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Are Megabats Flying Primates? Contrary Evidence from a Mitochondrial DNA Sequence

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Abstract

Bats (Chiroptera) are divided into the suborders Megachiroptera (fruit bats, 'megabats') and Microchiroptera (predominantly insectivores, 'microbats'). It had been found that megabats and primates share a connection system between the retina and the midbrain not seen in microbats or other eutherian mammals, and challenging but plausible hypotheses were made that (*a*) bats are diphyletic and (*b*) megabats are flying primates. We obtained two DNA sequences from the mitochondrion of the fruit bat *Pteropus poliocephalus*, and performed phylogenetic analyses using the bat sequences in conjunction with homologous *Drosophila*, mouse, cow and human sequences. Two trees stand out as significantly more likely than any other; neither of these links the bat and human as the closest sequences. These results cast considerable doubt on the hypothesis that megabats are particularly close to primates.

Introduction

Various phylogenetic schemes based on morphology have linked bats and primates, such as in McKenna's (1975) grandorder Archonta, which also includes the Dermoptera (flying lemurs) and Scandentia (tree shrews). Molecular systematists, using immunological comparisons and amino acid sequences, have found that bats are not placed particularly close to primates, and that they are not diphyletic (Cronin and Sarich 1980; Dene *et al.* 1980; Miyamoto and Goodman 1986), but have disagreed as to the correct placement. Unfortunately, one large study of protein sequences (Miyamoto and Goodman 1986) combined megabat and microbat prior to analysis, negating any test of bat diphyly or the place of megabats. Cronin and Sarich (1980) called for the use of DNA sequence data and the estimation of statistical sufficiency of results.

Pettigrew (1986) found that megabats and primates share a connection system between the retina and the midbrain not seen in microbats or other eutherian mammals. He hypothesised that (*a*) bats are diphyletic and (*b*) megabats are flying primates.

We chose mitochondrial DNA to test Pettigrew's hypotheses because it is relatively easily isolated and because several complete sequences are now available (Anderson *et al.* 1981, 1982; Bibb *et al.* 1981; Clary and Wolstenholme 1985; Roe *et al.* 1985). We were particularly interested in using the CO III sequence because studies involving mammals, an amphibian and a bird (see Clary and Wolstenholme 1985; Roe *et al.* 1985; Hawkins *et al.*, unpublished data) show that this gene evolves at an intermediate rate suitable for the time scale involved.

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Methods

DNA Characterization

Mitochondrial DNA was isolated from fresh tissue (liver, kidney, spleen, diaphragm and muscle) from a Sydney, Australia specimen of *Pteropus poliocephalus* using methods similar to those described by Lansman *et al.* (1981).

Digestion with a range of six-base restriction endonucleases yielded a size estimate of *c.* 18 500 bp.

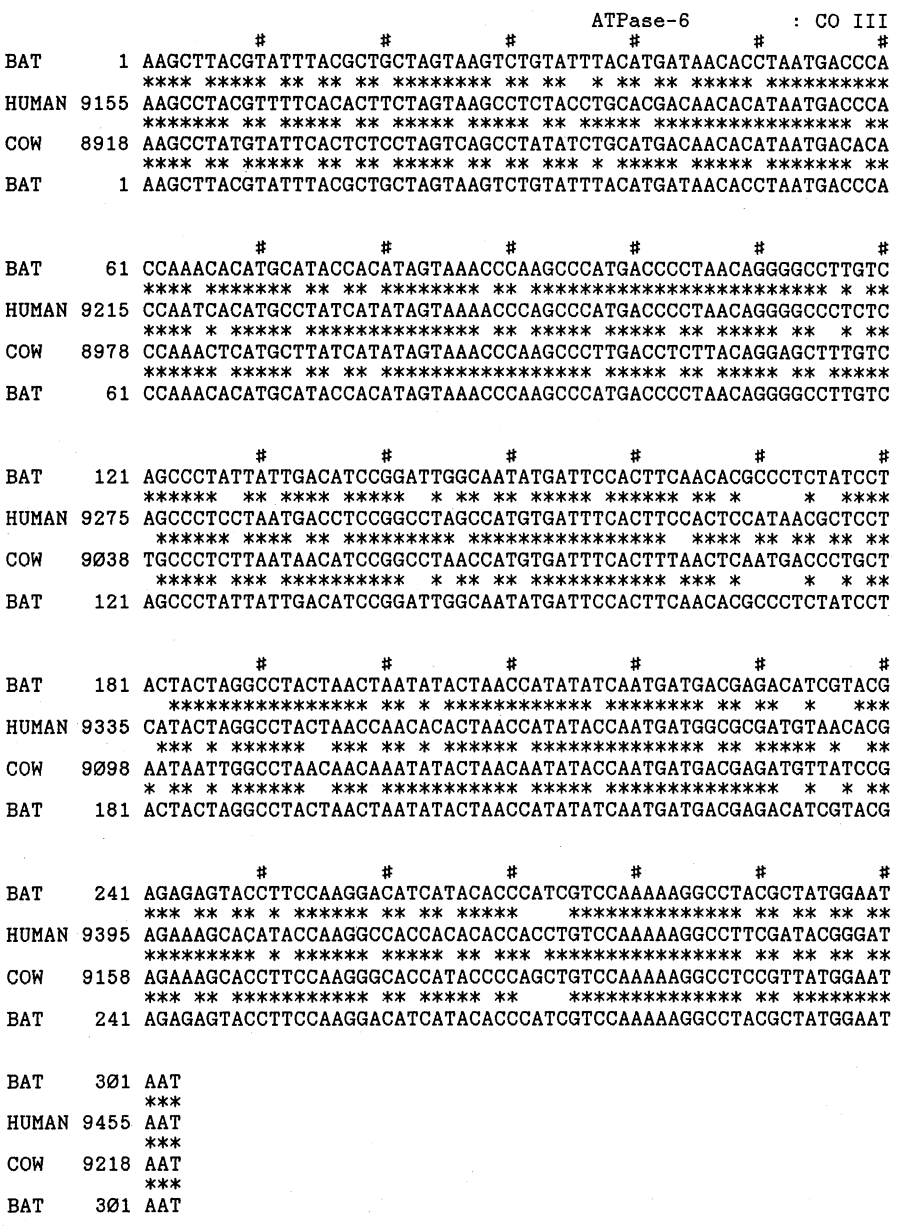

Fig. 1. Primary strand sequences of the cloned *Pteropus* sequences ('BAT'), compared with the homologous bovine ('COW') and human ('HUMAN') sequences: clone MAC CO III, with homology to bovine positions 8970–9221 (parts of the ATPase-6 and CO III genes).

Digestion with Hind III yielded four fragments, from which two clones were derived from a Hind III–Bam H1 digestion using standard methods (Maniatis *et al.* 1982). One of these was subcloned into M13 mp8, and subsequent sequencing carried out by the dideoxy method (Sanger *et al.* 1977).

Phylogenetic Analysis

The bat sequence data were used to evaluate the various possible trees involving the fruit bat and other sequences, including the human sequence. We used two basic methods of phylogenetic analysis, the well-known parsimony method and a maximum-likelihood method, which allows the statistical comparison of alternative trees. While extreme variation in evolutionary rates among lineages can lead to misleading results using the parsimony method (Felsenstein 1978; Lake 1986), the known variations in molecular evolutionary rates (Wu and Li 1985; Britten 1986; Vawter and Brown 1986) do not appear large enough to pose a problem for this method and, in any case, the maximum-likelihood method is believed to be insensitive to rate variations (Felsenstein 1981).

The two bat sequences were first aligned with the others using the ALIGN program in the GENEUS package (Taylor 1984; Harr *et al.* 1986) and then linked together for the phylogenetic analysis. The

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                    #         #         #         #         #         #
BAT       1 CCCCCCTAAATCCCCTGGGAGTTCCACTCCTAAATACATCAGTCCTATTAGCCTCAGGCG
            **** ********** * *** ********** ***** **  ** * ** ***** *
HUMAN  9571 CCCCGCTAAATCCCCTAGAAGTCCCACTCCTAAACACATCCGTATTACTCGCATCAGGAG
             *** ***** ****************** ** ***** ** **  ** * ** ** ****
COW    9334 ACCCACTAAACCCCCTAGAAGTCCCACTGCTCAACACCTCTGTCCTATTGGCTTCCGGAG
             *** ***** ***** * *** ***** ** ** ** ** ******** ** ** ** *
BAT       1 CCCCCCTAAATCCCCTGGGAGTTCCACTCCTAAATACATCAGTCCTATTAGCCTCAGGCG

                    #         #         #         #         #         #
BAT      61 TATCAATTACCTGAGGACACCACAGCCTAATAGAAGGTGATCGCAAGCCCATCGTACATC
            ******* *******  ***** ** *********    * ** ** *  **  * **
HUMAN  9631 TATCAATCACCTGAGCTCACCATAGTCTAATAGAAAACAACCGAAACCAAATAATTCAAG
            * ** ** ******** ** ****** ********    ******* ** *** * ****
COW    9394 TTTCTATTACCTGAGCCCATCATAGTTTAATAGAAGGGGACCGAAAGCATATATTACAAG
            * ** **********  ** ** **  ********** ** ** ****  **  ****
BAT       1 TATCAATTACCTGAGGACACCACAGCCTAATAGAAGGTGATCGCAAGCCCATCGTACATC

                    #         #         #         #         #         #
BAT     121 GTTTATTCACCACGATCCTTCTAGGTGGCTATTTTACCCTACTTCAAGCCTCAGAATACT
               *  * *  ** **  * ** ***  ************ ** ********** ****
HUMAN  9691 CACTGCTTATTACAATTTTACTGGGTCTCTATTTTACCCTCCTACAAGCCTCAGAGTACT
            * **  **** ** **   * * **  **** ** ** ** *************** ****
COW    9454 CCCTATTTATCACCATCACATTAGGAGTCTACTTCACACTACTACAAGCCTCAGAATACT
               **** * *** ***    **** * *** ** ** ***** ******************
BAT     121 GTTTATTCACCACGATCCTTCTAGGTGGCTATTTTACCCTACTTCAAGCCTCAGAATACT

                    #         #         #         #         #         #
BAT     181 ACGAGGCCCCGTTCACAATCGCAGATGGGGTATATGGGTCAACATTCTTTGTAGCTACCG
             **** * ** ***** **  * ** **  * ** ** ******** ******** ** *
HUMAN  9751 TCGAGTCTCCCTTCACCATTTCCGACGGCATCTACGGCTCAACATTTTTTGTAGCCACAG
              **  * ** ** ** ** ********  * ************ ******************
COW    9514 ATGAAGCACCTTTTACTATCTCCGACGGAGTTTACGGCTCAACTTTTTTTGTAGCCACAG
            * ** ** ** ** ** *** * ** ** ** ** ** ***** ** ******** ** *
BAT     181 ACGAGGCCCCGTTCACAATCGCAGATGGGGTATATGGGTCAACATTCTTTGTAGCTACCG

                    #         #         #
BAT     241 GATTCCATGGCCTTCATGTAATTATTGGATCC
            * ***** ** ***** ** ******** **
HUMAN  9811 GCTTCCACGGACTTCACGTCATTATTGGCTCA
            ********** ** ******** ***** **
COW    9754 GCTTCCACGGCCTCCACGTCATCATTGGGTCC
            * ***** ***** ** ** ** ***** ***
BAT     241 GATTCCATGGCCTTCATGTAATTATTGGATCC
```

Fig. 2. Primary strand sequences of the cloned *Pteropus* sequences ('BAT'), compared with the homologous bovine ('COW') and human ('HUMAN') sequences: clone SSBam5, with homology to bovine positions 9334–9606 (within the CO III gene).

analyses were performed using the DNAML version 3·01 (maximum likelihood) and DNAPARS version 3·0 (maximum parsimony) programs in the PHYLIP package (Felsenstein 1985). We used the average base frequencies for the four mammalian sequences for the maximum-likelihood analysis, and found by trial and error that transition–transversion ratios between 0·6 and 1·0 maximized the likelihood function.

Results

The two sequences from the CO III gene are shown in Figs 1 and 2; one has a small flanking section of the more rapidly evolving ATPase-6.

The two phylogenetic analysis methods yielded the same three trees as the most likely of the 15 possible rooted bifurcating trees using *Drosophila* as the outgroup (Fig. 3). Using the likelihood-ratio test as a conservative test of significance between trees (Sokal and Rohlf 1981: 695) shows that the two best trees (upper left in the figure) are not significantly different from each other but are significantly better than all the other trees. Neither of these trees links bats and humans, clearly rendering implausible any hypothesis of a close relationship between fruit bats and primates.

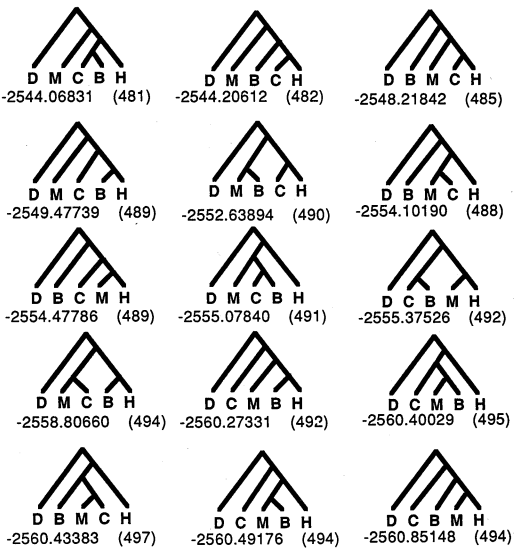

Fig. 3. The 15 bifurcating trees derivable from the data in Figs 1 and 2 analysed with the homologous *Drosophila* (D), mouse (M), cow (C) and human (H) sequences, using D as the outgroup and designating the fruit bat as B. The two bat sequences were aligned with the other sequences using the ALIGN program in the GENEUS package (Taylor 1984; Harr *et al.* 1986) and linked together for phylogenetic analysis. The analyses used both the maximum-likelihood (DNAML) and maximum-parsimony (DNAPARS) methods in the PHYLIP package (Felsenstein 1985). The natural logarithm of the likelihood and the number of steps under parsimony analysis for each tree are shown.

A frequent conclusion from protein sequence studies (e.g. that of Miyamoto and Goodman, 1986) has been that the mouse is closer to humans than is the cow. Our result contradicts this placement, but is consistent with some of the minimal trees found in other protein sequence studies (Penny *et al.* 1982; Penny and Hendy 1986), with globin gene sequence analyses (Smith *et al.* 1985), with conclusions from other mtDNA sequence studies (Hasegawa *et al.* 1985), and with the greater similarity of the cow than the mouse to human linkage relationships (Womack and Moll 1986). Wyss *et al.* (1987) stress the great current uncertainty from protein studies concerning this question, which we believe will probably be resolved using DNA sequence data.

We have not directly tested Pettigrew's conclusion of bat diphyly, which suggestion remains worthy of testing given the great immunological differences between the major bat groups (Cronin and Sarich 1980), although it should be noted that these authors regard bat monophyly as firmly established. But the logical basis of Pettigrew's proposal seems to rest largely on the supposed primate affinities of megabats, which our results contradict. Pettigrew's observation of the likelihood of convergence remains valid, but applicable to the

visual pathway and not to limb morphology, as noted by Martin (1986). Perhaps arboreal life (primates) and night flight without echolocation (megabats) impose similar demands on sensory capabilities.

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