

Resolution of Phylogenetic Relationships among Recently Evolved Species as a Function of Amount of DNA Sequence: An Empirical Study Based on Woodpeckers (Aves: Picidae)

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Synonymous substitutions in the 13 mitochondrial encoded protein genes form a large pool of characters that should approach the ideal for phylogenetic analysis of being independently and identically distributed. Pooling sequences from multiple mitochondrial protein-coding genes should result in statistically more powerful estimates of relationships among species that diverged sufficiently recently that most nucleotide substitutions are synonymous. Cytochrome oxidase I (COI) was sequenced for woodpecker species for which cytochrome *b* (cyt *b*) sequences were available. A pairing-design test based on the normal distribution indicated that cyt *b* evolves more rapidly than COI when all nucleotides are compared but their rates are equal for synonymous substitutions. Nearly all of the phylogenetically informative substitutions among woodpeckers are synonymous. Statistical support for relationships, as measured by bootstrap proportions, increased as the number of nucleotides increased from 1047 (cyt *b*) to 1512 (COI) to 2559 nucleotides (aggregate data set). Pseudo-bootstrap replicates showed the same trend and increasing the amount of sequence beyond the actual length of 2559 nucleotides to 5120 (2×) resulted in stronger bootstrap support, even though the amount of phylogenetic information was the same. However, the amount of sequence required to resolve an internode depends on the length of the internode and its depth in the phylogeny. © 2000 Academic Press

INTRODUCTION

The mitochondrial genome has been a particularly popular source of DNA sequence data for phylogenetic analysis of vertebrates. This is because mtDNA has a number of desirable properties that should lead to more efficient and reliable recovery of mitochondrial-

gene trees relative to nuclear-gene trees. These properties include a high substitution rate (Brown *et al.*, 1982), which is useful in studies of recently evolved taxa, and a highly conserved gene order, which lends itself to the design of PCR primers that work across a broad range of taxa (Kocher *et al.*, 1989). In addition, the mitochondrial genes are apparently not subject to recombination, although recently this has become a point of contention (Eyre-Walker *et al.*, 1999 versus Arctander, 1999, and Merriweather and Kaestle, 1999); recombination could erode the historical trace of lineages. On the other hand, mtDNA has some apparent shortcomings that reduce its potential for resolving phylogenetic relationships, viz., saturation by multiple substitutions at low levels of divergence (Irwin *et al.*, 1991; Graybeal, 1993; Meyer, 1994; Krajewski and King, 1995) and strong base composition bias (Collins *et al.*, 1994; Perna and Kocher, 1995). These two properties can result in weak phylogenetic signal, particularly for ancient evolutionary history (Moore and DeFilippis, 1997).

A distinct consideration is that gene trees, even if correctly resolved, may not correspond to the species tree as a result of lineage sorting (Avice *et al.*, 1983, 1990; Neigel and Avice, 1986; Hoelzer and Melnick, 1994). Mitochondrial genes have advantages and disadvantages in this regard. A clear advantage is that the mitochondrial-haplotype tree has a higher probability of congruence with the species tree than does a nuclear-gene tree (Moore, 1995), provided that the effective size of the male population is not severely reduced by some aspect of the species' mating system or population structure (Hoelzer, 1997; Moore, 1997). A property that can be either advantageous or disadvantageous to a phylogenetic analysis is that the mitochondrial genome, comprising 13 protein, 2 rRNA, and 22 tRNA coding genes plus a control region, is inherited as a single linkage group. The disadvantage is that, regardless of the number of mitochondrial genes sequenced, there can be only one *independent* estimate of the species tree. The advantage is that one could

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sequence additional genes from the *same* linkage group, thus bringing additional nucleotide characters to the analysis without fear that the additional genes might have distinct evolutionary histories as a result of lineage sorting or recombination.

An important question in designing an efficient phylogenetic analysis based on mitochondrial-gene sequences is: How much sequence is required to resolve the mt-haplotype tree under various circumstances? The answer depends on properties of the gene that affect patterns and rates of nucleotide substitution, for example base composition, transition–transversion substitution ratio, and structure of the substitution matrix (Yang, 1994), and on the evolutionary history of the group of taxa. The uncertainty about the amount of sequence required is clearly illustrated in the case of the human–chimp–gorilla relationships. Nei (1991) suggested that as many as 2600–3100 mtDNA nucleotides might be required, but Ruvolo *et al.* (1991) achieved good resolution with just 684 nucleotides from the mitochondrial cytochrome oxidase II gene. In theory, short internodes deep in the tree are most difficult to resolve (Saitou and Nei, 1986; Lanyon, 1988; Walsh *et al.*, 1999), and this was borne out in an empirical study (Moore and DeFilippis, 1997). This is unfortunate because it is likely that much diversity of the modern biota evolved in adaptive radiations characterized by numerous short internodes at the base of the tree.

In this paper we report nearly complete DNA sequences for the mitochondrial encoded cytochrome oxidase I (COI) gene for several species of woodpeckers (family Picidae, subfamily Picinae) and use those sequences, in combination with mitochondrial encoded cytochrome *b* (*cyt b*) sequences from a previous study on the same set of specimens (Moore and DeFilippis, 1997), to explore the relationship between the amount of DNA sequence and the level of phylogenetic resolution. We compare synonymous and nonsynonymous substitution rates in these two genes and report analyses designed to determine the effect of increasing the amount of sequence on resolution of internodes, particularly short internodes deep in the tree. Our conclusions are based on bootstrap proportions for specific internodes as a measure of statistical support. We compare bootstrap support based on *cyt b* alone (1047 bp), COI alone (1512 bp), and a data set that combines the two genes (2559 bp). In addition, we modified the standard bootstrap procedure so that we could create replicate data sets of varying sizes (400, 800, . . . , 5120 bp) by sampling nucleotide sites with replacement from the combined data set. Our purpose is to provide an empirical study of the effects on bootstrap support for inferred relationships that result from increasing the number of characters (nucleotide positions) available to the phylogenetic analysis. The woodpeckers are a relatively young group; the oldest bona fide fossil dates

to the Pliocene, approximately 6 MYBP (Short, 1974, 1982), which is an antiquity for which synonymous substitutions in mitochondrial protein-coding genes should be useful for recovering the phylogeny (Moore and DeFilippis, 1997; Moore *et al.*, 1999). Our results should be applicable to groups of similar antiquity, but their validity would be uncertain if applied to groups of more distantly related taxa in which most of the phylogenetic signal is in the form of nonsynonymous substitutions. However, our results could be applicable, at least qualitatively, to older groups if the nucleotide substitutions are neutral but occur in slowly evolving sequences, such as nuclear gene introns (Prychitko and Moore, 2000).

Obviously, the efficient application of molecular phylogenetic methods would benefit from studies that lead to a clearer understanding of the quantity and quality of DNA sequence required to achieve “significant” levels of statistical support. However, such studies are fraught with epistemic, methodological, and definitional problems. The epistemic problem is that very few known phylogenies exist that could serve as standards for testing and comparing methodologies. Moreover, the statistical properties of sampling distributions of topologies and of specific nodes inferred from DNA sequence data are complex and poorly known. Simulation studies have been useful in circumventing these problems, but it is always uncertain whether a simulation captures the essential properties of real evolved sequences. The study that we report here is based on real DNA sequence data, but suffers the shortcoming that the true phylogeny is unknown. Therefore, we cannot explore the effect that increasing the amount of sequence has on *accurately* recovering the true phylogeny—this is unknown—but we can explore its effect on convergence on a stable answer, which we measure by bootstrap support. With regard to definitions, we use the term *to resolve a phylogeny* or *node* to mean that the same answer is obtained in a high proportion of bootstrap replicates, which is distinct from claiming that the inferred topology is correct (or accurate). Unfortunately the term *to resolve* is taken by many to mean that the true phylogeny has been recovered, but in practice, it usually means that a particular topology or node has what is deemed to be significant statistical support—usually a high frequency of occurrence in bootstrap replicates. It is altogether possible that a particular tree inference procedure, for example maximum-parsimony, consistently arrives at the same *wrong* answer in bootstrap replicates as a result of bias, for example long-branch attraction. Through the remainder of this paper, we use the term *to resolve* to mean obtaining the same node, and we do not rigorously argue that it is a correct node. However, there is reason to believe that the inferred relationships among the woodpeckers, with the exception of the placement of *Campephilus haematogaster*,

accurately reflect the major phylogenetic features of this group (see Discussion).

The logic of the hypothesis that motivated this study is that, although COI and *cyt b* are expected to evolve at different total rates, their rates of synonymous substitutions are similar, as are their base compositions at synonymous sites. Moreover, most of the phylogenetically informative character-state changes in both genes are synonymous over the evolutionary history of recently evolved groups such as the woodpeckers. If these postulates are true, the synonymous sites for both genes are likely to be independently and identically distributed, or nearly so, in which case it would be useful to pool the sequences, anticipating better resolution of short, deep internodes. Specifically, in a progression from a short sequence (*cyt b*, 1047 bp) to a longer sequence (COI, 1512 bp) to the longest sequence (*cyt b* + COI, 2559 bp), the bootstrap support for specific internodes should increase.

The woodpeckers comprise the subfamily Picinae in the family Picidae, which includes two additional subfamilies, the Jynaginae (the wrynecks) and the Picumninae (the piculets). The relationships among the three subfamilies are relatively clear: the piculets are the sister group to the woodpeckers and the wrynecks are the sister group to the woodpecker–piculet clade (Goodge, 1972; Swierczewski and Raikow, 1981; Short, 1982; Burton, 1984; Tennant, 1991). In contrast, relationships among the major lineages within the woodpeckers are less clear. There appear to be two reasons for this: (1) The classical systematics of the subfamily has been based on two sets of characters, plumage and internal anatomical characters (primarily muscle, but some bone), which suggest conflicting phylogenetic relationships (Burt, 1930; Goodge, 1972; Swierczewski and Raikow, 1981; Short, 1982). (2) Once the basic “design” of the woodpecker evolved, a rapid radiation ensued in which the major lineages diverged over a short period of time (Moore and DeFilippis, 1997). Thus, the phylogeny is characterized by short internodes deep in the tree, and as noted above, this type of phylogeny is difficult to resolve with molecular data.

Although our primary goal was to determine the value of combining sequences from two similarly evolving mitochondrial protein genes for the purpose of resolving short internodes deep in the tree, our results bear upon some important unresolved relationships among woodpecker lineages, which we will discuss.

MATERIALS AND METHODS

Specimens

All the tissues used in this study were fresh-frozen; specimens are listed in Table 1, along with locale data. Tissues were donated by the Louisiana State University Museum of Natural Science or were taken from

specimens collected by William S. Moore and stored in his laboratory collection.

DNA Sequences

Total cellular DNA was isolated from frozen muscle or liver tissue according to proteinase-K/SDS methods adapted from Maniatus *et al.* (1982).

The genes were amplified via the polymerase chain reaction (PCR) according to standard protocols (Kocher *et al.*, 1989; Meyer *et al.*, 1990; Edwards *et al.*, 1991). The primers used for *cyt b* amplification are detailed in Moore and DeFilippis (1997). COI was amplified using primers designed by us and by David Mindell (University of Michigan). These are listed in Table 2 along with their nucleotide sequences and genome positions. PCR products were purified with Wizard PCR Preps (Promega) according to the manufacturer's protocol.

Double-stranded COI PCR products were sequenced directly using a dideoxy-chain termination protocol modified and synthesized from Sanger *et al.* (1977), Fawcett and Bartlett (1990), Barnard *et al.* (1994), and United States Biochemical/Amersham and Stratagene DNA sequencing protocols, with modified T7 DNA polymerase (Sequenase Version 2.0). The resultant dideoxy-chain termination mixtures were then run through 6% polyacrylamide gels and visualized by autoradiography.

Sequencing of *cyt b* is described in Moore and DeFilippis (1997); the *cyt b* sequences were reported in Appendix II of Moore and DeFilippis (1997) and are available from GenBank (U83282–U83302). The COI sequences are presented in Fig. 1 and are available from GenBank (AF272581–AF272598).

Data Analysis

We chose to sequence a single strand (light strand) of the COI gene for two specimens of each species rather than both strands for a single specimen. Low intraspecific divergence allows authentication of the sequences to the extent that nucleotides shared between the two sequences are almost certainly valid, and nucleotides that differ can be reexamined on the autoradiographs. Nucleotides that differed between intraspecific sequences and appeared anything less than single, distinct bands on autoradiographs were recorded as unknown (?). In many instances, there was substantial overlap between the sequences of contiguous amplification products from the same specimen; we further verified these nucleotides by direct comparison between the overlapping sequences. Sequencing two specimens provides a test for contamination of amplification products, which is a serious concern with PCR (Hackett *et al.*, 1995; Edwards and Arctander, 1996, 1997): Conspecific sequences should be very similar but usually not identical and should pair as sister taxa when both are entered in a phylogenetic analysis. Two cases exceptional to the procedure were *Veniliornis*

TABLE 1
Specimens, Locales, and Intraspecific Divergence

Subfamily Tribe Species	Common name	Locale	Voucher number ^c	Intraspecific divergence (substitutions/nucleotide, COI + cyt <i>b</i>)
Picumninae				
<i>Picumnus aurifrons</i>	Bar-breasted piculet	Bolivia	LSU 18254	0.0061
<i>Picumnus aurifrons</i>	Bar-breasted piculet	Bolivia	LSU 18479	—
Picinae				
Melanerpini				
<i>Melanerpes carolinus</i>	Red-bellied woodpecker	Kentucky, USA	WSU 8614	0.0021 ^a
<i>Melanerpes carolinus</i>	Red-bellied woodpecker	Alabama, USA	WSU 8625	—
<i>Sphyrapicus ruber</i>	Red-breasted sapsucker	California, USA	WSU 86148	0.0024
<i>Sphyrapicus ruber</i>	Red-breasted sapsucker	California, USA	WSU 86149	—
Campetherini				
<i>Picooides villosus</i>	Hairy woodpecker	Arizona, USA	WSU 86107	0.0044
<i>Picooides villosus</i>	Hairy woodpecker	California, USA	WSU 86144	—
Colaptini				
<i>Veniliornis callonotus</i>	Scarlet-backed woodpecker	Lambayique, Peru	LSU 5175	0.0010 ^b
<i>Veniliornis callonotus</i>	Scarlet-backed woodpecker	Lambayique, Peru	LSU 5178	—
<i>Veniliornis nigriceps</i>	Bar-bellied woodpecker	La Paz, Bolivia	LSU 1305	0.0390 ^b
<i>Veniliornis nigriceps</i>	Bar-bellied woodpecker	Pasco, Peru	LSU 8176	—
<i>Piculus rubiginosus</i>	Golden-olive woodpecker	Lambayique, Peru	LSU 5162	0.0025
<i>Piculus rubiginosus</i>	Golden-olive woodpecker	Lambayique, Peru	LSU 5222	—
<i>Colaptes auratus</i>	Northern flicker	Kentucky, USA	WSU 8618	0.0040
<i>Colaptes auratus</i>	Northern flicker	Arizona, USA	WSU 86101	—
Campephilini				
<i>Dryocopus pileatus</i>	Pileated woodpecker	Kentucky, USA	WSU 8615	0.0069
<i>Dryocopus pileatus</i>	Pileated woodpecker	Texas, USA	WSU 8634	—
<i>Campephilus haematogaster</i>	Crimson-bellied woodpecker	Esmeraldas, Ecuador	LSU 11786	0.0037
<i>Campephilus haematogaster</i>	Crimson-bellied woodpecker	Darien, Panama	LSU 2188	—

^a Based on only the COI sequence.

^b Based on only the cyt *b* sequence.

^c LSU, Louisiana State University; WSU, Wayne State University.

nigriceps and *V. callonotus*, for which limited resources permitted sequencing only one specimen of each species for COI; the two pair as sister species in all phy-

logenetic analyses, suggesting that the sequences are authentic.

For computational efficiency, especially with regard

TABLE 2
Primers Used to Amplify COI

Name	Sequence	Source
L6615	5' CCTCTGTAAAAAGGACTACAGCC 3'	Mindell
L6772	5' TTAGCCTCCTCATTCGAGCAGAATTGGG 3'	DeFilippis
L6958	5' AATAACATAAGCTTCTGACT 3'	Mindell
L7165	5' ACCGCCATCAACATAAAACCCCC 3'	Mindell
L7444	5' TACTCCGGAAAAAAGAACC 3'	Mindell
L7591	5' CAACCATAATCATTGCCATCCCAAC 3'	DeFilippis
H6835	5' GGTATAACTATGAAGAAGATTATTACGA 3'	DeFilippis
H7032	5' TTGCCAGCTAGTGGGGGGTA 3'	Mindell
H7539	5' GATGTAAAGTAGGCTCGGGTGTCTAC 3'	DeFilippis
H7774	5' GCCCCTATTGATAGGACGTAATGGA 3'	DeFilippis
H7956	5' GGGTAGTCCGAGTAACGTCG 3'	Mindell
H8191	5' CCAICITHAGGGTTTCGATTCCTCC 3'	DeFilippis

Note. Under Name, each primer is given a letter to designate the mitochondrial strand of synthesis (L = light and H = heavy) followed by the number of the 3' nucleotide binding site relative to the chicken mitochondrial genome (Desjardins and Morais, 1990). Under Source, Mindell indicates that the primer was designed by David P. Mindell; DeFilippis indicates that the primer was designed by Victor R. DeFilippis.

This test should be relatively powerful because the variance of the sampling distribution of the test statistic is diminished as the n_{ij} s increase. This test has the disadvantages that it can use only a subset of the data because it requires independent lineages and it is based on an approximation to the true distribution of differences. The test statistic is an approximation in two respects: normal distributions are used as approximations to the underlying binomial distributions and the σ_{ij}^2 s must be estimated. Nonetheless, because the n_{ij} s are fairly large, the approximation should be good. The test also assumes that the sequences are not so diverged that multiple substitutions at individual sites are significant.

The four independent pathways used to test the null hypothesis that COI and *cyt b* evolve at equal rates are indicated by dashed lines in Fig. 3.

Justification of Bootstrap Proportions

We use bootstrap proportions (P) to assess the strength of the phylogenetic signal in our data sets (Felsenstein, 1985) because they provide a measure of support for individual clades within the larger phylogeny, and they are conceptually similar to the probabilities of obtaining the correct tree in the simulation studies of Saitou and Nei (1986). Although the interpretation of bootstrap proportions remains controversial (see Sanderson, 1995 for a review; Hillis and Bull, 1993; Felsenstein and Kishino, 1993), their statistical properties are better known than those of other measures of phylogenetic strength.

The most literal meaning of a bootstrap proportion is that it is the proportion (percentage) of replicated applications of the tree algorithm (e.g., neighbor-joining), to bootstrap-resampled character data, in which a particular node appears. Bootstrap resampling entails taking replicate samples of the same size from the original sample by randomly drawing elements of the sample with replacement. However, if the resampled characters (columns of nucleotides from the aligned sequences) are independent and identically distributed, then a bootstrap proportion is an estimate of the proportion of topologies that have a particular node in a sampling distribution of topologies which would result if the evolutionary process was repeated over and over from the same starting conditions for the same class of characters and the same algorithm was used to compute the topology for each replicate. Most of the nucleotide variation upon which this study is based is synonymous and thus approximates the conditions of independence and identity of distribution (see below). It is also clear that the bootstrap proportion is a biased estimator, which tends to underestimate the true proportion for strongly supported nodes (Felsenstein and Kishino, 1993; Hillis and Bull, 1993).

A more biological interpretation of P was given by Felsenstein and Kishino, (1993): If P were not biased,

$(1 - P)$ would be the probability that the amount of data supporting the node would be in the sample if the node did not exist in the real phylogeny. But since P is biased, the correct statement is that the probability that the amount of data supporting the node would be in the sample if the node did not exist in the real phylogeny is less than or equal to $(1 - P)$. The bias appears to be quite strong when the node is strongly supported. For four OTUs, Hillis and Bull (1993) found that $P \geq 70\%$ corresponded to a probability $\geq 95\%$ that the node is real. Thus, for example, if a node has $P = 70\%$, it would be conservative to say that the probability that the set of sequences would contain that much support for the node, by chance, is less than or equal to 30% and in reality is probably less than or equal to 5% (Hillis and Bull, 1993).

Bootstrapping using 1000 replications was performed for the neighbor-joining and maximum-parsimony tree algorithms; bootstrapping was not carried out for the maximum-likelihood phylogenies because of insufficient computing resources.

To test the effects of increasing the amount of sequence on phylogenetic resolution further, we created additional sets of replicates based on sampling with replacement, just as in bootstrapping, but with sample sizes of 400, 800, 1200, 1600, 2000, 2560 (equal to COI + *cyt b*), 3600, and 5120 (equal to $2 \times$ [COI + *cyt b*]) nucleotides. This *pseudo-bootstrapping* was accomplished by modifying the code in the program BOOT in the PHYLIP program package such that samples of the desired size were drawn rather than samples of the original size, as is the standard bootstrap procedure (J. Felsenstein, personal communication). These samples were drawn from the aggregate (COI + *cyt b*) data set.

RESULTS

Intraspecific Variation

Percentage sequence divergence between the two specimens representing each species is provided in Table 1. Sequences for duplicate specimens were very similar for both *cyt b* and COI, with the exception of the two specimens of *V. nigriceps*, which differed by 3.9% for *cyt b* (COI sequence is not available for both specimens). The collecting locales for the two *V. nigriceps* are separated by considerable distance and the specimens represent distinct subspecies. The two specimens of *V. nigriceps* are paired as sister OTUs in phylogenies in which both are included and both are about equidistant from *V. callonotus*, thus confirming that neither is a contaminant amplification.

Phylogenetic Analysis

Phylogenies were estimated, using the best sequence for each species from each of the three data sets (COI, *cyt b*, and COI + *cyt b*), by three methods, neighbor-

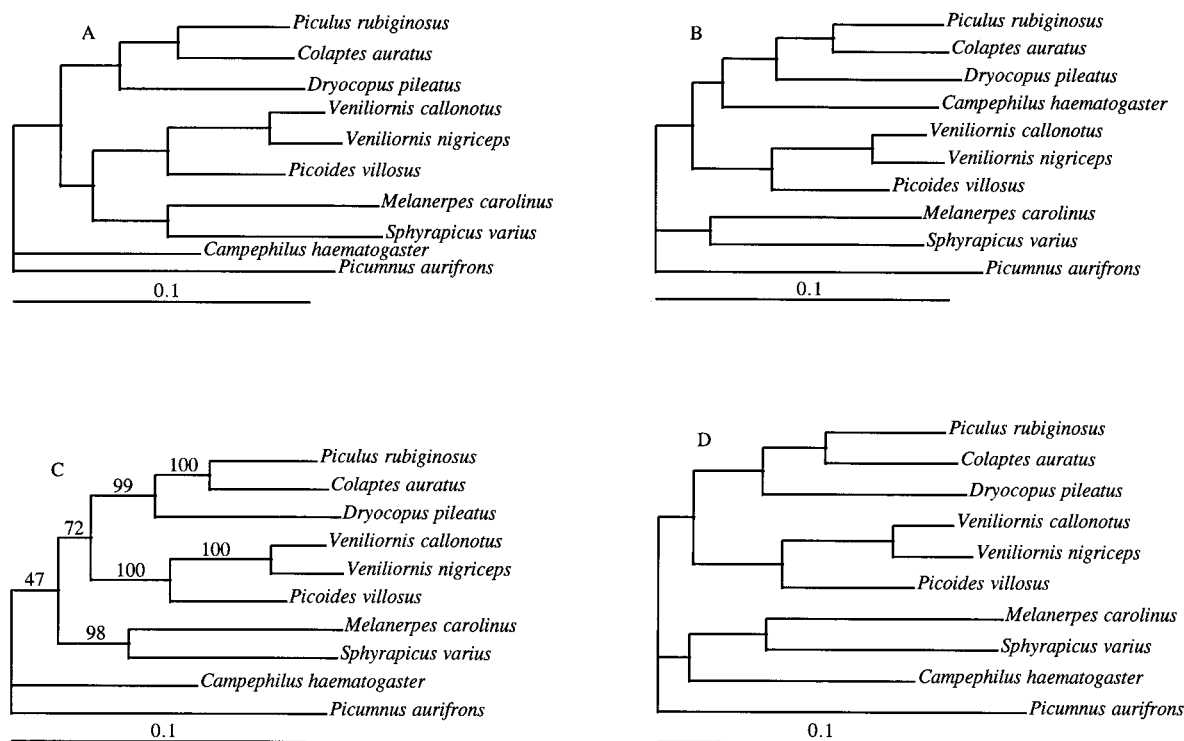


FIG. 2. Four topologies depicting woodpecker relationships that resulted from various combinations of data sets and phylogenetic algorithms. (A) The ML tree for the aggregate data set (COI + cyt *b*); topology A was also produced by the following combinations of data sets and phylogenetic algorithms: COI-ML, COI-MP, and COI + cyt *b*-MP. (B–D) Additional topologies observed among the other combinations of data sets and phylogenetic algorithms: (B) COI-NJ; (C) cyt *b*-ML, cyt *b*-MP, and COI + cyt *b*-NJ; (D) cyt *b*-NJ. Numbers above the internodes in C are bootstrap proportions for the NJ tree based on the aggregate data set (1000 replications). Branch lengths are maximum-likelihood estimates for each topology based on the aggregate data set and can be determined from the scale bar (substitutions/nucleotide).

joining, maximum-parsimony, and maximum-likelihood. Some clades appeared in all of the nine trees representing all combinations of data sets and tree methods, whereas others differed among trees. The maximum-likelihood tree for the aggregate data set (COI + cyt *b*) is presented in Fig. 2A. Three additional topologies appeared among the nine combinations; these are presented in Figs. 2B–2D. Three of all of the clades that appear in the nine analyses appeared in all nine analyses; i.e., they were inferred, without exception, by all of the algorithms for all of the data sets. These clades are ((*Colaptes*, *Piculus*), *Dryocopus*), which we will refer to as the Colaptini–*Dryocopus* clade; ((*Veniliornis callonotus*, *V. nigriceps*), *Picoides villosus*), which we will refer to as the *Picoides*–*Veniliornis* clade; and (*Melanerpes*, *Sphyrapicus*), which we will refer to as the Melanerpini clade. The three other topologies differ from that of the maximum-likelihood tree with regard to where *Campephilus* joins the tree and where the *Picoides*–*Veniliornis* clade joins the tree. Figure 2C is identical to the maximum-likelihood tree, except that the *Picoides*–*Veniliornis* clade is joined as the sister clade to the Colaptini–*Dryocopus* clade. In turn, Figs. 2B and 2D differ from Fig. 2C with

regard to the placement of *Campephilus*: in Fig. 2B *Campephilus* joins the tree near the base of the Colaptini–*Dryocopus* ancestral branch, whereas in Fig. 2D it joins the tree near the base of the Melanerpini ancestral branch. The ordering of the four topologies with regard to likelihood is 2A > 2B > 2C > 2D, but the differences among the likelihood scores for the four topologies are not statistically significant (Kishino–Hasegawa test; Kishino and Hasegawa, 1989; as implemented in PHYLIP 3.57c; Felsenstein, 1995). The topology of Fig. 2C is the same as that of the NJ tree for the aggregate data set; bootstrap proportions for 1000 NJ replicates are given above the internodes (branch lengths are maximum-likelihood estimates and can be determined from the scale bar given for each topology). Bootstrap support is strong for all of the internodes in the three stable clades noted above. In the topology of Fig. 2C, *Campephilus* is the sister group to all other woodpeckers, but this node is weakly supported (bootstrap proportions = 47%), which is consistent with the tendency of *Campephilus* to pair with other basal lineages in the other analyses. Also, in the aggregate NJ tree (Fig. 2C) the *Picoides*–*Veniliornis* clade joins with the Colaptini–*Dryocopus* clade, and this pairing oc-

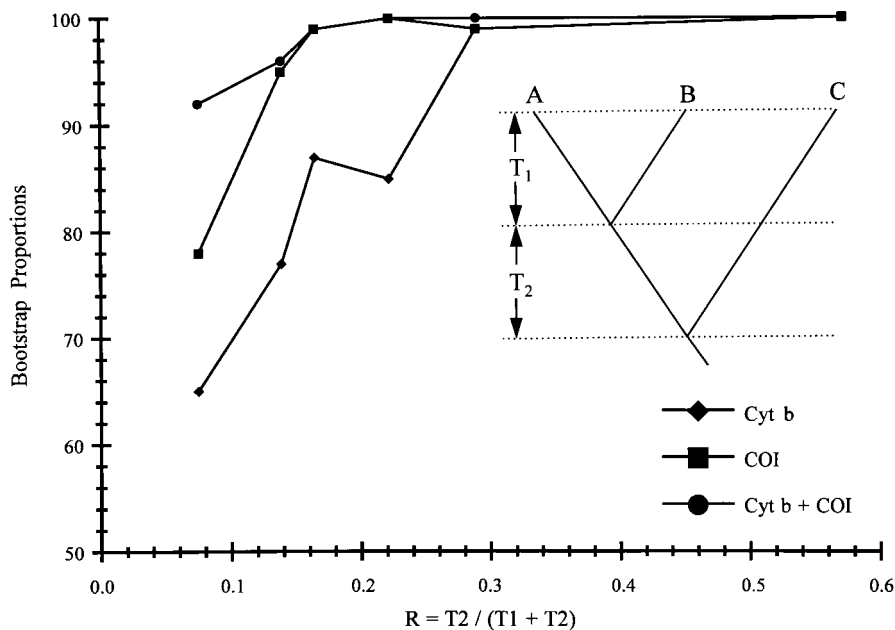


FIG. 4. Bootstrap support for internodes as a function of internode length. The columns of data points correspond to the internode numbers in Fig. 3 (from left to right: 1, 2, 3, 4, 5, 6).

Improved Resolution Results from Pooling COI and Cyt b

Based on theoretical (Saitou and Nei, 1986; Lanyon, 1988) and empirical (Moore and DeFilippis, 1997) studies, it is clear that the strength of the phylogenetic signal required to resolve the relationships among three species depends on the length of the internode, or the period of common ancestry, between the first and the second speciation events (T_2) and the amount of evolution beyond the second speciation event (T_1). These relationships are depicted in the hypothetical phylogeny in Fig. 4. When T_2 is short, the period of time when synapomorphies might arise in the common ancestor is limited, and when T_1 is long, the probability of a synapomorphic substitution being obliterated by a second nucleotide substitution is great. For these reasons, short internodes deep in the phylogeny are difficult to resolve. Saitou and Nei (1986) expressed the relationship between T_1 and T_2 as $R = T_2 / (T_1 + T_2)$; i.e., R is the fraction of the total evolutionary time between the first and the second speciation event. Using substitution patterns observed in mtDNA for the divergence of human, chimp, and gorilla, they showed that the probability of recovering the correct phylogeny would be close to 0.95 for a sequence of approximately 1000 bp if $R = 0.2$ but that 4200 bp would be required if $R = 0.1$.

Although the overall substitution rate of COI is slower than that of cyt *b*, the two genes evolve at the same rate with regard to synonymous substitutions, and most of the phylogenetic signal is contained in the synonymous substitutions. Thus, performing phyloge-

netic analyses on each of the two sequences and then on the pooled sequences allows us to explore empirically whether simply adding more data of comparable quality to the analysis results in greater resolution of short, deep internodes. The NJ tree for COI (1512 bp) alone, cyt *b* (1047 bp) alone, and the aggregate data (2559 bp) sets have the same topology when *Campephilus* is excluded from the analysis (Fig. 3). For each node, the bootstrap proportion was determined from 1000 replicates for each data set, and the length of T_1 and T_2 was calculated as described by Moore and DeFilippis (1997). R was then calculated for each node and the bootstrap proportion plotted as a function of R (Fig. 4). It is apparent that increasing the amount of sequence substantially increased bootstrap support for short, deep internodes; i.e., for low values of R . For example, for the lowest R value (internode 1, Fig. 3) the bootstrap support increased from 65 to 78 to 92% for cyt *b* (1047 bp), COI (1512 bp), and COI + cyt *b* (2559 bp), respectively.

To determine the effect of smaller, intermediate, and larger sequences on bootstrap support, we performed pseudo-bootstrap analyses as described under Materials and Methods. The results are plotted in Fig. 5. Although the stochastic variance is large for small values of R and short sequences, the trend is clear. Increasing the amount of sequence results in higher bootstrap support for all nodes, and the support for troublesome short, deep internodes is elevated from a level that most phylogeneticists would consider insignificant to levels that they would consider significant.

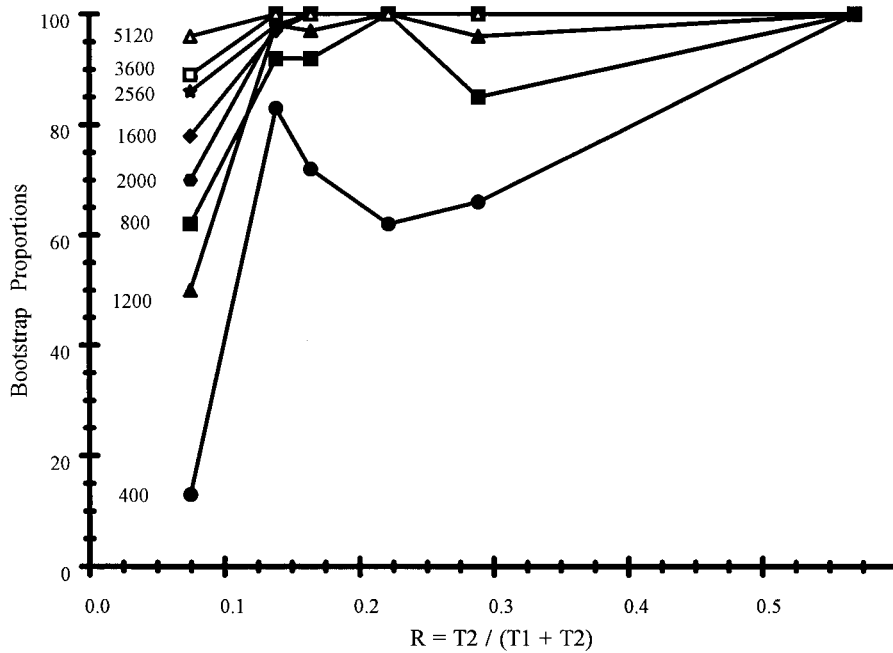


FIG. 5. Bootstrap support for internodes as a function of internode length, based on pseudo-bootstrap replicates. The columns of data points correspond to the internode numbers in Fig. 3 (from left to right: 1, 2, 3, 4, 5, 6). The number at the beginning of each plot indicates the length of the DNA sequence.

DISCUSSION

Variation in Topologies

Our discussion focuses on increased phylogenetic resolution resulting from increasing the amount of sequence and on phylogenetic relationships among woodpeckers. However, the variation in topologies (Figs. 2A–2D) resulting from different combinations of data sets and tree algorithms needs to be addressed first. The four topologies that result from the nine combinations of data and algorithms differ with regard to only two lineages, where the *Picooides-Veniliornis* clade joins the tree and where *Campephilus* joins the tree. The somewhat uncertain resolution of the *Picooides-Veniliornis* clade appears to stem from the ancestral lineage having shared ancestry with its sister group (either the Colaptini or the *Melanerpes-Sphyrapicus* clade) for only a short time early in the radiation of woodpeckers; i.e., its sister group relationship is defined by a short, deep branch. For example, assuming that Fig. 2C is the correct topology, the fraction of evolutionary history represented by the common ancestor of the *Picooides-Veniliornis* and the *Colaptini-Dryocopus* clades is $R = 0.13$. (Recall that Saitou and Nei, 1986 estimated that 1000 bp would be required to resolve a trichotomy if $R = 0.2$ and 4200 bp if $R = 0.1$.) The variation in where *Campephilus* joins the tree seems also to be a case of a short internode deep in the tree, but this case is more complex in that the majority of the analyses (seven of nine) indicates that *Campe-*

philus is the basal lineage of the woodpecker radiation. The high average divergence of *Campephilus* from all other woodpeckers apparently results in sampling error playing a dominant role in where its branch attaches to the network of other woodpeckers.

Although we are limited by the present analysis to arguing that increasing the number of characters results in increased statistical support for nodes, it is likely that the consensus (not shown) of the four topologies in Fig. 2 portrays correct relationships among these woodpeckers. Prychitko and Moore (2000) reported a phylogeny based on β -fibrinogen intron 7 (β -*fibint* 7) for a larger number of woodpecker species (13 + a piculet outgroup). The set of species contained in the present study is a subset of those in the nuclear-gene study of Prychitko and Moore (2000), so that phylogeny provides an independent test of the phylogenetic hypotheses. The β -*fibint* 7 gene tree differs from the topology in Fig. 2C in only two respects: *Campephilus* is the sister group to the Melanerpini (*Melanerpes* + *Sphyrapicus*), as opposed to being basal to all woodpeckers as in Fig. 2C, and the *Picooides-Veniliornis* clade is the sister group to this *Campephilus*-Melanerpini clade. There may be genuine incongruence between the mitochondrial-haplotype tree and the nuclear β -*fibint* 7 tree, but the level of bootstrap support for these two disparate nodes is not sufficient to conclude this. Congruence of the remaining nodes in the mitochondrial-haplotype tree and the β -*fibint* 7 tree would be highly improbable if this were not the

correct evolutionary history of these woodpeckers (Miyamoto and Fitch, 1995).

Amount of Sequence and Phylogenetic Resolution

Only a few theoretical (Saitou and Nei, 1986; Zharkikh and Li, 1993) and empirical (Cracraft and Helm-Bychowski, 1991; Cummings *et al.*, 1995; Zardoya and Meyer, 1996; Walsh *et al.*, 1999) studies have addressed questions about the relationship between amount of DNA sequence data and phylogenetic resolution. Our empirically based results are broadly consistent with theoretical predictions; *viz.*, increasing the amount of sequence improves phylogenetic resolution, and short internodes deep in the phylogeny are most problematic (Moore and DeFilippis, 1997). Moreover, our results suggest that substantial improvement in resolution can be gained for nodes where R is between approximately 0.08 and 0.2 by increasing the amount of sequence from 1047 nucleotides (cyt *b*) to 2559 (cyt *b* + COI), but where $R > 0.2$, 1047 nucleotides is sufficient to achieve strong bootstrap support, and there is little to be gained by increasing the number of nucleotides. This result is consistent with the theoretical studies of Saitou and Nei (1986) and Zharkikh and Li (1993).

The pseudo-bootstrap analysis supports and extends these results. First, when the internode is very short and deep (Fig. 5; $R = 0.074$), more than 2560 nucleotides are necessary to be reasonably confident of relationships (86%), whereas, for $R > 0.14$, comparable confidence can be achieved with many fewer nucleotides (approximately 800). Perhaps most importantly, the bootstrap proportion continued to increase for this shortest, deepest internode as the number of nucleotides was increased to 5120 ($R = 0.074$; 96%). As expected, however, the bootstrap proportion appears to approach 100% asymptotically, and it presumably would take many more than 5120 nucleotides to achieve 100%. Also, for $R = 0.074$, the order of bootstrap proportions does not increase as expected for increasing sample size. The sample of 400 nucleotides has the lowest bootstrap support, as expected, but then 1200 and 800 are reversed, as are 1600 and 2000. For all larger values of R , the bootstrap proportion increases as sample size increases, as expected. Presumably, this results from a larger variance in the sampling distribution of short, deep nodes.

In putting the pseudo-bootstrap analysis in perspective it is important to keep the sampling scheme in mind. The pseudo-bootstrap replicates of 5120 nucleotides were created by sampling with replacement 5120 times from the original aggregate sequence of 2559 nucleotides. Thus, the quality of the phylogenetic signal has not changed, nor has the signal-to-noise ratio; the higher bootstrap proportion is solely a result of a larger sample size. The implication of this empirical study is that if one could include more sequence con-

taining the same phylogenetic signal, one could achieve a more powerful statistical inference with regard to the existence of a particular node.

Our results are also consistent with empirical studies reported by Cummings *et al.* (1995) and Zardoya and Meyer (1996), based on mtDNA, in showing that large data sets have a better chance of resolving phylogenies than do small data sets. However, Cummings *et al.* (1995) reached the discouraging conclusion that efficient recovery of phylogenetic relationships requires a very large amount of sequence (8000+ bp) and that the nucleotides are best drawn as a random sample from the entire genome as opposed to a contiguous sequence. These two studies differ from ours, however, in an important respect; *viz.*, they examined ancient relationships among mammalian orders and vertebrate classes (50–400 MYBP), where most of the phylogenetic signal is expressed as amino acid substitutions, and synonymous positions are saturated by multiple substitutions. In contrast, our analysis is based on a radiation that is approximately 7–8 MY old (Moore and DeFilippis, 1997; Moore *et al.*, 1999), and the preponderance of substitutions is synonymous. We believe that the conclusion of Cummings *et al.* (1995) is an overgeneralization and an oversimplification that could obscure the fact that reliable phylogenies for many more-recent clades can be obtained from a relatively small amount of sequence. The actual amount of sequence required to resolve a trichotomy depends on several factors: the length of the internode relative to the length of daughter branches (R), the rate of substitution (which varies among genes), and the absolute age of the internode (Saitou and Nei, 1986; Moore and DeFilippis, 1997). Implicit in this argument is that phylogenetic analyses will often lead to “best” trees for which some relationships are consistently and strongly supported, whereas others are weakly and inconsistently supported as analytical conditions are varied (e.g., maximum-parsimony versus neighbor-joining). The expectation that all of the relationships among taxa included in an analysis will be resolved is often unrealistic, while at the same time it is of considerable interest that these difficult relationships involving short internodes suggest important evolutionary phenomena, such as adaptive radiations (Walsh *et al.*, 1999).

Much of the recent literature concerned with sampling strategies in molecular phylogenetics has focused on the effect of adding more characters (character sampling) as an alternative to adding more taxa (taxon sampling) (e.g., Goldman, 1998; Poe, 1998; Graybeal, 1998; Poe and Swofford, 1999; Johnson and Lanyon, 1999). Taken as a whole, this literature is often perplexing, with individual papers reaching disparate conclusions (e.g., Graybeal vs Poe and Swofford). This is in part because of the complexity of the data, the uncertainty that underlying assumptions are reasonably

satisfied, the enormity of possible outcomes, and the poor development of theory concerned with the sampling distributions of phylogeny estimations. In part, however, this perplexity stems from a failure to distinguish the problem of low statistical power from the problem of inconsistency (see also Penny *et al.*, 1992). In phylogenetics, inconsistency is the circumstance in which the estimation procedure converges on the wrong tree with increasing certainty as the number of characters increases (Felsenstein, 1978; DeBry, 1992). It is clear that adding additional taxa to "break up" long branches alleviates inconsistency, at least in some circumstances (Hendy and Penny, 1989; Graybeal, 1998). However, adding more characters would exacerbate problems caused by inconsistency. On the other hand, by extrapolation from statistical theory, one would expect that increasing the number of characters should result in greater statistical power, that is greater ability to distinguish short, deep internodes (Walsh *et al.*, 1999). Our results are consistent with this expectation in that increasing the number of characters did increase the bootstrap support for short, deep internodes. This conclusion is consistent with the few theoretical (Saitou and Nei, 1986; Walsh *et al.*, 1999) and empirical (Omland *et al.*, 1999; Johnson and Lanyon, 1999) studies that have addressed this issue. We emphasize, however, that our analysis was based on pooling very similar data sets comprising mostly synonymous substitutions from two mitochondrial encoded protein genes. The results may not apply when data sets are pooled from genes with very different rates and patterns of substitutions; this remains to be determined. The effect of increasing taxon sampling on statistical power is largely unknown. Omland *et al.* (1999) found that adding several subspecies for each species in a phylogenetic analysis increased bootstrap support for interspecific nodes. This is not a case of alleviating inconsistency by adding taxa but seemingly increasing statistical power. On the other hand, we have seen cases in which adding additional taxa that "break" an internode uniting, say, two species, actually decreases the bootstrap proportion for nearby nodes because, with the randomness of bootstrap resampling, the new taxon associates occasionally with various taxa in its vicinity of the tree (unpublished analysis).

Woodpecker Phylogenetics

It is generally agreed that the piculets (subfamily Picumninae) are the sister group to the woodpeckers (Short, 1982). Based on this belief we designated the piculet *Picumnus aurifrons* the outgroup for purposes of rooting our trees, and midpoint rooting of the neighbor-joining tree also results in *P. aurifrons* being positioned as the outgroup. Short (1982) divided the woodpeckers into six tribes, four of which are represented in the present study: Melanerpini (*Melanerpes carolinus*), Campetherini (*Picooides villosus*), Colaptini (*Colaptes*

auratus, *Piculus rubiginosus*, *Veniliornis callonotus*, and *V. nigriceps*), and Campephilini (*Campephilus haematogaster* and *Dryocopus pileatus*); the two tribes not represented in this study, Picini and Meiglyptini, are restricted to the Old World.

Some relationships that are consistently and strongly supported in our analysis are at odds with this classification. First, the genus *Veniliornis* does not form a clade with other species assigned to the tribe Colaptini (*Colaptes* and *Piculus*), but rather forms a clade with the genus *Picooides*; this clade was supported by 100% of 1000 bootstrap replicates in the neighbor-joining analysis of the combined data set (Fig. 2C). Second, the genus *Dryocopus*, which Short (1982) combined with *Campephilus* in the tribe Campephilini, forms a clade with the two colaptine genera *Colaptes* and *Piculus*, which is at odds with its inclusion in Campephilini. The relationship between *Dryocopus* and *Campephilus* has long been a point of contention. Plumage patterns are somewhat similar among all species in both genera and strikingly similar between certain species of *Dryocopus* and *Campephilus* (Bock and Miller, 1959; Bock, 1963; Cody, 1969; Short, 1982). On the other hand, the structure of the foot is very similar between *Dryocopus* and *Colaptes*, whereas that of *Campephilus* is very different from either of these (Bock and Miller, 1959; Bock, 1963). The sister group relationship between *Dryocopus* and the colaptines was supported by 99% of 1000 bootstrap replicates (Fig. 2C).

Our analyses of mtDNA sequences also bear on the long-standing argument of the relationship between the sapsuckers (*Sphyrapicus*) and the genera *Melanerpes* or *Dendrocopos* + *Picooides* (presently lumped into the single genus *Picooides*; Short, 1982). Swierczewski and Raikow (1981), based on cladistic analysis of hind limb myological characters, concluded that sapsuckers are the sister group of *Picooides* (*sensu* Short), whereas Short and Morony (1970), Short (1982), Goodge (1972), and Tennant (1991) concluded that sapsuckers are closely related to *Melanerpes*. Our analysis strongly supports the latter hypothesis, with an increase in statistical support (node 2, Fig. 3; second column of data points from left, Fig. 4) from 77 to 95% resulting from increasing the amount of DNA sequence from 1047 bp (cyt *b*) to 2559 bp (cyt *b* + COI).

CONCLUSION

The properties of the mitochondrial genome and especially the protein-coding genes suggest a useful strategy for resolving phylogenetic relationships among species belonging to relatively recently evolved vertebrate groups. The strategy is to sequence multiple protein-coding genes from the mitochondrial genome and to combine the data in a single pool for phylogenetic analysis. The pooled data would constitute a

large sample of characters that approach the ideal for phylogenetic inference of being independently and identically distributed (Felsenstein, 1985; Sanderson, 1995; Swofford *et al.*, 1996) because most of the variation involves synonymous substitutions. The large pool of nucleotide characters should lead to a statistically powerful inference of the gene tree. Pooling is justified because most variation results from synonymous substitutions, which accrue at the same rate in COI and *cyt b* and should be similar for all mitochondrial encoded protein genes, and because the mitochondrial genome comprises a single linkage group, which obviates the worry of pooling data from gene trees with distinct evolutionary histories. Finally, the mitochondrial-haplotype tree, if correctly resolved, has a greater chance of congruence with the species tree than does a nuclear-gene tree (Moore, 1995). In sum, sequencing multiple protein-coding genes should result in a high probability of resolving the mitochondrial-haplotype tree, which in turn has a relatively high probability of being congruent with the species tree. This strategy should be successful in resolving relationships among taxa that evolved in the past 9 MY or so if the generation time is short and for more ancient groups if the generation time is longer (Moore and DeFilippis, 1997).

APPENDIX 1

As two DNA sequences diverge from an ancestral sequence, they accumulate nucleotide differences. The number of nucleotide sites that differ between the sequences after an elapsed time is a random variable (X) that has a binomial distribution, provided that the elapsed time is short enough that multiple hits are negligible, i.e., $X \sim B(n; p)$, where p is the probability of a nucleotide mutating over the time elapsed since common ancestry and n is the number of nucleotides. It is possible to calculate an approximate probability that the number of differences (X) between alleles was drawn from a binomial distribution with the same p as that governing the divergence of alleles at a second nonhomologous gene that shared the same evolutionary history (e.g., *cyt b* and COI of the mitochondrial genome). Moreover, the probability distribution of X can be approximated by the normal distribution, provided that n is large. This provides a foundation for statistically comparing the rates of divergence of two (or more) different genes by relating the genetic distance between them to the binomial distribution and then approximating the binomial distribution by a normal distribution.

Suppose that we have a phylogeny for DNA sequences that are in the same linkage group; i.e., there is no crossing over between genes within the linkage group so that all genes in the linkage group have the same evolutionary history. This is the case with the

genes that comprise the mitochondrial genome. Further, suppose that the phylogeny is well resolved such that two or more independent pathways of divergence can be chosen from the phylogeny; i.e., we can identify pairs of lineages leading from ancestral nodes to terminal branches that share no branches in common (see Fig. 3). Let X_{11} be the number of nucleotides that differ in the first gene of the terminal taxa of the first independent pathway; let n_{11} be the number of nucleotides in the sequence that could be substituted, each with probability p_{11} ; then $X_{11} \sim B(n_{11}; p_{11})$. Similarly, let $X_{12} \sim B(n_{12}; p_{12})$ be the binomial distribution for the number of nucleotides that differ in the second gene of the first independent pathway; let $X_{21} \sim B(n_{21}; p_{21})$ be the corresponding binomial distribution for the second pathway, first gene; let $X_{22} \sim B(n_{22}; p_{22})$ be the corresponding binomial distribution for the second pathway, second gene, etc. The estimated genetic distance between the terminal taxa for the j th gene of the i th independent pathway is $\hat{p}_{ij} = X_{ij}/n_{ij}$; X_{ij} and \hat{p}_{ij} approach normal distributions as n_{ij} increases. With the application of expected value operators, it can be shown that $E[\hat{p}_{ij}] = p_{ij}$ and $Var[\hat{p}_{ij}] = p_{ij}(1 - p_{ij})/n_{ij}$. The difference between estimated substitution rates of the two genes for the same independent pathway (i) in the phylogeny is the random variable $d_i = \hat{p}_{i1} - \hat{p}_{i2}$, which is also approximately normally distributed; i.e., $d_i \sim N[\mu_{d_i}, (\sigma_{d_i}^2)]$, where $\sigma_{d_i}^2 = p_{ij}(1 - p_{ij})/n_{ij}$ for $j = \{1, 2\}$. Moreover, $\mu_{d_i} = 0$ if the divergence rates of the two genes are identical. If k independent pathways can be identified in the phylogeny, a new random variable, $D = \sum_{i=1}^k d_i$, can be defined which also is approximately normally distributed, viz., $D \sim N[\mu_D, \sigma_D^2]$, where $\mu_D = \sum_{i=1}^k \mu_{d_i}$ and $\sigma_D^2 = \sum_{i=1}^k \sigma_{d_i}^2 = \sum_{i=1}^k (\sigma_{d_{i1}}^2 + \sigma_{d_{i2}}^2)$. Again, as a null hypothesis, if the rates of substitution are the same for both genes over all of the k independent pathways, then $\mu_D = 0$.

Thus, to test the null hypothesis that the rate of substitution for two genes is the same over k independent pathways, sum the observed differences in proportions of sites substituted in the two genes over the k pathways ($\sum_{i=1}^k (\hat{p}_{i1} - \hat{p}_{i2})$). Sum the estimated binomial variances for each gene over the k pathways ($\sum_{i=1}^k (\hat{\sigma}_{i1}^2 + \hat{\sigma}_{i2}^2) = \sum_{i=1}^k [\hat{p}_{i1}(1 - \hat{p}_{i1})/n_{i1} + \hat{p}_{i2}(1 - \hat{p}_{i2})/n_{i2}]$). The statistic $Z = \sum_{i=1}^k (\hat{p}_{i1} - \hat{p}_{i2}) / \sum_{i=1}^k (\hat{\sigma}_{i1}^2 + \hat{\sigma}_{i2}^2)$ has approximately a standard normal distribution and the null hypothesis should be rejected if $Z \geq Z_{\omega/2}$.

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