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Evolution

Phylogenetic relationships among megabats, microbats, and primates

(molecular systematics/Chiroptera/sequence alignment/rates of evolution)

DAVID P. MINDELL*†, CHRISTOPHER W. DICK‡, AND ROBERT J. BAKER§

*Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221; ‡School of Natural Sciences, Hampshire College, Amherst, MA 01002; and §Department of Biological Sciences, Texas Tech University, Lubbock, TX 79409

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ABSTRACT **We present 744 nucleotide base positions from the mitochondrial 12S rRNA gene and 236 base positions from the mitochondrial cytochrome oxidase subunit I gene for a microbat, *Brachyphylla cavernarum*, and a megabat, *Pteropus capestratus*, in phylogenetic analyses with homologous DNA sequences from *Homo sapiens, Mus musculus* (house mouse), and *Gallus gallus* (chicken). We use information on evolutionary rate differences for different types of sequence change to establish phylogenetic character weights, and we consider alternative rRNA alignment strategies in finding that this mtDNA data set clearly supports bat monophyly. This result is found despite variations in outgroup used, gap coding scheme, and order of input for DNA sequences in multiple alignment bouts. These findings are congruent with morphological characters including details of wing structure as well as cladistic analyses of amino acid sequences for three globin genes and indicate that neurological similarities between megabats and primates are due to either retention of primitive characters or to convergent evolution rather than to inheritance from a common ancestor. This finding also indicates a single origin for flight among mammals.**

The world's bats (order Chiroptera) can be readily separated into two groups on the basis of morphological and behavioral characters: (*i*) suborder Megachiroptera (megabats), comprised of the single family Pteropodidae, and (*ii*) suborder Microchiroptera (microbats), encompassing all the other families (1). The megabats include the large fruit-eating bats found only in the Old World, whereas the microbats are primarily insectivorous and occur in the New World as well. Whether these two taxa represent a natural, monophyletic group, or whether the megabats represent an early offshoot of the primate lineage, unrelated to the microbat lineage, is unclear. Morphological features of the forelimbs related to flight suggest a sister relationship for the bats (2); however, shared features of neural pathways related to vision unite megabats and primates (3, 4). Resolution of this issue will improve our understanding of the origin(s) of flight among mammals, as well as the evolutionary history of mammalian orders including the primates.

Here we present 744 nucleotide base positions from the mitochondrial 12S rRNA gene and 236 base positions from the mitochondrial cytochrome oxidase subunit I (COI) gene for a microchiropteran, *Brachyphylla cavernarum*, and a megachiropteran, *Pteropus capestratus*, in phylogenetic analyses with homologous DNA sequences from *Homo sapiens, Mus musculus* (house mouse), and *Gallus gallus* (chicken). We use information on relative rates of sequence change to establish phylogenetic character weights and consider alternative rRNA alignment strategies in finding that this mtDNA data set clearly supports bat monophyly.¶

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METHODS

Molecular Techniques. Single-stranded DNAs were obtained with asymmetrical PCR amplifications from total genomic DNA preparations (5), and sequences were determined by dideoxynucleotide chain termination (6) with Sequenase T7 DNA polymerase (United States Biochemical). Primers used for 12S rRNA were L613 (5′-ACACAAAGCATGGCACTGAA-3′), H887 (5′-GTGACCGCGGTGGCTGGCAC-3′), L905 (5′-TGTGCCAGCCACCGCGGTCA-3′), L1091 and H1478 (7), and H1133 (5′-AAAAGCTTGTGCTCGTAGTTCTCCTGGCG-3′). Primers used for COI were L6704 (5′-TACTCCGGAAAAAAAGAACC-3′) and H7216 (5′-GGGTAGTCCGAGTAACGTCG-3′).

Evolutionary Rates and Character Weights. We made all possible pairwise comparisons (n = 55) of 12S rRNA sequences for 11 animal species, plotting percentage sequence difference against estimated time since divergence (Fig. 1). Slope of curves reflects rate of evolutionary change. Leveling off of curves is due to multiple substitutions at single nucleotide base positions known as saturation with change and indicates reduced amounts of phylogenetic information for taxonomic divergences of the ages shown (8). Percentage difference in 12S rRNA transitions increases most rapidly among species that have diverged from a common ancestor within the past 20 million years and thereafter increases more slowly than transversions (Fig. 1*A*). The slope of the curve for transitions becomes more level after ≈85 million years with average values of 11%, 12%, and 15% for divergences of 85, 350, and 600 million years of age, respectively (Fig. 1*B*). The curve for 12S rRNA transversions decreases in slope after 85 million years to a lesser degree, with average values of 9%, 12%, and 17%, respectively, for the same divergence times. Thus, transition differences in comparisons of 12S rRNAs will carry relatively little phylogenetic information for species divergences over 20 million years or so. The slower rate of transversion substitutions indicates a greater amount of phylogenetically useful information over a longer time period.

Analysis of COI sequences for the same 11 species shows that third nucleotide positions within COI codons become saturated with change for species divergences at least as recent as 85 million years ago (Fig. 1*C*). Conversely, the percentage difference curve for first and second COI codon positions indicates that these sites are changing much more slowly and are relatively unsaturated with change over periods as long as 600 million years. Considering all substitutions, the 12S rRNA and COI sequences have similar rates of evolutionary change (including decreases in rate due to saturation) with 0.27% and 0.25% per million years over the

Abbreviation: COI, cytochrome oxidase subunit I.
†To whom reprint requests should be addressed.
¶The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M81134–M81139).

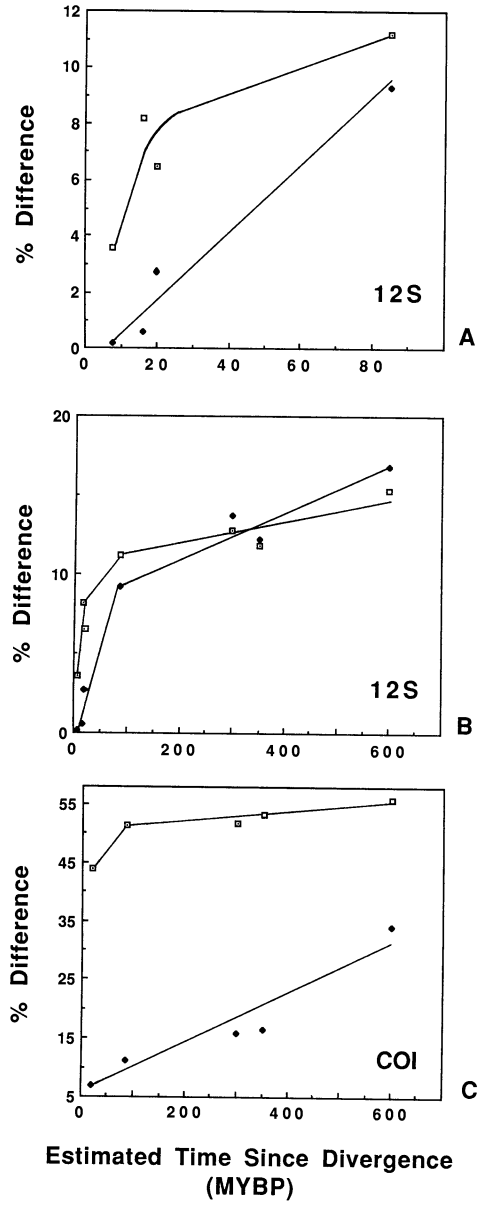


FIG. 1. Percentage difference versus estimated time since divergence for pairwise comparisons among 11 species for mitochondrial small subunit (12S) rRNA (*A* and *B*) and mitochondrial COI DNA sequences (*C*). Species included and data sources are *Drosophila yakuba* (9) (fruit fly), *Xenopus laevis* (10, 11) (frog), *Bos taurus* (12) (cow), *Giraffa camelopardalis* (13) (giraffe), *Sus scrofa* (13) (pig), *M. musculus* (14), *Rattus norvegicus* (15) (rat), *H. sapiens* (16), *Pan troglodytes* (17) (chimpanzee), *Pongo pygmaeus* (17) (orangutan), *G. gallus* (18). Points represent averages for species comparisons having the same estimated divergence times. (*A* and *B*) For 12S rRNAs, % difference is calculated as 100{1-[no. of identical nucleotides/(no. of shared base positions + total no. of gaps)]} in which each gap is counted conservatively as a single character regardless of its length in base positions. Diamonds, transversions; squares, transitions. (*C*) For COI sequences, % difference is (no. of substitutions at the given codon position)/total no. of the given codon positions in the gene). Squares, all substitutions at third codon positions; diamonds, all substitutions at first and second codon positions. Estimated time since divergence in millions of years before present (MYBP) for various species pairs are 600 for *Drosophila* with vertebrates, 350 for *Xenopus* with other vertebrates, 300 for birds with mammals, 85 for comparisons among mammalian orders, 20 within mammalian orders, and variously <20 for primate divergences (17).

past 85 million years and 0.06% and 0.05% over the past 600 million years, respectively.

A recent estimate for minimum age of a common ancestor for Primates, Scandentia (tree shrews), Dermoptera (flying lemur), and Chiroptera is 68 million years old (19). The oldest known fossil bat (*Icaronycteris index*) has been attributed to microchiroptera and is 50–55 million years old, and the oldest species attributable to the megachiroptera (*Archaeopteropus transiens*) is ≈30 million years old (20). Due to incompleteness of the fossil record, fossils provide only minimum estimates of age for taxa and their divergences from each other. However, these minimum age estimates, together with the information on evolutionary rates of change for various types of mitochondrial 12S rRNA and COI sequences (Fig. 1), allow us to make an a priori assessment of the reliability or weight to be accorded to those various types of nucleotide change. Namely, because of their relatively rapid rates of change, 12S transitions and COI third codon position changes, especially transitions, should be accorded less weight in these particular phylogenetic analyses. This is not meant to imply that there is no phylogenetic information at all in those character categories. Rather, our aim is to minimize homoplasious (convergent or parallel) character change in the data set.

Sequence Alignment and Phylogenetic Analysis. Parsimony analyses were conducted with PAUP (21) using two different outgroup taxa, *G. gallus* and *M. musculus*, in alternative analyses to examine their effect on phylogenetic hypotheses. *G. gallus* is more reliable as an outgroup because the lack of consensus regarding branching pattern among eutherian mammal orders (see ref. 22) leaves the possibility open that *M. musculus* is an ingroup member. The minimum age estimate for a common ancestor of birds and mammals is 300–310 million years old (19). This is considerably older than the ingroup divergences; however, evolutionary rate curves (Fig. 1) show that 12S transversions and COI first and second codon positions remain potentially informative over these periods of time.

Alignment of DNA sequences is a crucial step in phylogenetic analyses, in assessing which base positions are homologous, and thus are directly comparable. The 12S rRNAs lack reading frames and have many small insertion or deletion events, making alignment difficult relative to protein-coding genes. In addition, alignments of more than two species sequences are sensitive to the order in which those sequences are added to the overall group alignment (23, 24). Thus, because homology relationships denote shared descent, DNA sequences should be input in a multiple alignment bout in decreasing order of phylogenetic relationship (23). This order will often be unknown, and, in such cases, alternative alignments with differing species input orders can be analyzed. If resultant phylogenetic trees are identical, then the relationships may be considered unbiased by alignment input order. We have taken this approach in aligning the 12S sequences from the two bats and *H. sapiens*.

Three different input orders are possible in aligning sequences for the three ingroup species: (*B. cavernarum,H. sapiens*)*P. capestratus*, (*B. cavernarum,P. capestratus*)*H. sapiens*, and (*P. capestratus,H. sapiens*)*B. cavernarum*. In each case, sequences for the first two species enclosed in parentheses were aligned, and a consensus sequence was made from that alignment in which all variable nucleotide sites were given the appropriate IUPAC (International Union of Pure and Applied Chemistry) ambiguity code. This consensus sequence was then aligned with the third ingroup species, which was then aligned with the first two species' sequences accordingly. Another consensus sequence was made, as described above, from these three sequences combined, and then the outgroup was aligned to this second consensus sequence. We used the algorithm of Myers and Miller (25) for pairwise alignments, with parameters for open

gap cost and unit gap cost both set to 10, the same cost assigned to a single substitution.

RESULTS AND DISCUSSION

Pairwise comparisons of 12S rRNA sequences (Fig. 2) among five species range from differences of 19.4% to 29.0% (Table 1). The least divergent pair includes the two bats, and the most divergent pairs involve various species of mammals with the chicken. Similarly, percentage difference values in COI sequence pairwise comparisons ranged from 16.5% to 24.1%, with the smallest difference found between the two bats and the largest difference found in comparisons of different mammals and chicken.

Three sets of relationships are possible among the three ingroup taxa. To choose among them, we constrained parsimony analyses to each possible topology and compared the number of character changes (parsimony score) required for each (Fig. 3). Based on 12S rRNA transversions the most parsimonious tree unites the two bats as sisters relative to *H. sapiens*. These relationships were found in separate analyses with both outgroups (chicken and mouse) and all three alignment sets, showing the result not to be an artifact of alignment order or outgroup used. Analyses reported below use chicken as the outgroup. Most-parsimonious scores of 172, 175, and 197 for 12S rRNA transversions for the three different alignment sets are 11, 8, and 10 steps shorter than the shortest tree uniting *Brachyphylla* and *Homo*, and 8, 10, and 6 steps shorter than the shortest tree uniting *Pteropus* and *Homo*. Sister status for the two bats is supported by 20, 19, and 21 synapomorphies for the three different alignment sets, whereas sister relationships between *Homo* and *Brachy-*

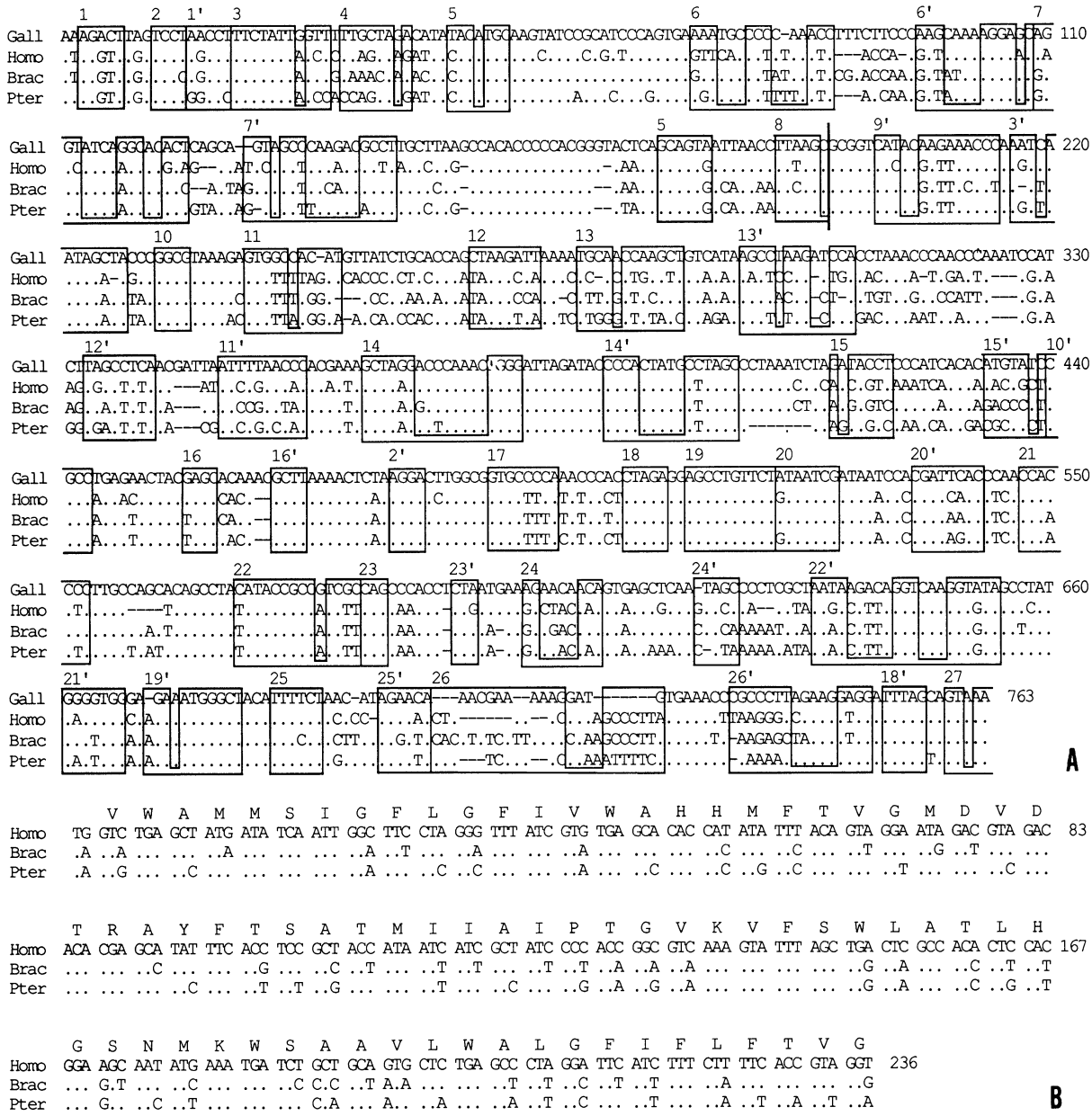


FIG. 2. Aligned sequences for portions of the mitochondrial 12S rRNA gene (*A*) and mitochondrial COI gene (*B*) for *B. cavernarum* (Brac), a microbat, and *P. capestratus* (Pter), a megabat, variously with *H. sapiens* (16) (Homo) and *G. gallus* (18) (Gall) sequences. Dots, identity with top sequence; dashes, gaps invoked to maintain alignment. (*A*) For 12S rRNA, regions postulated to form double-stranded stems are boxed. Numbers at the upper left corner of boxes denote stem location (following ref. 17). Alignment was determined by first aligning *Brachyphylla* and *Pteropus*, followed by *Homo* and *Gallus*, as described in the text [(BP)H]. Vertical line after base position 195 denotes location of a hiatus in this otherwise continuous sequence.

Table 1. Pairwise comparisons for species DNA sequences from the mitochondrial 12S rRNA and COI genes

Species compared	Shared base positions*		Identical base positions		Total no. of gaps		% difference	
	12S	COI	12S	COI	12S	COI	12S	COI
Brac/Pter	716	236	587	197	12	0	19.4	16.5
Brac/Homo	716	236	573	192	13	0	21.4	18.6
Brac/Mus	720	236	559	197	14	0	23.8	16.5
Brac/Gall	724	236	534	180	19	0	28.1	23.7
Pter/Homo	713	236	572	192	14	0	21.3	18.6
Pter/Mus	712	236	564	193	12	0	22.1	18.2
Pter/Gall	712	236	526	184	21	0	28.2	22.0
Homo/Mus	716	236	550	185	13	0	24.6	21.6
Homo/Gall	716	236	530	179	23	0	28.3	24.1
Mus/Gall	717	236	527	186	25	0	29.0	21.2

Percentage difference values are calculated as described in Fig. 1. Brac, *B. cavernarum*; Pter, *P. capestratus*; Homo, *H. sapiens*; Mus, *M. musculus*; Gall, *G. gallus*.
*Number of identical base positions plus number of substitutions.

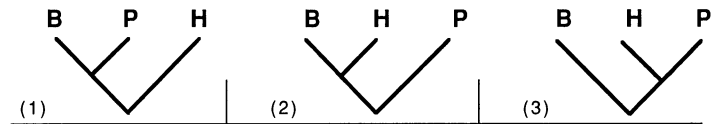
phylla or *Pteropus* are supported by only 9, 12, and 14 and 12, 9, and 14 synapomorphies, respectively. Thus, the number of shared derived characters uniting megachiroptera and microchiroptera as sisters is 2.2–1.5 times larger than the number of shared derived characters supporting the alternative sister relationships. If each synapomorphy had a 50% chance of supporting either of two trees being compared (a random distribution), then the binomial probabilities for the observed distribution of synapomorphies are [based on the (BP)H alignment] as follows: 0.031 for (*Brachyphylla,Pteropus*) versus (*Brachyphylla,Homo*), 0.108 for (*Brachyphylla,Pteropus*) versus (*Pteropus,Homo*), and 0.332 for (*Brachyphylla, Homo*) versus (*Pteropus,Homo*). This is not a critical test, however, because the inherent assumption of rate constancy does not hold. The (*Homo,Pteropus*)*Brachyphylla* alignment set yielded the longest trees for each of the three possible topologies (Fig. 3).

We repeated all analyses with gaps coded, first, as missing characters (Fig. 3) and, then, as new (fifth) character states, and we found similar levels of support for the tree uniting bats as sisters. We combined all five study taxa (Table 1) in a single parsimony analysis for 12S rRNA, using an alignment determined with the CLUSTAL program (26) in which sequences are input in order of overall similarity. We found the most parsimonious tree based on transversions to unite bats as sisters, with nine fewer steps and eight more synapomorphies supporting the node in question compared to the tree uniting *Pteropus* and *Homo*. *Mus* was consistently placed outside the bat and primate clade with *Gallus* as the outgroup.

Lake's (27) phylogenetic invariants method attempts to compensate for effects of differences in evolutionary rate and saturation with change among homologous sequences by estimating the number of informative transversion differences along the central branch and discounting one class of parallel (uninformative) transversions in peripheral branches of an unrooted tree for four taxa. Using each of the three different alignment sets, as described above, we found the topology uniting the bats as sisters to be supported, with probabilities of 0.001, 0.008, and 0.001, respectively (Fig. 3). Probabilities for the other two topologies were not significant. Lake's method is sensitive to the ratio of transitions to transversions and requires a balance among various classes of transitions and transversions (28), and we place more emphasis on the fact that these findings are congruent with the parsimony analysis than on exact significance values.

rRNAs contain considerable amounts of within-molecule base pairing and several studies have revealed a tendency for stems (paired regions) to show change on one side that is matched by change on the other side, maintaining nucleotide base complementarity (17). This presents a potential problem, as only independently varying characters are appropriate for phylogenetic analyses. To examine this potential bias, we have overlaid the 12S rRNA sequences for the study taxa on the secondary structure model for *Escherichia coli* (29) as modified for the common chimpanzee (17). This extrapolation is tentative, as extensive experimental evidence for deducing secondary structure has been done only for *E. coli*. However, there does appear to be a common architectural core in rRNA secondary structure across diverse taxa (30).

For the rRNA sequence analyzed (Fig. 2) similar numbers of base positions occur in hypothesized stems (n = 372; 49%) and loops (n = 391; 51%), and overall numbers of substitutions relative to the outgroup (*G. gallus*) are similar within hypothesized stems and loops, being 90 and 104 for *Homo*, 90 and 108 for *Brachyphylla*, and 90 and 112 for *Pteropus*, respectively. In comparing the two bats to *H. sapiens* sequence, however, a slightly greater than expected number of hypothesized stem region substitutions are compensated (33% expected, 42% observed; χ^2 = 3.035; degrees of freedom = 1; P = 0.0815). Twenty shared derived characters



ALIGNMENT ORDER →	(B,P)H	(B,H)P	(H,P)B	(B,P)H	(B,H)P	(H,P)B	(B,P)H	(B,H)P	(H,P)B
PARSIMONY SCORES:									
12S rRNA									
All chars.	380	388	415	391	396	425	379	382	375
Transv. only	172*	175*	197*	183	183	207	180	185	203
COI									
All chars.		111			113			113	
Transv. only		54			54			57	
12S rRNA PHYL. INVAR.	0.001	0.008	0.001	1.000	0.754	0.727	0.180	0.289	0.146

FIG. 3. Lengths for each of three possible phylogenetic trees (parsimony scores) for mitochondrial 12S rRNA and mitochondrial COI sequences (Fig. 2), using, for 12S rRNAs only, each of the three possible alignment orders for *B. cavernarum* (B), *P. capestratus* (P), and *H. sapiens* (H) with *G. gallus* as an outgroup (see text). Parsimony scores are given for analyses under two weighting schemes, equal weight for all substitutions, and zero weight for transitions. Asterisks, score for the most-parsimonious tree topology under the given alignment order for transversions-only analyses, as they are considered most reliable in light of rapid change for transitions (see Fig. 1). Binomial probabilities are given for Lake's phylogenetic invariants analysis of the 12S rRNAs.

support the sister relationship for the two bats in the most parsimonious tree, and seven (35%) of these occur in hypothesized stems. However, only two of these represent compensatory change (positions 550 and 664; Fig. 2), and, thus, the potential bias of compensatory change in paired regions in the most parsimonious tree appears minimal. Parsimony analyses based on transversions at hypothesized stem sites only and at loop sites only both still found the tree with bats as sisters to be the most parsimonious.

In COI sequence analyses, we found a total of 127 nucleotide differences among the three ingroup species; 121 (95%) of these occurred at third codon positions, 6 occurred at first codon positions, and 0 occurred at second codon positions. There are 44 differences (over all codon positions) in pairwise comparisons of *Homo* with both *Brachyphylla* and *Pteropus*, and 39 differences between *Brachyphylla* and *Pteropus*.

Two different most-parsimonious trees, each requiring 54 character changes, were found for COI transversions (Fig. 3), one uniting the two bats and the other uniting *Brachyphylla* and *Homo*. Analysis of all characters resulted in a most-parsimonious tree uniting the two bats; however, evolutionary rate determinations (Fig. 1) indicate that the many third codon position transitions (52% of all third position substitutions) included are saturated with change and potentially misleading. Furthermore, the alternative tree topologies require only two additional steps. Thus, parsimony analyses of COI sequences in Fig. 2 are less informative than the 12S sequences, due to the fact that most change was limited to saturated third codon positions. However, if we consider the six first codon position differences found, and their slower rate of change, we find that there were no such differences between the two bats, and three such differences (one transition and two transversions) between *Homo* and each of the bats, in broad agreement with the 12S rRNA sequence analyses.

Intraspecific polymorphism underlies the difference between gene trees and species trees and is a potential source of inaccuracy; however, levels of sequence difference among *Pteropus, Brachyphylla*, and *Homo* (Table 1) are all at least 10-fold greater than difference levels found in rapidly evolving D-loop sequences for seven *H. sapiens* individuals (31), indicating that intraspecific polymorphism is low relative to differences among the study sequences. Furthermore, intraspecific polymorphisms were unlikely to be used in our phylogenetic analyses based on slowly evolving substitution types.

In summary, we find monophyly of order Chiroptera to be supported by the mitochondrial sequence data. The 12S rRNA transversions provide the clearest phylogenetic inference, with *Brachyphylla* and *Pteropus* united as sisters by 8–11 more synapomorphies than found in less-parsimonious, alternative topologies. Fewer informative COI sequence characters are available; however, the most reliable category of COI change (first codon position substitutions) also supports monophyly of Chiroptera. These findings are congruent with numerous morphological characters, including details of wing structure as well as cladistic analyses of amino acid sequences for three different globin genes from each of two megabat and four microbat species (32). This congruence also indicates that the neurological similarities between megabats and primates are either due to convergent evolution rather than to inheritance from a common ancestor, or that they represent shared primitive rather than shared derived features. In either case, they do not appear phylogenetically informative. The mitochondrial DNA characters analyzed here are from genes known to be homologous across the study taxa, whereas the homology of sequences underlying the neurological similarities is uncertain, and our analyses incorporate increasing knowledge of sequence character evolution, with synapomorphies representing base substitution types occurring at demonstrated and appropriately slow rates.

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