

Molecular phylogeny of the superorder Archonta

(cytochrome oxidase subunit II/molecular systematics/mitochondrial DNA)

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ABSTRACT The superorder Archonta has been hypothesized to include primates, tree shrews, bats, and flying lemurs as descendants of a common ancestor. More recently, a diphyletic origin for bats has been proposed. To evaluate these hypotheses, the nucleotide sequence of the mitochondrial cytochrome oxidase subunit II gene was determined from a bushbaby (*Galago senegalensis*), flying lemur (*Cynocephalus variegatus*), tree shrew (*Tupaia glis*), spear-nosed bat (*Phyllostomus hastatus*), rousette bat (*Rousettus leschenaulti*), and nine-banded armadillo (*Dasypus novemcinctus*) and was compared with published sequences of a human, cow, and mouse. Phylogenetic analyses of the sequences give evidence that primates, tree shrews, and flying lemurs have a recent common ancestor but that bats are genealogically distant. The monophyletic origin of bats is supported. Contrary to interpretations based on morphological data, tree shrews are shown to be no more closely affiliated with primates than are flying lemurs. Analyses of the cytochrome oxidase subunit II gene give marginally more support to a Dermoptera–Scandentia clade than to a Dermoptera–Primates clade.

Determination of phylogenetic relationships among eutherian mammals has been difficult and often controversial. No single hypothesis of eutherian higher-level relationships has received widespread support (1). Relationships are contested even when one focuses on a small subset of eutherian orders. For example, the superorder Archonta initially consisted of Primates plus four additional orders, Scandentia (tree shrews), Dermoptera (flying lemurs), Chiroptera (bats), and Macroscelidea (elephant shrews) (2). Subsequently, the monophyly (shared ancestry) of this superorder has been both rejected outright (3–5) and confidently affirmed (6), with the only recent consensus being the exclusion of Macroscelidea. Relationships among both orders and suborders in Archonta are uncertain, and, as of late, even the monophyly of Chiroptera has been questioned (7, 8). This latter hypothesis suggests that the order Chiroptera is diphyletic such that the suborder Megachiroptera is a sister to Primates and the suborder Microchiroptera is several branches away in the eutherian phylogeny. If this hypothesis is correct, flight and all its requisite adaptations evolved twice among mammals, an event that would represent one of the most striking examples of convergent evolution ever recorded for mammals.

Although nucleotide and/or amino acid sequences have been used to formulate several hypotheses pertaining to eutherian relationships (9, 10), no molecular study using discrete characters has been published that includes all major lineages in the superorder Archonta. Here we examine nucleotide sequence variation from the mitochondrial cytochrome oxidase subunit II (COII) gene in order to address three primary questions: (i) is the superorder Archonta monophy-

letic, (ii) what is the sister group to Primates, (iii) do Megachiroptera and Microchiroptera share a common ancestry?†

MATERIALS AND METHODS

Liver tissue was collected from *Galago senegalensis*, *Cynocephalus variegatus*, *Tupaia glis*, *Phyllostomus hastatus*, *Rousettus leschenaulti*, and *Dasypus novemcinctus*, and mtDNA was isolated via cesium chloride/propidium iodide gradient centrifugation (11). The mtDNA was brought to equilibrium with a solution of 100 mM Tris-HCl, pH 8.0/50 mM EDTA via dialysis.

Double- and single-stranded copies of the COII gene were amplified from isolated mtDNA via the PCR (12, 13) using *Taq* DNA polymerase (Perkin-Elmer/Cetus) and 25–30 temperature cycles (95°C, 1 min; 45°C–55°C, 1 min; 72°C, 1.25 min). Oligonucleotide primers for amplifying and sequencing the COII gene were located in the tRNA^{asp} (L7553, 5'-AACCATTTTCATAACTTTGTC-3') and tRNA^{lys} (H8320, 5'-CTCTTAATCTTTAACTTAAAAG-3') genes that flank the COII gene. Other sets of primers internal to the COII gene (L7900, 5'-AAGACAATAGGCCACCAATGATAC-3'; L7826, 5'-CCTTATCCTAATTGCCCTACC-3'; H7966, 5'-CGGAGTTCTCCTGGTTTTAGGTC-3'; H7862, 5'-AAGGGAGGGATCGTTGAC-3') were also used for amplification and sequencing. Numbers in the primer names indicate the positions in the reference sequence (*Homo sapiens*) (14) of the 3' bases, and the prefix H or L refers to the heavy or light strand. Double-stranded DNA containing the 3' terminal 466 bases of the COII gene of *Tupaia glis* was produced by PCR using *Thermococcus litoralis* (Vent) DNA polymerase (New England Biolabs) and was inserted into the pUC19 plasmid via blunt-end ligation. Nucleotide sequences were determined via the dideoxynucleotide chain-termination method (15).

RESULTS AND DISCUSSION

The COII gene sequences of *Galago* (order Primates), *Cynocephalus* (order Dermoptera), *Tupaia* (order Scandentia), *Phyllostomus* (suborder Microchiroptera), and *Rousettus* (suborder Megachiroptera) were combined with existing sequences of human (14), cow (16), and mouse (17), and these combined data were used in all subsequent phylogenetic analyses. All phylogenetic trees were rooted with the armadillo (*Dasypus novemcinctus*), because the order Edentata is generally regarded as being divergent relative to the other orders of Eutheria (6, 18).

Several different analytical approaches were used to evaluate phylogenetic relationships. Although somewhat different tree topologies were obtained with these approaches (Fig. 1), most of these discrepancies can be explained by the

Abbreviation: COII, cytochrome oxidase subunit II.

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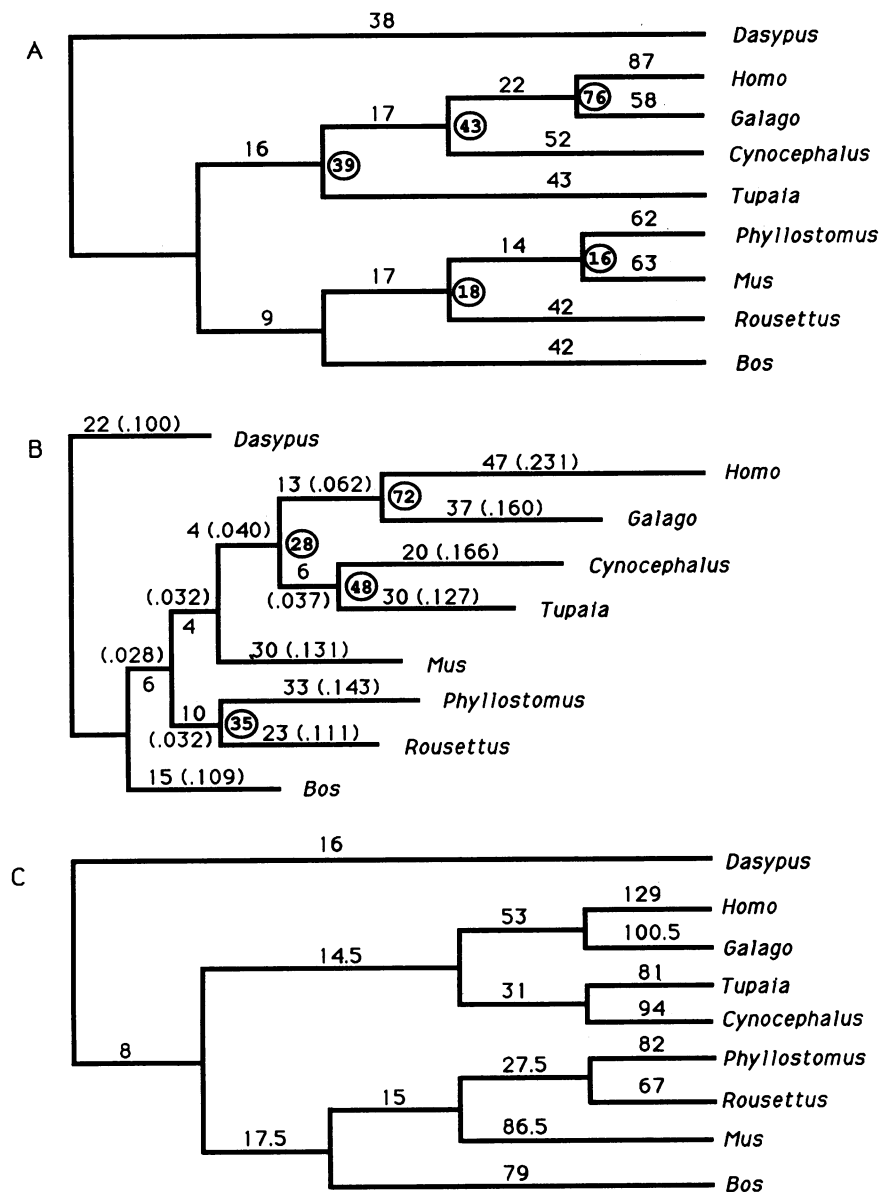


FIG. 1. Phylogenetic hypotheses derived from analyses of COII gene sequences. (A) Maximum parsimony (19) tree based on equal weighting of all nucleotide substitutions constructed using the branch and bound option of PAUP 3.0N (20) with the edentate *Dasylops* as the outgroup. Minimum possible branch lengths are shown. The tree required 874 steps and had a consistency index of 0.549, excluding autapomorphies. The number of times select nodes were present after bootstrap resampling (21) of the data (100 replicates, branch and bound option; PAUP 3.0N) are shown in circles. (B) Tree produced by maximum-likelihood analysis (22) (branch lengths shown in parentheses) using the observed base frequencies in the substitution process [DNAml of PHYLIP, version 3.3; global branch swapping (23)] and maximum parsimony (PAUP 3.0N, branch and bound) analysis of only transversions (minimum branch lengths shown). The maximum-parsimony tree required 383 steps and had a consistency index of 0.433, excluding autapomorphies. Bootstrap values for select nodes derived from transversion parsimony analysis are shown in circles (100 replicates, branch and bound; PAUP 3.0N). (C) Tree produced using the dynamically weighted parsimony procedure of Williams and Fitch (24) [program WTSUBS (25)] with existential weighting of transformations. Uniformly weighted tree length is 887. The length along the weighted path is 955.5. Branch lengths shown are weighted values.

necessity to consider some form of character weighting with respect to the increased probability of multiple substitutions at the third base position, the transition/transversion ratio, the frequency of particular nucleotides, and the probability of change at each nucleotide position.

When total substitutions (equally weighted) were used in a maximum-parsimony analysis (19), the monophyly of Archonta and Chiroptera was not supported, and Dermoptera was found to be a sister to Primates (Fig. 1A). If one uses a bootstrap analysis (21) to evaluate the consistency or reliability of particular nodes, most values are small (<20%) with the exception of the clade consisting of the orders Scandentia, Dermoptera, and Primates.

Two expectations of mtDNA sequence divergence are that the transition/transversion ratio decreases and transition substitutions approach saturation as evolutionary divergence increases (26). Therefore, several different methods of character weighting were used to evaluate the results derived from total substitutions. A consideration of only transversions at all positions in a maximum-parsimony analysis supported the monophyly of Chiroptera, and Dermoptera and Scandentia were shown to form a clade that was sister to Primates (Fig. 1B). Although the consistency of particular lineage associations evaluated by bootstrap analysis was low, the values supporting chiropteran monophyly were higher

than those obtained by using total substitutions. The dynamically weighted parsimony method of Williams and Fitch (24) was used in an attempt to incorporate a more complex weighting scheme into the parsimony analysis. Relative to the orders of Archonta, two very similar topologies were produced, depending on whether transformation costs were given a weight inversely proportional to the frequency of co-occurrence of two nucleotides at a site (existential weighting) (Fig. 1C) or to the sum of the number of ways each pair of nucleotides could occur at a site (combinatorial weighting). The topology produced by combinatorial weighting was similar to that of Fig. 1C, differing only by the placement of *Bos*, sister to the bats, and *Mus*, sister to all of the ingroup taxa. Transformation matrices were allowed to assume asymmetrical values, which is justified by the use of a well-established root to determine polarity. The topologies produced were unaffected by whether the weight given to each site, based on its number of inferred changes, was an inverse linear or quadratic proportion, due to the fact that the same changes were involved in either case. Because the transformation matrices derived by dynamic parsimony gave proportionally large weights to most transversions, these analyses produced topologies very similar to that based on strict transversion parsimony, in that the bats were monophyletic

and Dermoptera and Scandentia were a sister clade to Primates.

The relationships depicted in Fig. 1B also were supported by a maximum-likelihood determination (22) of the phylogeny most likely to have resulted in the observed data based on the relative frequency of each nucleotide and a transition/transversion bias. In the case of the maximum-likelihood analysis, several transition/transversion ratios were used (1.0–2.5), and all values produced the same tree topology, with a value of 1.5 maximizing the log-likelihood. This ratio was used in all maximum-likelihood analyses. Under the maximum-likelihood model, a 3-fold higher rate of change also was specified for the third codon position relative to the first and second positions (a rate close to that empirically observed). The phylogeny resulting from this analysis was essentially the same as that observed in Fig. 1B except that *Bos* was shown to be sister to Chiroptera. Finally, the reliability of the nodes produced by the maximum-likelihood analyses was examined via bootstrap resampling of the data [DNAdist of PHYLIP 3.3 (23); 100 replicates] and calculation of pairwise distances between the taxa under the maximum-likelihood model. Trees were then constructed by the Fitch–Margoliash method (27) (Fitch of PHYLIP 3.3). The resultant consensus tree (Consense of PHYLIP 3.3) produced from this analysis differed from Fig. 1B only in the placement of Dermoptera as sister to Primates. The nodes uniting *Homo–Galago*, Primates–Dermoptera, and Primates–Dermoptera–Scandentia were present in 77, 50, and 70 of the trees, respectively. Chiropteran monophyly was supported in 36 of the trees.

Two methods were used to evaluate the resultant trees derived from the COII gene sequence data (Fig. 1) with respect both to one another and to phylogenetic hypotheses proposed on the basis of morphological features (Fig. 2). The trees corresponding to the morphological hypotheses were constructed by placing constraints on the topologies of parsimony analysis and determining the shortest tree consistent with these constraints. First, the test devised by Templeton (29) was used to determine which topologies were best

supported by the data within a parsimony framework. By this method, no distinction could be made among the topologies shown in Fig. 1, but the hypotheses derived from morphological data had significantly less support. Second, the criteria of Kishino and Hasegawa (30) were used to statistically test the difference in log-likelihood of the hypothesized topologies. These tests produced results similar to those of the Templeton test. The two topologies consistent with the hypothesis of Pettigrew *et al.* (8) could be unequivocally rejected, and the difference in log-likelihood of the second hypothesis (6, 28) was just barely contained within its 95% confidence interval. Two major conclusions can be drawn from the tests presented in Fig. 2. First, the diphyletic origin of bats as proposed by Pettigrew and colleagues (7, 8) is not supported. Second, the monophyly of Archonta and the relationships among archontan orders derived from the postcranial skeleton and other anatomical features (6, 28) is very unlikely on the basis of the COII gene data.

Traditionally, Dermoptera and Chiroptera are grouped by several characters (1, 2, 6) into a clade sister to Primates and Scandentia (28). Here Primates, Scandentia, and Dermoptera are established as a clade, but Chiroptera appears to be part of an earlier split, a conclusion corroborated by immunological data (31) and nucleotide sequence data from the mitochondrial 12S rRNA gene (32). This result is especially surprising in light of previous inferences of a close relationship between bats and dermopterans (6, 33). Although some of the morphological features used to support a chiropteran–dermopteran clade are associated with a gliding/flying lifestyle and thus may be convergent similarities, other independent synapomorphies involving the basicranium also are refuted. The two principal characters that support an archontan clade are the structure of the sustentacular facet of the astragalus and the presence of a pendant penis. However, both of these characters are quite variable within each order of Archonta, particularly among microchiropterans, which have lost the sustentacular facet and some of which possess a sheathed penis that is similar to the primitive condition (6). This variability casts doubt on the quality of these features as

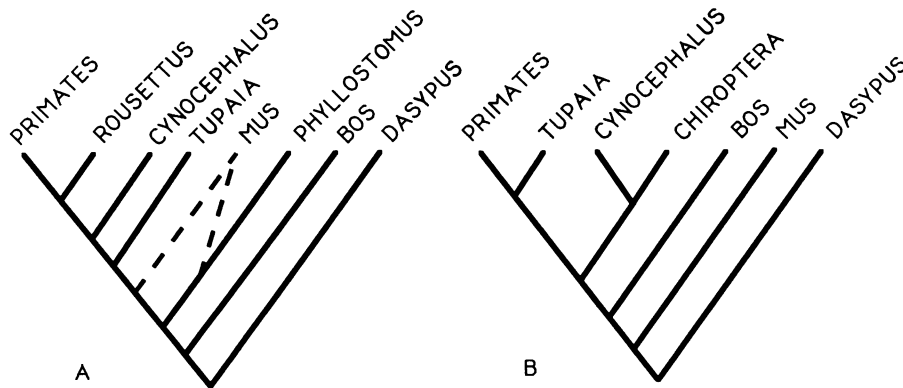


FIG. 2. Morphological hypotheses for the relationships of archontan taxa and statistical comparisons of the various topologies shown in this paper. (A) Topology consistent with hypotheses of Pettigrew and colleagues (7, 8). The tree was constructed in PAUP 3.0N (branch and bound option) (20) under the topological constraint {{{(Homo, Galago), Roussettus}, Cynocephalus), Tupaia}. Two equally parsimonious topologies (901 steps) were produced that differed only in the placement of *Mus*, as indicated by dashed lines. (B) Topology consistent with the morphological data of Novacek and Wyss (6) and Wible and Covert (28), constructed in PAUP 3.0N under the constraint {{{(Homo, Galago), Tupaia}, [(Phyllostomus, Roussettus), Cynocephalus]}. (C) Statistical comparison of the support for various hypotheses of archontan taxa based on COII gene sequences. The hypotheses are compared within the framework of parsimony and maximum likelihood by using the criteria of Templeton (29) and Kishino and Hasegawa (30), respectively. Topologies with significantly less support ($P < 0.05$) than the best tree are denoted by asterisks.

C	TOPOLOGY	* STEPS DIFFERENT (SD)	LN LIKELIHOOD DIFFERENCE (SD)
	FIGURE 1A	----	8.15 (13.43)
	FIGURE 1B	9 (7.82)	----
	FIGURE 1C	13 (6.86)	7.43 (9.15)
	PETTIGREW ET AL. (Fig. 4a)	27 (9.23)*	44.54 (15.97)*
	WIBLE, COVERT, NOVACEK AND WYSS (Fig. 4b)	27 (8.31)*	40.28 (16.88)*
		20 (10.10)*	29.45 (15.28)

phylogenetic characters at the level of interordinal comparisons.

The precise relationships among Primates, Dermoptera, and Scandentia are not resolved by our data, but it is clear that Scandentia is no more closely related to Primates than is Dermoptera. The two hypotheses presented in Fig. 1 for these three orders have rather significant ramifications for the presumed primitive morphotype of Primates. Tree shrews historically have been chosen to represent the primitive morphotype for modern primates (34), a conclusion apparently based on their retention of primitive skeletal traits and "trends" of morphological modification similar to those of Primates. In reality, it has been extremely difficult to demonstrate a special relationship of tree shrews to any group of placental mammals, much less to Primates in particular. Although tree shrews possess traits in common with many other taxa, these traits are present in a confusing combination of primitive and parallel states (3, 35, 36). The present indication that tree shrews are not the closest relatives to Primates renders their use as prototypic primates invalid in a phylogenetic sense. As another matter, it has recently been demonstrated on the basis of well-preserved fossils that paromomyids are actually members of the ancestral stock of Dermoptera (37, 38). This is consistent with a close relationship between Primates and Dermoptera (31). However, the weighted phylogenetic analyses presented in Fig. 1 would argue against this conclusion, because a sister-group relationship between Dermoptera and Scandentia seems more likely with the COII gene sequence data. To our knowledge, this relationship has not been proposed previously.

Monophyly of Chiroptera is supported by both weighted parsimony analysis and maximum-likelihood analysis of the nucleotide sequences of the COII gene (Fig. 1 B and C). This conclusion is further supported by immunological data (31), by nucleotide sequence data (32), and by many morphological features (33, 39) unassociated with flight. Also, in concordance with data from the cytochrome oxidase subunit III gene (40), absolutely no evidence is found for a close relationship between Primates and Megachiroptera. The fact that strict monophyly of Chiroptera is not supported by equally weighted parsimony analysis of total nucleotide substitutions (four additional steps are required to achieve monophyly) (Fig. 1A) is not surprising given the high degree of homoplasy expected for transitions within a mitochondrial gene when examining divergences as ancient as these. In addition, a tree representing the phylogeny of mammalian orders is likely to exhibit very short internodes relative to terminal branches. This is reflected by the typical portrayal of the eutherian radiation as a polytomous bush at the end of the Mesozoic (1). Because the oldest microchiropteran fossil (*Icaronycteris*) (41) is at least 50 million years old, the two suborders of Chiroptera must have shared a comparatively short common ancestry and, in a chronological sense, are almost as divergent as mammalian orders. The COII gene data are consistent with short internodes and an ancient divergence between Megachiroptera and Microchiroptera.

Pettigrew and his associates (7, 8) are the main proponents of a diphyletic origin for bats. They base their analyses on neural morphology, proportional lengths of forelimb bones (metacarpophalangeal index), and amino acid sequences of B-hemoglobin. However, the results drawn from the hemoglobin sequences are sufficiently ambiguous and so contradictory to other well-founded hypotheses of mammalian relationships as to be of highly questionable utility. Furthermore, the metacarpophalangeal index serves only to distinguish between Micro- and Megachiroptera and provides no evidence for alternative relationships between the suborders or with other orders. Therefore, neural anatomy provides the strongest evidence to support their hypotheses. However, the weight of these characters seems decreased in view of the

larger and more diverse sets of data supporting monophyly. In addition, the manner in which the neural characters have been sampled, coded for analysis, and polarized has recently been called into question (42–44).

Although this study is based on a single gene, it does make possible several important observations relative to the evolution of mammalian orders. First, the order Chiroptera is shown to consist of two divergent groups, Megachiroptera and Microchiroptera, separated by amounts of change comparable to those seen between other orders of archontans. The suborder Megachiroptera does not share a common ancestry with Dermoptera or Primates. Nevertheless, the magnitude of difference separating the two suborders of bats suggests that the evolutionary history of bats is old, with the separation of the two major lineages occurring over a relatively short period of time. This idea receives support from other recent studies of chiropteran evolution (45), and, if correct, a large amount of molecular data will be needed to confirm conclusively the monophyly of Chiroptera. Second, these data do not support the monophyly of the superorder Archonta as currently recognized, because both suborders of Chiroptera are shown to be quite divergent from other archontan orders. Third, the orders Primates, Dermoptera, and Scandentia are shown to represent a monophyletic clade. There is no evidence to support either a Scandentia/Primates or Dermoptera/Chiroptera clade. If these two observations are accepted, the evolution of morphological characters defining these two clades must be reinterpreted.

The evolutionary history of mammals is characterized by morphological diversity, the origin of novelty over short evolutionary time periods, and a large degree of convergent and parallel evolution. The controversies surrounding the superorder Archonta are the result of conflicting interpretations of molecular evolution and the relationships derived from particular morphological characters. The data presented here provide a consistent pattern for the relationships among archontan orders and bring into question interpretations derived from particular morphological characters while at the same time being congruent with other morphological data. If one is to resolve the persistent controversies surrounding the mammalian radiations, the use of independent data from both molecules and morphology is requisite, and the ultimate test of any resultant phylogeny should be congruence.

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