divergence time intervals will provide more robust rate comparisons, reducing the impact of any one taxon; however, the relative rate differences found remain tentative and should be reassessed as more data becomes available.

Apparent advantages of character rate comparisons based on branch lengths are as mentioned above (points 2 and 3), and it would be useful to know if the same patterns are seen in both types of comparison. We performed such comparisons and found that the relative rate estimates determined in a phylogenetic context show patterns similar to those from direct pairwise comparisons (Figures 2 and 3; see below for methods). For example, in the 13 mitochondrial protein-coding gene graphs, both estimates of change decrease in rate, suggesting multiple hits, after about 100 my. Estimates of evolutionary rate for categories of character change are generally not done in the context of a phylogeny because the phylogeny is unknown. Several methods have been used to estimate rates iteratively in phylogenetic analyses (31, 121); however, these focus on individual characters and do not pertain directly to learning about variable rates for particular categories of sequence change.

CONSTRAINTS AND PATTERNS FOR MOLECULAR RATES

Recognizing mutation and fixation as two fundamental steps in evolution, we summarize primary constraints influencing rates of change at these two levels (Table 1). Variation in mutation rate can stem from differences in replication


COI
ND2
ATP6
Cytb
1st
2nd
3rd
% seq difference
time
0.25
0.2
0.15
0.1
0.05
0
200
400
600
800
1000

(a)

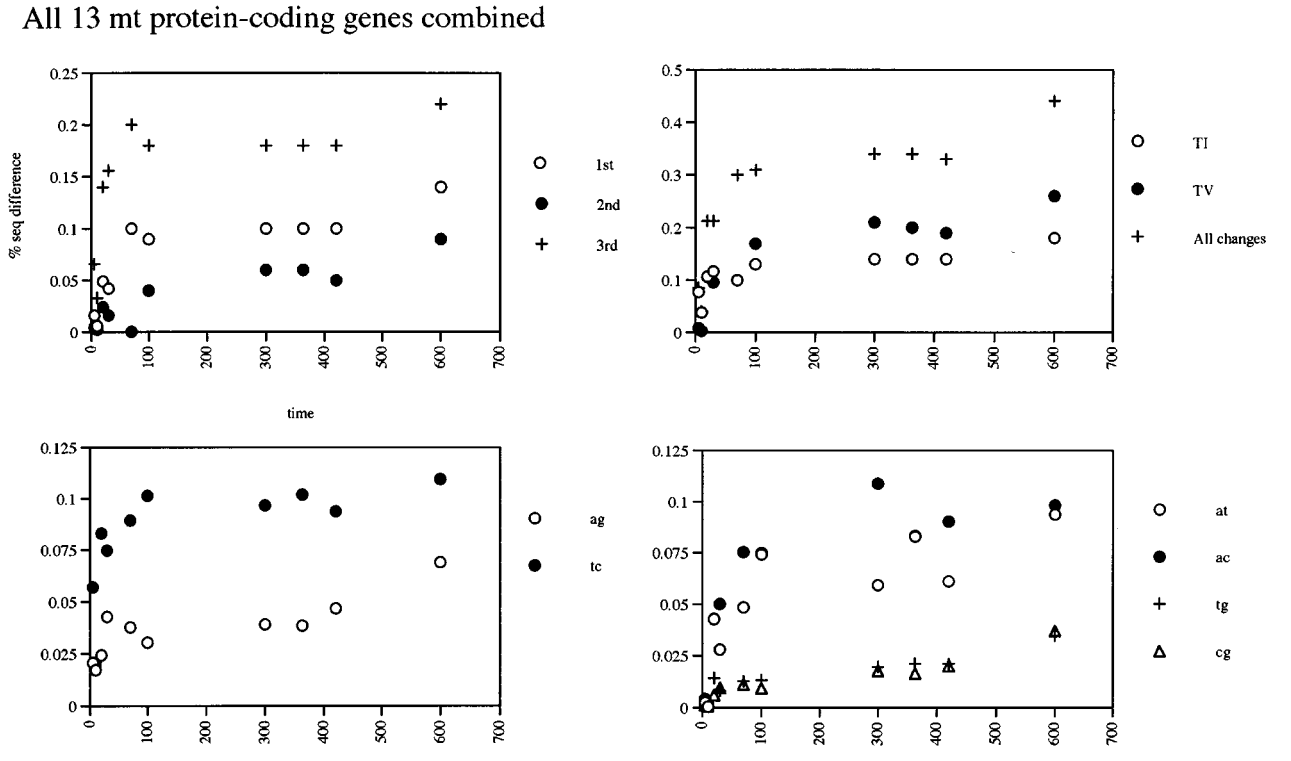


Figure 2 Comparative absolute rates of sequence change based on direct comparisons of species pairs. Plots show percentage DNA sequence difference versus estimated time (mya) since divergence for (*a*) four mitochondrial protein-coding genes, and for (*b*) 13 mitochondrial protein-coding genes combined (broken down by codon position and types of change; TI, transitions; TV, transversions). Comparisons involve the following 12 taxa (with database accessions numbers): *Cyprinius carpio*, carp, X61010; *Crossostoma lacustre*, loach, M91245; *Xenopus laevis*, frog, X02890, M10217, X01600, X01601; *Gallus gallus*, chicken, X52392; *Didelphus virginiana*, opossum, Z29573; *Mus musculus*, mouse, V00711; *Rattus norvegicus*, rat, X14848; *Homo sapiens*, human, V00662; *Balaenoptera physalus*, fin whale, X61145; *Balaenoptera musculus*, blue whale, X72204; *Phoca vitulina*, harbor seal, X63726, S37044; *Halichoerus grypus*, grey seal, X72004. Estimates of divergence times are based on fossil and biogeographic evidence as summarized in Benton (7).

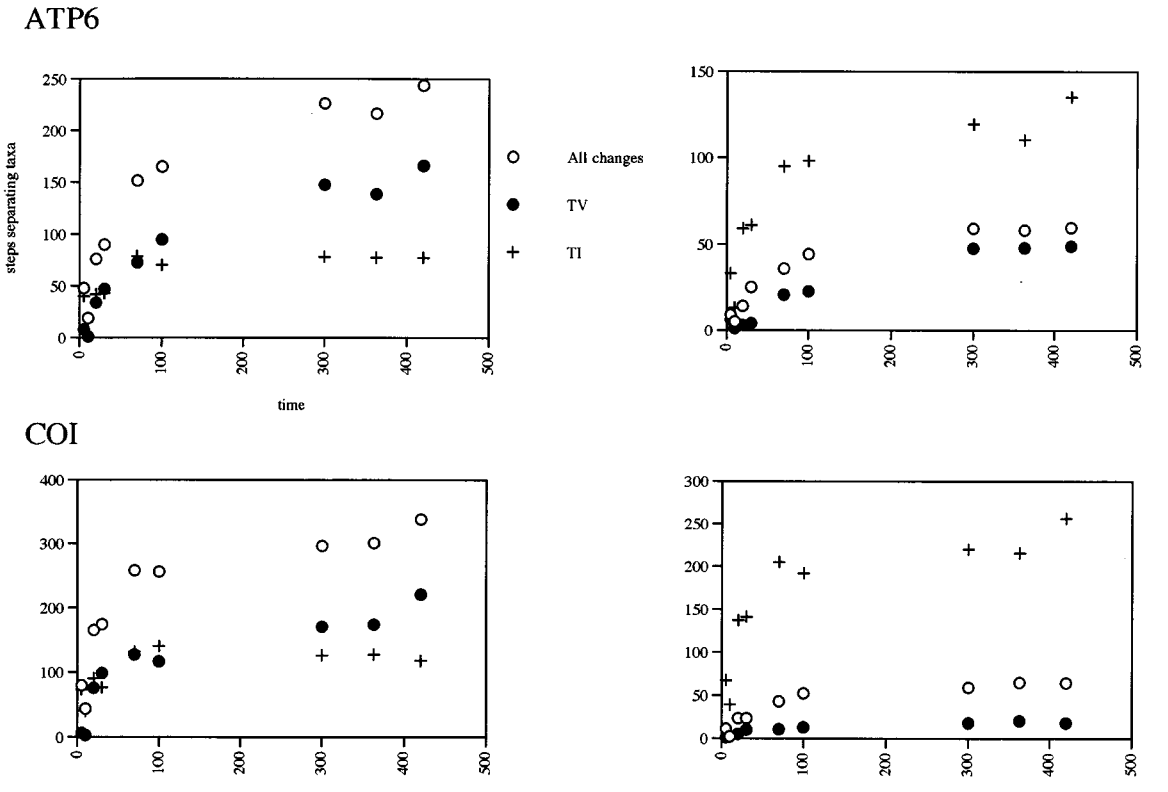


Figure 3 Comparative absolute rates of sequence change based on phylogenetic tree branch lengths. Plots show total numbers of character changes (counted from the two branch tips back to their first shared node) separating species pairs, versus estimated time since divergence for four individual mitochondrial protein-coding genes, and for 13 mitochondrial protein-coding genes combined. Comparisons involve the same 12 taxa as in Figure 2.

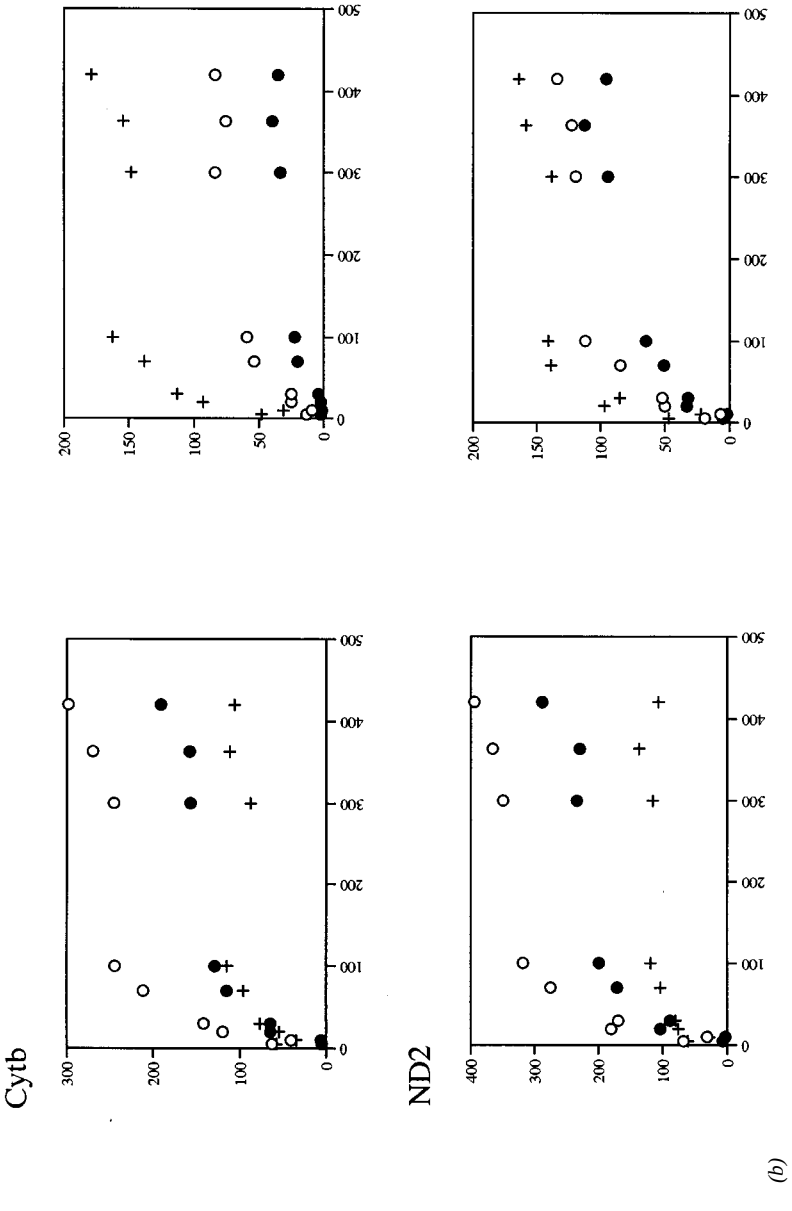
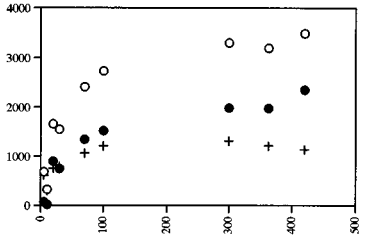


Figure 3 (*Continued*)

All 13 mt protein-coding genes combined

(c)



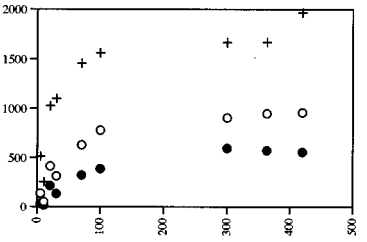


Figure 3 (*Continued*)

fidelity (different polymerase types), replication repair (different mechanisms and different enzymes), replication frequency, exposure to mutagens (especially DNA-damaging oxygen free-radicals), and the initial conditions of differential codon and nucleotide base composition for genes and taxa. These factors and others may influence variation in both the overall rate and the rate for particular kinds of change. The likelihood that one or more mutations become fixed is influenced by a set of continuous and overlapping constraints, including the genetic code, secondary or tertiary structure, gene function, population size, frequency of cladogenesis, and natural selection.

Variation in the nature of these constraints across taxa and over time contributes to patterns of variation in rate of molecular sequence evolution for different taxa and different kinds of character changes. However, determining which of the multiple overlapping constraints are responsible, and to what degree, may be difficult or impossible. So-called patterns must be considered as hypotheses to be judged on their individual merits for the set of taxa or genes in which they are proposed. In preliminary analyses, we characterized covariation among various types of character change (by codon position and by each of the six possible base substitution pairs) using principal component analysis, and we found that no single category of change served well as a proxy for a larger subset.

Recognizing that patterns in rate stem from variation in the constraints on mutation and fixation (Table 1), we consider patterns among taxa as characterizing groups whose members have similar sets of constraints, rather than characterizing individual clades. The need for this is demonstrated by numerous observations of rate differences among closely related taxa differing in the nature of their constraints (e.g. 8a, 9, 23, 37, 77, 126). Further illustrating a decoupling between patterns of rate variation and clades are studies showing that two or more sets of homologous genes need not show the same pattern of rate variability across the same set of organisms (76). That is, constraints vary

Table 1 Overlapping constraints and their hypothesized effects on evolutionary rates for molecular sequences among taxa.

Variable constraints	Hypothesized effect
A) ON MUTATION RATE	
Replication rate	Less frequent replication yields slower rates of change (11, 68, 89)
Replication and repair efficiency	Higher efficiency yields slower rates of change (49)
Polymerase type	Polymerases with lower error rates yield slower rates of change (e.g. low fidelity reverse transcriptase yields extremely fast rates for retroviruses compared to eukaryotes with higher fidelity nuclear-encoded DNA polymerases (24, 112))
Exposure to mutagens	Reduced exposure to mutagens, such as DNA-damaging oxygen free-radicals, yields slower rates of change (71, 105, reviewed in 95)
Base composition and codon usage	Unequal nucleotide base frequencies (8, 62, 94, 84, 58) and unequal codon usage (46, 84, 102) bias the frequency of alternative substitutions (e.g. initial low frequency of G yields relatively slow rate of G->T, A, or C)
B) ON FIXATION RATE	
Genetic code	Degeneracy of code contributes to slower rates for nonsynonymous than synonymous change, transversions than transitions, and progressively faster rates at second, first and third codon positions in many taxa.
Secondary and tertiary structure	Functional/structural importance and conservation across taxa is correlated with slower rates of primary sequence character change (123, 22, 48)
Gene or protein function	Functional importance (presence of a large proportion of sites of large effect) is correlated with slower rates of sequence character change in unchanging environments (reviewed in 67) and faster rates in changing environments (42a; see Directional selection below)
Purifying selection	Selection pressure against deleterious mutations yields slower rates of change. E.g. more stringent cellular environments such as extreme or fluctuating body temperatures are correlated with slower rates of change in some taxa (1, 6, 76, 114); relaxation of selection pressure is associated with faster rates in others (53)
Directional selection	Increased selection pressure for adaptive change yields faster rates of evolution, particularly for nonsynonymous substitutions (37, 57, 107)
Population size	Smaller effective population sizes can yield faster rates of change (23, 88, 82a)
Cladogenesis rate	Increased lineage splitting and isolation can preserve variation that might otherwise be lost, yielding faster rates of change (41, 77, 73, see 8a)

within and among genes as well as across taxa. Although the members of any particular clade, and the genes within any particular genome, may share similar constraints on mutation and fixation rates, this should not be assumed a priori.

With these caveats in mind, some general trends among clades, reflecting their variable constraints, are apparent. Mitochondrial and nuclear rates are reported as slower in primates, particularly humans, than in rodents (44, 68, 100, but see 26), slower in some birds than in some mammals (11, 59, 76), and slower in some fish, amphibians, and nonavian reptiles than in some mammals and birds (1, 4, 70, 117). Analogous rate differences have been observed within and among many other taxa, including plants (42, 124), bacteria (64), and viruses (24, 25). The broad trends noted among higher-level taxa entail slower rates in those taxa with one or more of the following: lower replication rates, decreased exposure to mutagenic oxygen free-radicals (decreased metabolic rate), higher replication fidelity, and greater purifying selection pressure. Variation in such constraints within and among taxa can yield exceptions in the trends (126). Effective population size and rate of cladogenesis are especially prone to within taxon variation and can influence rate variability. Patterns in rate heterogeneity for various kinds of molecular sequence character change also stem from multiple constraints (Table 1) and have recently been reviewed by Simon et al (108).

APPLICATION OF RATE VARIABILITY

Practice

We provide analyses to assess the ability of various weighting schemes to recover phylogenetic signal. Because it is not possible to know when any particular phylogenetic hypothesis is accurate, we use parsimony and corroboration among data from the largest available mt-DNA set to choose among competing hypotheses. We use the shortest tree based on the largest data set as a proxy for the most accurate tree and as a standard in comparing performance of alternative weighting schemes for various data sets. We analyze a set of 12 vertebrate animals (see Figure 2 for taxon names and GenBank accession numbers) representing taxa for which morphological and molecular analyses have been done and for which entire mitochondrial genomes are available (13, 19, 47, 54, 87).

We aligned amino acids for all 13 mitochondrial protein-coding genes using Clustal W (115) and imposed this alignment on the DNAs using DNA Stacks (27). Direct pairwise comparisons were done using MEGA (63), and branch lengths based on unambiguous changes only were determined for absolute rate comparisons using MacClade (69) and the most highly corroborated tree from our own and previous analyses (see references above). We conducted 100 replicate heuristic parsimony analyses, with randomized ordering of taxon

addition, for five mitochondrial DNA data sets (all 13 protein-coding genes combined, ATP6, COI, Cytb, ND2) with each of four character weighting schemes (equal weight for all characters, transversions only, codon positions 1 and 2 only, codon position 1 and 2 transversions only) using PAUP (113). Loach (*Crossostoma lacustre*) and Carp (*Cyprinius carpio*) were designated as sister outgroup taxa. Character rescaled consistency indicies were used in iterative weighting bouts on analyses of all characters and of codon positions 1 and 2 only.

Using the 13 mitochondrial protein-coding genes combined, we found the same single most-parsimonious tree with all four weighting schemes (Figure 4), and this tree has the same topology as the most-parsimonious tree based on the entire mitochondrial genome (19). The 13 genes combined represent the largest data set in our analyses and is the least sensitive to alternative weighting schemes. This lack of sensitivity to alternative weighting schemes further indicates the robustness of this topology using congruence as an optimality criterion (119).

Does unequal weighting for individual genes, based on comparative absolute rates, improve congruence with our 13-gene-tree? The comparative absolute rate plots show both third codon position changes and TIs decreasing in rate after about 100 my (Figures 2 and 3). If these kinds of character change include proportionally more homoplasy, down-weighting them a priori should improve resolution for divergences that occurred 100 mya or more, involving the Frog, Chicken and Opossum. For the three individual genes (ATP6, COI, Cytb) in which equal weighting for all characters gives trees incongruent with the 13-gene tree, down-weighting does improve congruence for those older divergences (Figure 4), and the answer to the above question is "yes." The most stringent weighting scheme that we apply, using codon position 1 and 2 TVs only, brings Frog, Chicken, and Opossum into congruence with the 13-gene tree.

However, these unequal weighting schemes do not always bring placement of more recently diverged taxa, specifically Human, into congruence with the 13-gene tree. Divergence of Human from other mammalian orders is estimated to be about 70 mya, and the incongruence found suggests that unequal weighting yields a net loss of phylogenetic signal. Short internodes such as those thought to separate most mammalian orders are particularly susceptible to change under alternative weighting schemes, given the relatively small number of characters supporting them.

ND2 analyses differ from those for the other three individual genes in that both equal weighting for all characters and TVs only yield the 13-gene tree, but the other weighting schemes, involving codon positions 1 and 2 only, yield an incongruent placement of Chicken. The most stringent weighting, with codon positions 1 and 2 TVs only, does return Human to the position congruent with the

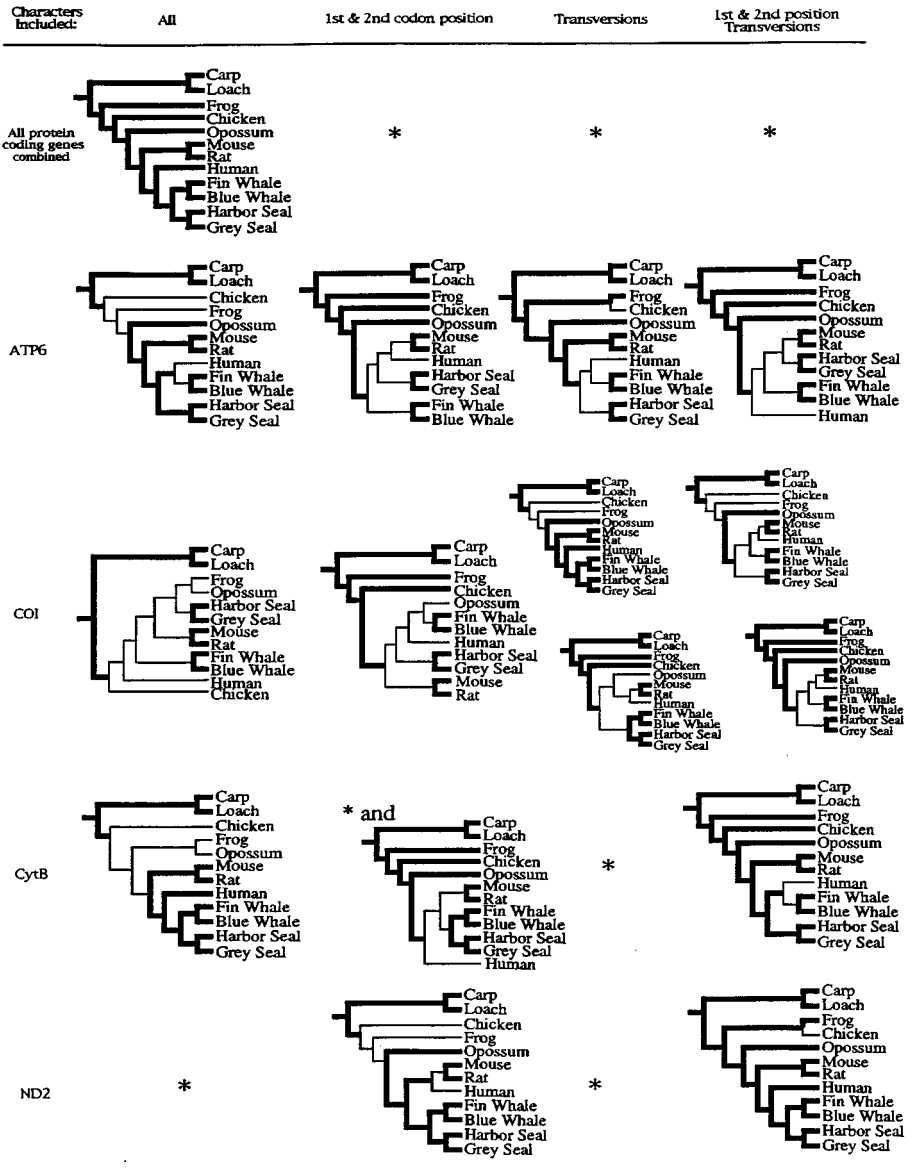


Figure 4 Most-parsimonious trees for five mitochondrial DNA data sets under each of four alternative weighting schemes. See Figure 2 for full taxon names. Asterisks (∗) denote same topology as shown in the top left tree for all 13 protein-coding genes combined, all characters equally weighted (our most highly corroborated tree). Thin branches denote departure in phylogenetic placement relative to the most highly corroborated tree.

13-gene tree, though Chicken and Frog are sisters (Figure 4). Again, unequal weighting appears to yield a net loss of phylogenetic information. This is indicative of a general and persistent problem. Unequal weighting schemes may often yield a net increase in phylogenetic signal, though there are exceptions. We see the problem as involving lack of refinement in generalizations about relative rates of change for different kinds of characters that inform unequal weighting.

Theory

INDEPENDENCE OF SYSTEMATICS Consideration of weighting as something separate or additional reflects an operational view, with weighting perceived as an optional procedure to be done before, during, or after initial phylogenetic analysis. However, given that a decision not to weight characters is equivalent to deciding a priori that all characters deserve equal weight in phylogenetic analysis, it can be seen that weighting of characters, whether explicit or implicit, is an inextricable component of all phylogenetic analyses. Selection of taxa and characters for study from the set of all organisms and all potential characters is one of the first forms of weighting applied in all phylogenetic analysis, regardless of whether characters are collected de novo or compiled from previous analyses.

Equal weighting for all characters is consistent with the potential for all characters to be informative of phylogeny and the need to provide initial consideration of all characters. However, equal weighting as a stipulation for all studies conflicts with the view that not all characters are equally informative of phylogeny and also with the weight of evidence reviewed here on variable constraints and rates of change for molecular characters (Table 1), which supports that view. This support derives from the finite number of possible character states available and the inevitability of homoplasious similarity among taxa for rapidly evolving characters.

In an early recognition of the variable information content of different characters, Darwin (20) noted that the less any part of the organism is concerned with special habits, the more important it becomes for classification. He further suggested that characters found to be constant throughout large groups of species be given greater weight than more variable characters. In the same vein, Farris (30:587) states, "It is argued quite validly that a character which changes slowly is the best kind of character for discerning the evolutionary relationships of organisms." The notion of giving greater weight to characters changing less often, in order to reduce homoplasy, is widely accepted, though the means for determining weights is less clear. As noted by Eldredge & Cracraft (29:12), systematists have been able to avoid addressing the issue directly by recognizing "all nonconvergent characters [as] relevant to defining monophyletic groups at

some level." However, this assumes an ability to identify nonconvergent characters and to apply them at the appropriate taxonomic level.

Character weights based on phylogenetically determined measures of homoplasy (31, 43) make no claims independent of phylogeny about the information content of characters. Other weighting schemes, whether based on measures of character compatibility (93, 101) or, as is more common, various estimates of comparative absolute rate (Figures 2 and 3; e.g. 54, 74) or frequency of cooccurrence of alternative states at homologous sites (62, 118), do make claims that are not derived from character distributions on a phylogenetic hypothesis. This apparent independence leads to the criticism of having to assume some knowledge of evolution not directly determined by phylogenetic analysis (43). This criticism is the same as that stated at the beginning of this review: putative knowledge about evolutionary process, not determined in a phylogenetic context, should not be used in estimating phylogeny because those assumptions weaken the empirical basis of analyses. If this criticism were applicable in the case of relative rate estimates, it would be a compelling reason not to use them as weights. However, we note four problems with the criticism that relative rate estimates are simply assumptions or hypotheses of evolutionary process, independent from, and inappropriate for, phylogenetic analysis:

1. The criticism entails misunderstanding of the source of physical-chemical constraints in dismissing them simply as evolutionary hypotheses. The effects of fundamental physical constraints, such as the genetic code, the initial base composition, and the replication rate (Table 1), do not rely on any particular evolutionary theory or phylogenetic hypothesis. They help to circumscribe physical limitations on sequence character change across all organisms, rather than describing relative frequencies for change that are unique to certain taxa, at certain times, and under certain historically variable conditions. As a consequence, not all character changes are equally probable.

2. The criticism asks systematists to impose a clean separation between evolutionary theory and phylogenetic practice (for justification of this separation see 10, 92) that, ultimately, cannot be achieved. Theory—our notions of how evolution has proceeded—impacts cladistic methods via choice of study taxa, choice of data sets, choice of outgroups, character polarity determinations (whether by outgroup or ontogenetic criteria) (see 60), character weighting (whether equal or unequal), and determination of which characters to compare across taxa (homology assessment). Absolute separation of theory and practice would seem to require random choice among all taxa and characters when initiating a study. Not only are theory and practice

integrated, they are mutually informing, and barring theory from informing practice would entail loss of evidence. Science is a cumulative enterprise. As Frost & Kluge (40:267) point out, "It is the reconciliation. . . of the results of several discovery operations that suggests global explanations," and we suggest that analyses of rates of character evolution represent a set of discovery operations. We agree that untested or weakly supported notions about evolution should not be used in phylogeny estimation. However, well-corroborated hypotheses about relative rate for the same characters and taxa whose relationships are being studied are useful and in some cases necessary. Several studies indicate the success of unequal character weighting in recovering "known" phylogenies, whether those phylogenies are well corroborated and "noncontroversial," simulated, or result from manipulation of populations in a laboratory (2, 3, 51, 56, 80 and see below).

3. Assessment of relative severity for other constraints does entail evolutionary hypotheses in many instances (e.g., the degree of directional selection for nonsynonymous change in some taxon, or the potential influence of speciation frequency on accumulation of character change within some clade); however, these hypotheses are amenable to testing, often in a phylogenetic context (e.g., 17, 77, 99).

4. Relative rate estimates based on discrete character branch lengths (Figure 3) are determined in the context of a phylogeny. Relative rate estimates based on pairwise comparisons cannot be justified in this way, although we have demonstrated their general similarity to rate estimates based on branch lengths in some instances (Figures 2 and 3).

A useful approach to character weighting is to assess relative rates of change for various character types as outlined above (though we consider only a small set of all the potential treatments), accord greater weight to types that are less common than expected in order to reduce homoplasy in the data set, and then apply a phylogeny-based iterative weighting method, focusing on homoplasy reduction in further analyses. Rate estimates should be based on the data set at hand and not on estimates of absolute or relative rates estimated for other taxa and other genes. The actual weight accorded to various characters is less important than their relative values. Iterative weighting methods (31, 121), while appealing, remain sensitive to the initial weights applied and can be misleading when data sets have high levels of homoplasy (50). This can be seen in our analyses for COI and Cytb including all characters. For these, we used individual character consistency indices as weights in successive bouts of parsimony analysis, and we found trees incongruent with the best corroborated

tree based on whole mitochondrial genomes and our analyses of all 13 protein-coding genes combined (Figure 4).

Just as all phylogenetic analyses entail decisions of weighting, whether equal or unequal, all weighting schemes entail decisions of how to apply those weights across taxa and across characters. Application of weights based on comparative absolute rate curves assumes that general trends in relative rate inferred apply to all taxa and characters involved, and at some level this assumption will be violated. Violations may stem from a variety of sources, such as taxon-specific (or gene-specific) differences in base composition, sequence (and sub-sequence) function, and severity of selective effects. Such violations may underlie anomalous results from analyses such as ours for ND2 (Figure 4). That is, heterogeneity in evolutionary rate occurs across scales, and attempts to reduce homoplasy by shifting emphasis away from some characters can enhance the effects of homoplasious similarity in other characters. For example, down-weighting codon position 3 can enhance effects of any homoplasious similarity at positions 1 and 2.

TOTAL EVIDENCE, TOTAL DATA, AND THE LAW OF LARGE NUMBERS Considerable debate has focused on two related issues: 1) the relative merits of partitioning and combining data sets, and 2) taxonomic versus character congruence (12, 15, 16, 21, 28, 61, 79, 81). Partitioning of data sets for separate analyses is often associated with a preference for taxonomic over character congruence in estimating phylogeny. However, this is not required. Unequal weighting is a form of partitioning, giving differential weight to different sets of characters, yet character congruence is used rather than taxonomic congruence. Similarly, a preference for character congruence has become associated with a directive to use and equally weight all available data. This association is also not obligatory.

We distinguish between total data and total evidence. A total data approach accords equal weight to all characters regardless of their degree of homoplasy. A total evidence approach considers all characters as potentially informative but can use successive approximations (14, 31) and character analyses and rate estimates as discovery operations and sources of evidence in determining the relative information content of character sets, and it can apply weights accordingly. In this view, total evidence includes the characters themselves and our improving understanding of their history of change. This is in keeping with our view that theory and practice of systematics can be mutually informing and that informed unequal weighting can enhance phylogenetic accuracy.

The total data approach with equal weighting of all characters may be appropriate 1) in the absence of evolutionary rate heterogeneity (and the underlying variation in constraints,) (Table 1) or 2) if the law of large numbers can be shown to apply as a generality for phylogenetic analysis and if sufficient data

are available. The law of large numbers states that in a series of independent trials, the frequency of a given event will tend toward the probability of the event in any one trial (39). Beyond such formal definitions, however, the law of large numbers is used to suggest that, as independent data accumulate, anomalies in the data will tend to cancel out or decrease in relative frequency. In the context of phylogenetic inference this might be paraphrased as supposing that as more and more data are included in analyses, the phylogenetic signal will dominate noise.

Numerous studies, including ours (Figure 4), demonstrate that larger data sets can perform better in recovery of a well-corroborated tree or a known tree based on simulation or laboratory manipulation. However, this observation does not necessarily mean the law of large numbers is well applied to phylogeny in all instances. The constraints outlined in Table 1, varying across taxa and characters sets, will have decidedly nonrandom effects on patterns of homoplasy accumulation, and it should not be assumed that homoplasies will always cancel out for any particular set of taxa and characters (see 65). The related assumption that homoplasy will always be randomly distributed on phylogenetic trees seems questionable, also due to the variability of constraints in Table 1. The addition of equally weighted character sets with high levels of homoplasy will not necessarily enhance phylogenetic resolution, and short internodes, due to similar divergence times, are particularly sensitive to additional homoplasy.

However, it appears that a threshold for number of DNA characters does exist at which phylogenetic accuracy is very likely with equal weighting of most characters. Cummings et al (19) found that parsimony trees from random samples of 7000 equally weighted mtDNAs (excluding the rapidly evolving D-loop region) yielded the same tree for 10 taxa as did the whole mitochondrial genome about 90% of the time. In a numerical simulation study of four taxa with equal rates of evolution Hillis et al (51) found that equally weighted parsimony analyses yielded the known tree 100% of the time with about 1500 bases. Parsimony analyses with unequal weighting required even fewer characters in recovering the known tree 100% of the time. Differences between these two studies suggest that larger numbers of taxa and (nonidealized) unequal rates of sequence change may require larger amounts of sequence data.

CONCLUSIONS

There have been recurrent calls to keep the practice of systematics separate and free from evolutionary theory in order to ensure its objectivity (10, 109). Presently, such prescriptions are mostly ignored, in part because the complete separation of practice and theory would leave systematists without the ability to choose study taxa and characters from the vast array of candidates. Systematists

seek to avoid assumptions in their methods, yet the growing body of evidence in studies of organismal and molecular evolution indicate some patterns in rates of change that could be used to reduce confounding effects of homoplasy. Panchen (91:243) put the challenge succinctly, "[I]f it can be established that cladistics can yield a natural classification only in particular cases by the use of evolutionary hypotheses based on data extrinsic to the synapomorphy scheme, then phylogenetics would have to be reconsidered." We interpret his "evolutionary hypotheses" to include unequal character weights based on evolutionary rate comparisons and "phylogenetics" to include methods of unequal weighting. If our highly corroborated 13-gene tree in Figure 4 is acceptable as a standard for assessing a "natural classification," then we have demonstrated, as have many others, that "evolutionary hypotheses" can "yield a natural classification" where equal weighting does not.

Debate regarding the proper relationship between systematics practice and our understanding of evolution is continual. We see one particular issue that will be of increasing interest in the near future as data sets grow to include tens of thousands of characters for each taxon; that issue is the validity, however or not, of the law of large numbers in phylogenetic inference. If valid, as more and more data are included, phylogenetic signal will come to dominate noise, in all instances. Faith in this law of large numbers has been professed (implicitly or explicitly) by the pheneticists and systematists formerly known as pattern (or transformed) cladists, who share the ideal of theory-free systematics. We do not rule out the possibility that tens of thousands of bases may reduce or even eliminate the need for historical biological knowledge in applying unequal weighting schemes or other models in phylogenetic analysis for some taxa sets. Our analyses showing robustness of the largest data set (12,120 bases) to alternative weighting schemes (Figure 4) are consistent with predictions of the law of large numbers. However, it seems that biology will remain relevant in systematics for a number of reasons.

Minimally, biology (including our understanding of evolutionary rate heterogeneity) matters if we want to: 1) match sets of taxa with characters likely to be historically informative (neither invariant nor rife with homoplasy); 2) avoid comparing characters with different histories due to reticulations, horizontal transfer (21, 72), or lineage sorting (82); 3) be alerted to the possibility of long-branch attraction problems (33); 4) avoid the assumption inherent in the law of large numbers that homoplasy will tend to cancel itself out (or be randomly distributed in phylogenetic hypotheses); and 5) understand the cause of the hierarchy of taxa in nature as inheritance of DNA/RNA and descent with modification.

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