Flying Primates: 25 years on.
Jack Pettigrew

Flying Primate Update:
It is 25 years since I published the first paper, in *Science*\(^1\), on the “flying primate” hypothesis, so I thought that it might be appropriate to post an update.

One would be hard put to find any scientist these days who is prepared to give the hypothesis much credence. An exception would be my colleague in South Africa, Paul Manger, who has painstakingly put together a strong case for flying primates from independent sources in the various modulatory brainstem nuclei, which show that megabats have a fundamentally primate brain compared to the insectivore brainstem of microbats.\(^2,3\).

The reason for the unpopularity of the thesis is the weight of DNA evidence that appears to refute it\(^4\). However, molecular biologists working on bat DNA seem to have overlooked the possibility that DNA can also undergo convergent evolution, like the convergent scenarios in brain and skin which are repeatedly put forward in discussions about bat evolution\(^5\). There are a large number of characters supporting each side of the argument, but it has to be admitted that those linking megabats and microbats (“bat monophyly”) are most easily explained by convergent evolution that has been driven by the powerful and all-encompassing selection pressure that accompanies mammalian flight. In addition to all the physical changes to morphology that are required for aerial, powered flight, unique metabolic changes take place, such as the increased temperature that follows the 20X increase in energy output\(^{16a-c}\).

Birds have the most highly modified genome of any vertebrate. For example, birds have 4 H isochoresses compared to the 3 found in mammals. H isochoresses have elevated levels of G+C content, with avian H4 having 100% G+C that is so “sticky” that conventional DNA sequencing is impossible. These changes have evolved in response to elevated temperature and metabolic rate.

Metabolic demands seemed to have resulted in genomic changes in both birds and bats (In support of the idea that high metabolism causes genomic modifications, a BLAST search of a bat mtDNA sequence can turn up a bird sequence amongst the homologues!). Such widespread consequences of flight can easily be overlooked by enthusiasts of bat monophyly, who have sometimes used them as dubious claims for uniting megabats and microbats phylogenetically. Take the case for hooked milk teeth. These extraordinary prehensile teeth are found only in neonatal microbats and megabats, but they are not unequivocal evidence for monophyly because they could easily have been invented independently in the two kinds of bats to improve the neonate’s purchase on the teat of a highly mobile mother. Detailed morphology of the hooked teeth from the two kinds of bats supports this interpretation because there is strong evidence of convergence in the form of many differences, including the structure of the hook. Another example is the genome size reduction associated with flying birds. Bats have a genome size reduction that is often taken as a shared derived trait supportive of monophyly. The details are actually just as likely, perhaps more likely, to have been the result of convergent evolution of this flight-related feature. Since convergence is consistently overlooked in this way by
proponents of bat monophyly, it would not be surprising to find that an even less obvious candidate has been overlooked…..DNA itself.

I deal below with the convergent evolution of DNA in the two kinds of bats that I think has been falsely used to argue for their monophyly. But first I summarise the evidence for “flying primates” that has continued to accumulate steadily in many systems, including molecular studies that do not involve DNA sequence with which they conflict, a problem with the DNA evidence that is not generally discussed. It is as if DNA is somehow superior in this controversy, despite the rare, but well-documented, examples where DNA is actually more misleading about phylogeny than other molecular evidence. The alternative molecular evidence forms a coherent, meaningful, interlocking biological whole that contrasts with the somewhat unidimensional DNA sequence data whose phylogenetic proposals have not so far informed us much about the biology of bats. Taken in isolation from other data, DNA sequence data can support preposterous associations, like the rhinolophoid-megabat link that DNA proposed for the first time. This link is an unlikely union of megabats (large-brained, highly visual, phytophagous, short-metacarpal wings etc ……54 differences) with the most widely different group of microbats (rhinolophoids are small-brained, non-visual, the most acoustically-specialised animals on the planet because of Doppler shift compensated echolocation, insectivorous, long metacarpal wings and more than 50 other differences), while excluding the remaining majority of the microbats. This DNA-based association has no other support from anywhere within the biology of bats. The puzzling connection is just one of many DNA results that are the likely result of convergent evolution in the DNAs of two unrelated flying mammals with similar, high metabolism. The effect may be exaggerated in this case because rhinolophoids and megabats both lack torpor in most species, so that the life-time integral of high body temperature would not have been relieved intermittently as it would have been in bat species that go into torpor.

Limitations of DNA evidence: DNA has been enormously helpful, and convenient, in the vast majority of phylogenetic reconstructions, but are the current tools sharp enough to reveal all of the traps, like the gross errors committed by DNA in rare cases like Dictyostelium and Amphioxus detailed below. Should we put all of our weight behind this method in the rare cases when its results conflict with every other approach, and when it fails to illuminate reliably even a single feature of natural history? In the case of bat phylogeny, DNA even conflicts with other kinds of molecular data. The fact that protein sequence data support “flying primates” while nucleotide sequence data from the same genes do not\(^7\), can be taken to mean that there is a problem accepting DNA data from bats without qualification. It is becoming clear exactly what this problem is, as I show here\(^17\).

Consolidating the Brain Data that support “Flying Primates”. Recent reviewers have tended to ignore the main points of my recent submissions and instead trot out old arguments that make one wonder whether they had even read the manuscript in hand. One such tired old argument posits that the striking similarity between complex details of the retino-tectal wiring of megabats and primates is a case of convergent evolution. In trying to assess who is right about a “he said, she said” argument of convergent evolution used by both sides, one needs to understand both the details of the proposed convergence (there should be differences between the two cases if they are really independent, like the reversed order of processing in the retinas of the convergent simple eyes of cephalopods and vertebrates),……. As well as the selection pressures operating to bring about the convergence. Bob Martin was one of the first to put the view that the primate and megabat midbrain patterns were convergent, arguing that the unique midbrain decussation of primates had also arisen in megabats because both groups of mammals were highly visual and were subject to similar selection pressures in the “fine branch niche”. I never found this argument compelling because the megabat wing precludes much dextrous interaction with fine branches, but it is possible that megabat ancestors were dextrous in this way before the wing evolved. Martin’s interpretation is made very unlikely by the colugo, which also has the hemi-decussated retinotectal pathway\(^9\), but certainly does not live in the fine branch niche, as anyone will attest who has heard it in the forest, crashing rather clumsily into trees during its long nocturnal glides. I have already detailed the many derived features shared between colugos and megabats that support a colugo-primate-megabat
One of these is behavioural, an aspect of phylogenetic reconstruction that is now usually ignored, despite its success with bird phylogeny in the hands of Konrad Lorenz. When colugos and megabats defaecate, they reverse the usual upside-down posture to hang demurely by their thumbs, a posture that contrasts starkly with the “slice” executed by microbats in the dorsiflexed position without releasing their feet from the perch. DNA now supports the predicted link between the colugo and primates that we first showed with brain data. DNA does not reveal the predicted specific between megabats and colugo. This partial failure of the prediction from “flying primates” can be possibly be explained by the highly modified DNA of bats, which would have distanced megabats even further from the 86 Mya split between colugo and primates. It has only been with some difficulty and controversy that the very distant DNA s of colugo and primate have been established as sisters, so it would not be surprising to find that metabolism-modified DNA of megabats was still out of reach. If it is accepted for the moment that the megabat genome has been highly modified during millennia of high temperature metabolism and so can be an unreliable way to check relationships with more normal genomes like primates and colugos, phylogeny seems to be a much better way to account for the presence of the decussated retinotectal pathway in primates, colugos and megabats than does convergent evolution. Sharper DNA tools for the detection of convergence may enable a better test of whether DNA from two unrelated flying mammals has converged, or whether the brains of megabats and primates have converged.
Brain Data First Confirmed Primate Status of Colugo:
The colugo, or gliding “lemur”, has played an important role in the current debate as it fits all the necessary criteria for it to be the progenitor of megabats, including a Paleotropical distribution. For example, colugos are the only living gliding mammals to have a gliding membrane, or patagium, that encloses all the digits of one limb so that it would have been a fairly simple development to have been converted into a bat patagium. The colugo’s niche in the abundant leaf-world of the canopy also makes ecological sense if it is not to compete for fruit with its hypothetical megabat descendants, so explaining why it is has not progressed from the gliding mode to flight after the estimated 86 million years since its divergence from the primate lineage.

Because early anatomists placed the colugo, alongside megabats, in the primates, the colugo provides an independent line of evidence for the flying primate hypothesis.

Colugo phylogeny has always been controversial when based upon the DNA evidence. In view of the arguments here that bats have highly modified DNA as a result of millennia of mutational pressure from high levels of aerobic metabolism, we should perhaps not expect ringing support from DNA for a megabat-colugo association. In fact, the molecular evidence is also quite contentious surrounding the claim that the Dermoptera (a mammalian order containing a very divergent group of colugos) is the closest sister group to primates. It is noteworthy that the tarsier, which no one doubts as a primate, often appears outside the primate+colugo clade. This supports my own argument that the tarsier is not an anthropoid, but branched off at the base of the primate tree and so can require care to align precisely with primates. Tarsier and the colugo share a unique arrangement in the 6 layers of the lateral geniculate nucleus that is not seen in any other primate, a brain trait that tends to cement the colugo-primate link. Some studies have claimed that the tree shrews should occupy that sister branch instead of the colugo, but the DNA evidence for the primate link is strongly supported by these much older data from brain and behaviour. Colugos have the highly specific pattern of connections between retina and midbrain found otherwise only in primates and megabats and there is a suite of behavioural traits that uniquely link megabats and colugos, such as motor patterns for terrestrial locomotion and defaecation. Incidentally, these are traits absent from microbats, who have complex, but completely different, patterns.

It is likely that even small mutational biases could present challenges for DNA-based phylogeny when divergences are as
old as the colugo-primate split at 86 Mya. So we might predict that this difficulty would be even greater if megabats came off the colugo lineage, and so would have a comparable divergence time which would then be exaggerated by a much more dramatic mutational bias related to flight. The fact that brain and behaviour give clear support for the colugo-primate link, when the DNA evidence was contentious and only now reaching acceptance, gives some hope that the megabat-primate link based upon brain data will eventually be vindicated by DNA also when there is better understanding of such mutational biases.

Brain Stem Executive Systems of Bats:
The argument of neural convergence in unrelated mammals that are all subjected to the selection pressures of the “fine branch niche” can be completely side stepped by studying other complex neural systems that are not part of the visual system. The widely-arborising brainstem systems involved in high-level executive functions use a variety of different neurotransmitter/neuromodulator molecules that aid in their characterisation in the lab. Such systems have been shown to have similarity in the number, location and morphology of their nuclei when the comparison species are related phylogenetically, but are so complex overall that similarity by chance or by convergence is not possible when the taxa are unrelated. Each of these systems has a relatively small number of neurons that contrasts with their extraordinarily widespread arborisations, that reach every part of the brain. They are therefore thought to be involved in executive functions involving their concerted action on many regions of the brain, as might be expected if they were associated with emotion, for example. In the formulation of Seymour Kety, some of these systems are involved in the detection and signalling of the timing of events of great survival value, such as hunger, thirst, sex and danger, with the system playing a role in communicating such times to the brain so that it can take action (e.g. by storing information that might be useful during a recurrence). Paul Manger and colleagues in South Africa have studied four such systems using antibodies specific for Acetyl Choline, Dopamine, Serotonin, Orexin etc. From the phylogenetic point of view, the results are quite clear cut. All the different brainstem systems of megabats are virtually identical to those of primates. In contrast, microbat brainstems are very different, and most similar to insectivores, sensu latu. Because insectivores are polyphyletic, with possibly as many as four separate lineages, present work involves the investigation of many different insectivores to see if one lineage is closer to microbats. These brainstem data strongly support the earlier retinotectal data that first led to a reformulation of the flying primate hypothesis (whose history is old, going right back yo Linnaeus). Both independent sets of data make it impossible to ignore the possibility that megabats represent an early branch of the primate lineage that has evolved flight independent of the invention of flight by microbats.

Bats Giving a Bad Name to Phylogeny from Brain Characters?:
One of the unfortunate consequences of the “flying primate” controversy is the cloud it has cast over the use of brain characters to reconstruct phylogeny. If well-polarised characters with well-understood distributions like the primate pattern of retinotectal decussation appear to fail, it casts a pall over the use of brain characters in general.

A Microbat Sister Group from Brain Organisation:
There is overwhelming evidence for derived brain characters shared between megabats and primates. But the flight-related characters shared between megabats and microbats tended to offset the "flying primate" hypothesis because there seemed to be no clear sister group of the microbats that could distance them from megabats.

That has changed now, as a result of a detailed study of "Insectivores".

It is well recognised that insectivores are polyphyletic, with Afrotherian insectivores such as the Golden Mole having no special relationship with the shrews, for example. Instead this analysis, only the soricid shrews shared derived brain characters with microbats, not Afrotherian insectivores or hedgehogs. Soricids have primitive kind of echolocation that is
not seen in other insectivores, suggesting that this may link soricids to microbats.

Nervous system characters have long history of illuminating phylogenetic questions because of their diversity and stability. In the present case, such characters are unambiguous in separating the megabats from microbats, but also in providing plausible sister groups for each, with the microbats aligned with soricid insectivores and the megabats aligned with primates.


Visualising the Primate Retino-tectal Decussation:
It can sometimes be difficult to present neurobiological data in the same striking way as it is first experienced and collected in the lab. This is partly true of the primate pattern of retinotectal decussation. In contrast, new anatomical tracers can give a cogent picture that requires no further additions or qualifications, as seen below for the retinotectal decussation of the megabat, *Rousettus*.

![Image of retinotectal decussation](http://www.uq.edu.au/nuq/jack/Update.htm)

Different coloured fluorescent tracers had been injected into each tectum and the retinotectal ganglion cells so labelled by retrograde transport. The pattern of labelling shows a vertical separation around the midline, of opposite colored neurons, representing the opposite midbrain tecta. This provides incontrovertible evidence for the specialised, partially-
decussated retino-tectal pattern of connections in *Rousettus*, in contrast to the less direct interpretations of the neurophysiology initially used to define this primate trait and the errors that can mask the specialised trait, such as damage at the injection site in temporal retina that is responsible for the ipsilateral pathway. *Rousettus* thus conforms exactly to the primate pattern. A number of technical errors explain why this species of megabat was at first thought to be an outlier from all the other megabats studied. The new data obtained with this labelling method shows, graphically and without argument, that *Rousettus* conforms to the primate pattern of retinotectal connections, like all other megabats studied. (from Pettigrew et al. 2008 pdf)

**Convergence of DNA:**
Schoolboys know that one can readily place vertebrates within their phylogeny using gross features of their brain. An avian brain is easily recognisable for what it is and quite distinctive from a mammalian brain, for example. For this reason, it is hard to believe that the myriad details of modern neuroscience cannot be as generally useful in phylogenetic reconstruction as they are in specific examples, such as electric fish, where they have proved very useful. Rather than questioning the use of brain in phylogenetic reconstruction, it is now time to look closely at DNA in those cases when it has become an outlier that is in conflict with a wide variety of other methods for phylogenetic reconstruction. In the case of bats, virtually every other method is in conflict, even other molecular methods. So there is good cause to examine the possibility that bat DNA has undergone convergent modifications that have misled us into thinking that the two kinds of bats have similar DNA by virtue of common lineage, rather than by virtue of convergence as a result of similar, high metabolism life style.

Since it is possible to clear up the confusion caused by the erroneous conclusion that one species of megabat is an outlier that fails to conform to the primate pattern, a major question then arises concerning the overwhelming body of DNA sequence data that conflict with the flying primate hypothesis by linking microbats and megabats more closely than megabats and primates. Despite the widespread use of DNA for phylogenetic reconstruction, it is not uncommon for conflict to arise between the DNA phylogeny and phylogeny of the same taxa given by other kinds of data. Only rarely is this conflict explained, but I can show here that there is a plausible explanation for the conflict between the DNA evidence and all the other evidence, except for the flight-related characters. In fact, a simple statement of this thesis is that the DNA characters linking megabats and microbats are also flight-related, not evidence for a phylogenetic connection.

1. Conflict between Protein-sequence and DNA-sequence phylogenies of bats:
One indication that there is a problem with DNA-based phylogeny of a taxon is provided when there is a conflict with protein sequences translated from the same data. This may seem odd, since DNA sequence determines the protein sequence, but a DNA codon has more room to deviate or “wobble” without changing the encoded amino acid, and such deviations can sometimes be extreme enough to distort the inferred phylogeny. My favourite example of this is the slime mould *Dictyostelium*, which is a eucaryote with a very large body of DNA sequence available, as it is a model system for molecular biologists. Many megabytes of DNA sequence say unequivocally that *Dictyostelium* is a prokaryote, aligning with one branch of the Bacteria! If instead, one translates the DNA into amino acid sequence, *Dictyostelium* now moves 3-4 major nodes up the tree of life to align with yeasts and other eucaryotes with which it belongs, rather than with prokaryote bacteria. DNA has obviously made a mistake, but can that be explained? The likely explanation is that this DNA is highly modified, with one of the highest A+T contents (85%) in biology. The prokaryotes whose sequence “attracts” *Dictyostelium* also have high A+T content, with the result that there are coincidental A or T substitutions that mislead the reconstruction into the association with bacteria that also have a high A+T.12

Another example where DNA is misleading about phylogeny and where protein sequence data gives a more accurate phylogeny concerns the mitochondrial genome of Amphioxus, the cephalochordate lancelet. DNA gives a tree where Amphioxus lies outside the echinoderms! While echinoderms are an accepted sister taxon to cephalochordates, it is a
complete departure to suggest that Amphioxus should lie even further away. By examining the amino acid sequence, the investigators showed that Amphioxus lay with other cephalochordates, as expected.

We obviously need some other source of information to distinguish coincidental substitutions from the shared substitutions that genuinely indicate a phylogenetic relationship. Translating the DNA codons into amino acid reduces the problem and gives a phylogeny that is closer to reality, but note that an extreme bias in DNA can also lead to a bias in translated amino acids that can also distort phylogenetic reconstruction, although not to the same degree as with DNA.

I have pointed out this problem repeatedly in the past with respect to bat phylogeny, but have not often been taken seriously. In response to my critical comments, lip service is sometimes paid to the problem of base compositional bias, as if this will deal with the general problem of DNA convergence.

Try this thought experiment: You have two identical DNA sequences that have come from different organisms that are not thought to be related. How can one distinguish between the two following alternatives? 1. That the two DNA molecules are similar by evolutionary convergence: 2. That the two DNA molecules share a common ancestor with the same sequence. The answer is that it is logically impossible to make the distinction on the basis of a comparison between the two molecules alone. More information is required about the evolutionary context.

Bernardi and colleagues are the leaders in understanding the evolution of the molar properties of DNA, having discovered the isochore phenomenon. Their lab has provided evidence for gene conversion, in evolution, of high A+T regions into high G+C regions. Such a process would vitiate attempts to reveal it by a simple-minded measure of base composition. I have recommended that all DNA-based phylogenies be tested against a protein-sequence based phylogeny, a simple matter to undertake by translating the DNA sequence. I am not aware that this has been done by any of the recent proponents of DNA-based phylogenies of bats. Their reluctance can be understood, both because of the inevitable conflict that arises between the two sets of sequences, from microbats and megabats, and also because there may also be slight differences between the amino acid sequence assumed from the DNA sequence and the actual amino acid sequence of the in vivo protein. However, the basic test, phylogenetic reconstruction with both DNA and translated amino acid sequence, can easily be carried out as a first step on the abundant sequence data available. In every case so far, when this test is carried out, there is a mismatch between the DNA-based and protein-based phylogenies that conflicts with the hypothesis of bat monophyly. A quantitative measure can be derived from the DNA sequence data that explains the mismatch and predicts the degree of convergence. This quantitative measure of DNA convergence is greatest for just those controversial associations, such as the megabat-microbat association. The greatest measure of convergence is seen for the rhinolophoid-megabat association, which is not supported by any data set and which most investigators of bats have found unlikely, but which have members with very high temperatures over time because they lack torpor and may therefore have independently undergone the most extreme DNA modifications (see below).

If one takes the published amino acid sequences from bats, leaving aside for the moment inferences about amino acid sequence based on DNA sequence, all fail to yield a phylogeny of bats that is consistent with the phylogeny given by DNA. This is true for the following proteins: - prestin, haemoglobin, foxp2, opsin, rhodopsin. FoxP2 is of special interest. First, it does not support bat monophyly and instead, clearly gives the flying primate phylogeny. Second, it comes from the H3 isochore where G+C content is highest and the A+T mutational bias is least, suggesting that a more reliable phylogeny emerges when one avoids DNA sequence that is high in A+T content. I made this suggestion previously, when I showed that support for bat monophyly from four DNA sequences was based upon a 4:1 bias of A+T substitutions. If these were ignored and the phylogenetic reconstruction instead carried out using G+C substitutions, the same sequence data support the flying primates hypothesis!

While some of these protein sequences are consistent with flying primates, this is not a realistic expectation, even though
they all fail to support monophyly. This is because the amino acid sequences are not completely immune from the genomic modifications affecting the DNA. For example, genes with a high concentration of A+T also have more amino acids in the FYMINK group (the six amino acids that have codons with a bias to A or T). The amino acid phylogeny may not be as distorted by peculiar events in genomic evolution as the DNA phylogeny, but failure to get the same tree using DNA and protein sequence translated from the DNA sequence should be ringing alarm bells.

A Quantitative Approach to DNA Convergence:
There are different approaches to the detection of a convergence. In the classical case of the simple eye of vertebrates that has converged on the simple eye of cephalopods, even without knowledge of the completely independent vertebrate and invertebrate lineages neuroscience spotlights the convergence effortlessly by virtue of the inside-out arrangement of the retina of one compared to the other. Such specialist knowledge may not always be available. For example, there is still much to learn about the DNA changes that occur following genomic evolution, such as isochore formation, conversion to G+C bias of A+T base composition bias, etc. We therefore do not know enough about these modifications to be able to detect differences between microbat DNA and megabat DNA that could reveal convergence, even if they are present. A more general method for detecting convergence involves phylogenetic reconstruction that reveals the converging taxa by virtue of their wide separation on the tree compared with the contrasting closeness given by their similarities. In the case of the independent evolution of the two simple eyes, abundant information places the vertebrate eye on a branch that is widely separated from the cephalopod branch despite the closeness implied by their similarity.

These days, a major source of phylogenetic information comes from DNA, so how can one use it to detect convergence of DNA itself? I have already alluded to the many problems that could be explained by convergence of megabat and microbat DNA, such as the shared high metabolism of microbats and megabats, the mismatch between their protein and DNA phylogenies, the dozens of differences in brain and behaviour except for flight-related characters etc. But how could this convergence be measured?

Ultrametric Geometry of DNA distances:
The evolutionary distance between two DNA molecules can be measured in two ways:-- directly, by counting the differences, and indirectly by estimating the distance using other related DNA molecules.

Imagine that you are part of a team of four, A,B,C,D, scattered around a field and each armed with a laser positioning device that enables all six distances between team mates to be measured (AB, BC, AC, BD, AD, CD). Now suppose that it appears that one of these distances, CD, has been compromised, perhaps by a small obstacle that interferes with line of sight between C and D. Even without the direct CD measurement, it can be estimated from the geometry of the other five measurements that include C and D. This is slightly nasty geometry, but it is otherwise computationally straightforward and leads to a precise value for the questionable sixth measurement using the other five measurements . The same ultrametric geometry can be used to calculate a sixth questionable DNA distance from the other five distances when you want to know about the relationship of four DNA molecules. I used it to make an indirect measurement of the distance between two DNA molecules from their distances to two other molecules and to compare this with the actual distance. When I did this for hundreds of comparisons, the estimated and measured distance were the same (within a percent or two), as expected, but not for bats (which could be more than 50% different!) . The rhinolophoid-megabat distance showed the biggest anomaly, with the actual measured distance less than half of the distance estimated using the other nearby taxa. This is the signature of convergence, as the taxa appear to be much closer based directly on DNA sequence than was indicated by the geometry of DNA sequence information from the surrounding, less- biased, phylogenetic context.
Quantitative Evidence for Convergent DNA is Most Obvious in the Megabat-Rhinolophoid Microbat Comparison: Note that the anomaly was greatest in the same pairing that is probably the most questionable of all the recent proposals from DNA sequence studies of bats. Incorporating the larger, calculated rhinolophoid-megabat distance into programs for phylogenetic reconstruction, such as PAUP and Maximum Likelihood, eliminates this bizarre association as well as failing to provide any support for the monophyly of bats.

Table 4 shows these effects in some real measurements from bats. Note that Estimated and Measured distances between a variety of mammal DNA (not shown) are usually equal, as expected. But when the comparison is between a megabat and a microbat DNA (as in the Table, where the comparison bats are the megabat, *Pteropus*, and the microbat, *Rhinolophus*), the Estimated distance, calculated from ultrametric geometry using neighbouring taxa, is much greater than the actual Measured distance. This is true for many genes, as shown in the Table and conforms exactly to expectations.
of convergent DNA. In other words, the DNA of microbats and megabats is similar in a way that cannot be accounted for their phylogeny. The similarity is thus probably a result of the similarity between selective pressures that have been operating on the evolution of both kinds of bat DNA because of the high temperatures and metabolic rate of powered flight.

**Table 4:**

**Magnitude of the Error of the Rhinolophus-Pteropus Distance.**

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Japanese Group Also Find DNA Convergence in Bats: A comparable approach to detect DNA convergence has been developed by another lab in Japan\(^1\), except that in this case they were able to make the indirect estimate of distance with the help of a sophisticated computer algorithm that permitted a much larger number of pairs than the total of four that I used to simplify the calculation. Their aims and conclusions were the same as mine, with dramatic changes in the constructed phylogeny of bats when DNA distances corrected for convergence were used. They explicitly proposed that their approach could be used as a test for convergence between DNA molecules and found strong evidence for this in bat DNA.

This approach might find wider application in making fine adjustments to distances of other taxa than bats, but I never saw such big discrepancies between the measured and estimated distances as I saw for bats. This is presumably because bat DNA is subject to a unique selection pressure—sustained very high temperature (flight increases bat body temperature by 5 C deg, or ~14\%)\(^{16a-c}\) that is known to lead to the evolution of complex widespread genomic changes such as the isochores described by Bernardi and colleagues. The exact details of these modifications remain to be worked out, but their effect in artificially shortening the measured distance between megabat and microbat DNA is a convergence that has happened independently in the two lines of bats, as we see from the large discrepancy between the estimated and measured megabat-microbat distances.

**Complete Genomes of Selected Bats:**
The evidence of convergent DNA that I have provided could be verified and elaborated using the complete genomes of some bats. This would be the best way to reveal the extraordinary genomic modifications of bats. For example, it has
been known for more than 30 years that megabats have the highest A+T content of any vertebrate. Despite this long history, little has been added to our knowledge of any features of genomic evolution, such as isochore evolution, that might accompany these dramatic changes in base composition of bats. Even if one discounts the possibility that megabat DNA and microbat DNA have converged, a possibility so extraordinary that it deserves definitive investigation, the evidence of extreme, bird-like, genomic modifications in bats warrants complete genome determinations in at least one species of megabat and one species of microbat.

There have been no proposals of this kind that have been followed up, despite the increasingly economical costs of whole genome determination. A wide variety of mammals now have complete genomes revealed, so one can ask why there has been such a delay in bats, where the possibilities for understanding the impact of temperature on genomic evolution are arguably more significant than in any other mammal. I wonder whether part of the problem is the smug acceptance of the DNA sequence on bats so far, that has the underlying assumption that no more needs to be done to test the alternative viewpoints on bat phylogeny, an assumption that I commonly encounter in reviews that are deaf to the arguments for bat genomic modifications.

Whole Bat Genomes:

Two years after I made the above plea, Science published side-by-side whole genomes of a microbat, Myotis, and a megabat, Pteropus. The emphasis in this paper is the remarkable, convergent, similarity that has evolved independently in various parts of the immune system of both kinds of bats under the selective effects of flight. This is an extraordinary finding, but does confirm my own prediction that the metabolic consequences of flight will impact the genome. The authors explain the result in terms of the increased need to repair bat DNA because of the increased level of damaging ROS produced by heightened aerobic metabolism. This unexpected convergent effect on the two immune systems is indirect and complex compared to the more direct oxidative effects of ROS that I have raised, such as greater sensitivity of the guanine base to oxidation compared to the other three bases, with a resultant misreading of 8-oxoguanine as adenine and increase in the AT content. There are more flow-on effects, such as C-conversion, elucidated by Bernardi's group that help to explain the wholesale modifications of bat DNA at the level of base composition.

If one uses just the published sequences of these convergent immune genes to erect a phylogeny, monophyly of bats is not supported because the gross similarities in the overall organization of the genes, such as the duplications and multiplications, are not mirrored in the pattern of SNPs.

This is an extraordinary example of the selective effects of increased metabolism. I do not think it was widely predicted that the increased load of ROS species in bats would have to be protected in just these immune genes. Despite this support for my prediction, I was disappointed that the bulk of the unannotated genes were given uncritically to phylogenetic algorithms that came up with party-line monophyly without bothering to check for convergence using any of the many possible tests. In other words, phylogeny was not tested using a comparison of the amino sequence phylogeny with the DNA sequence phylogeny using the same gene, a common source of conflict for many genes in bats, such as foxP2. Nor was DNA convergence tested using the quantitative checks detailed above.

If DNA convergence was found in completely unexpected immune genes, protected at some distance from the primary effects of increased metabolism and ROS, what about DNA that is closer to the firing line, like the dramatic changes in base composition that have been shown to accompany increased body temperature and accompany the DNA of both kinds of bats?

Earlier Whole Genome Studies of Bats:
Long before the development of high through-put DNA sequencing, whole genome studies of a number of megabat and microbat species has confirmed the foregoing arguments about DNA convergence using DNA-DNA hybridization. This
technique tends now to be superseded by sequence studies, but it does enable comparison of whole genomes, chopped into convenient fragments, that give a global measure of similarity between the DNA of 2 taxa. These studies confirmed earlier work that bat DNA, from both megabats and microbats, has a large mutational bias toward A+T. This can be explained by the greatly increased aerobic metabolism associated with flight and the particular sensitivity of DNA (and the base G in particular) to oxidation by ROS. Megabat DNA has a record A+T bias for mammals, but microbats are also subject to the same bias, with the rhinolophoids approaching the level seen in megabats.

The results showed that the greatest similarity between taxa (measured by melting point of the DNA--DNA hybrid) was seen between megabat DNA and rhinolophoid DNA, like the same anomalous association between these widely-divergent taxa seen in DNA sequence studies. This is a preposterous association that is not supported by a single fact from the biology of bats. (note: The reply to my assertion from enthusiasts about bat monophyly was very weak: viz:-"They are also united by residence in the Paleotropics". Well, what about those Paleotropical families of microbats that are not part of this paraphyletic arrangement that splits the microbats despite the evidence against it (e.g. Nycteridae, Craseonycteridae). The artifactual nature of the association was revealed by an experiment that is particularly-well suited to DNA-DNA hybridization studies of large fractions of the genome. Since high A+T DNA "melts" (separates into two separate strands) at a lower temperature than DNA with high G+C content, one can use this fact to produce fractions of the whole genome which are enriched for G+C. When such fractions are used, most comparisons between pairs of mammals are unchanged, but the close similarity between different bat DNAs disappears. Megabat and rhinolophoid DNA now retreat to be as far apart as any pair of mammals.

DNA hybridization studies on this large scale are no longer fashionable and the three papers on bat DNA are not often read, nor understood (judging by the misquotes). Nevertheless, they provide prima facie evidence for an artificially close distance between megabat and microbat DNA that is attributable to A+T bias.

References and Notes:


He said, She said Convergent Evolution:
The large number of derived characters that link megabats and primates, instead of being support for the flying primate scenario, are often written off as examples of convergent evolution, without any compelling explanation in terms of the similar selection pressures that must have been suffered by the primate and megabat lineages to bring this about. Moreover, it is difficult to find the small differences between megabat and primates wiring diagrams that should be apparent in unrelated but convergent lineages, like the opposite wiring of the retina that is found in the independent inventions of the simple eye by cephalopods and vertebrates. Convergent evolution is a key concept here, because it has been advanced by both sides of the argument about bat evolution. Since virtually all of the characters that link microbats and megabats are associated with flight, and since flight exerts massive selection pressure on both physical form and on metabolism, convergent evolution of two different flying mammals is a stronger argument than unexplained convergent evolution of the nervous systems and other anatomy of primates and megabats. Moreover, we find many detailed differences between the flight apparatus of megabats and microbats that conform to expectations about convergent evolution. For example, a simple measurement on the wing bones of both kinds of bats completely separates them, without overlap, strongly suggesting that they have separate origins despite the broad similarity that is required for function as a wing.

Bringing DNA into the argument about convergence is a relatively new idea that may bring the usual scorn and condescension from those who consider that the case for bat monophyly is sewn up and that convergent DNA is implausible. In fact, this idea has a long history going back to Alan Wilson, who showed that incautious use of molecular data to reconstruct phylogeny placed a leaf-eating monkey as a sister taxon to the cow! Wilson was very much aware of the trap of convergence, but there are many egregious errors in this field where an erroneous phylogenies have been seriously proposed. I pointed out one such error concerning the flying primate hypothesis, which was rejected on the basis of a subjective alignment of DNA sequence that ignored a more parsimonious alignment, with fewer gaps, that
supported the opposing, flying primate hypothesis. Such unconscious biases are common in discussions about bat evolution, where even the title of a paper can reveal them. “…..with convincing evidence for bat monophyly” is one such title where the authors reveal that they are convinced without putting the case to the readership, which is the usual body to decide whether the case presented is “convincing” or not.

There have been many studies of bat DNA. The earliest involved small fragments of DNA. Some of these appeared together along with a derogatory piece in Science. The timing of 4 studies suggested collusion between the investigators, who were all proponents of monophyly, but they all had the same embarrassing error which none had picked up. It was well known that bat DNA has marked compositional bias, with megabats having the highest levels of A+T known in vertebrates and some microbats not very different. All the substitutions claimed in support of monophyly had a four times as many A+T substitutions as G+C substitutions. This effect was replicated in all 4 studies and only served to reinforce what was already known about the compositional bias found in both microbats without providing any support for monophyly. A later computational study tried to pour cold water on this embarrassment for proponents of monophyly, but was unsuccessful because it proposed, but ultimately lacked, a model for the evolution of such a compositional bias.

The present-day exponent of bat sequence studies is Emma Teeling. With her colleagues she has increased the amount of bat sequence available by orders of magnitude and is a vigorous proponent of bat monophyly based on this large data set. Merely increasing the amount of DNA sequence available does not necessarily help, as shown by the studies of Amphioxus and Dictyostelium, where huge amounts of DNA merely served to confirm the error. In fact, the work on bat DNA is fundamentally flawed because it relies on a number of assumptions that may be approximately valid for most mammals but are certainly not true for bats.

All algorithms for reconstructing phylogeny from DNA sequence have problematical features that make them vulnerable in studies of bat DNA. First, they are based upon a proposed model of DNA evolution that may be appropriate for most mammals but not for bats. It is not easy to come up with a suitable model for the evolution of bat DNA, since it is complex and has been studied only cursorily, but the widespread genomic modifications of bat DNA show that a new one is needed. The extraordinary properties of bat DNA have been recognized for more than 50 years since Bernardi and colleagues showed that megabat DNA has the highest A+T content of any vertebrate. Microbats show a similar, if not so large, shift so one can imagine many coincidental A or T substitutions between megabat and microbat DNA that would be treated as support for monophyly by the algorithm. **Note that it is logically impossible to tell whether similarity of this kind between two DNA molecules is the result of common ancestry or the independent acquisition of increased A+T substitutions.** Some alternative approach would be needed to provide some phylogenetic context that could distinguish between these two possibilities, which is impossible from a consideration of the two sequences alone.

Teeling pays lip service to the issue of base composition by measuring it in each sequence, but this is not an adequate approach. First of all, despite her claims, most bat DNA sequences are biased toward A+T. In a survey of 15 genes that had been sequenced from bats, I found that 13 had elevated A+T (as high as 80% in GC3). The two remaining genes were from the H3 isochore and had very high GC (88% in vWF) that would pose a similar problem with coincidental substitutions to that posed by high A+T. Second, Bernardi’s group has shown that extreme A+T biases can be converted in evolution to C+G once a threshold has been reached. For this reason, convergent similarities between unrelated DNA molecules might be undetectable by a simple measure of base composition. If the approach is limited to the pair of most similar DNA sequences, as all present phylogenetic reconstruction algorithms are, there is no way to tell if they got that way from a common ancestor or have independently acquired the similarity……convergence of DNA in other ways. A second related problem is that construction of trees by the various algorithms always starts with the pair of taxa that are measured to be closest. I often asked the originators of these programs, like Walter Fitch and David Swofford, why there was no alternative that enabled the approach to a solution by starting far away from it, instead of with the closest pair. My motivation for avoiding the closest pair was my recognition that this would distort all later analysis if
the members of the starting pair were artificially close. Approaching the construction of the tree from a distance might avoid the problem of bias if the initial starting point is a pair that is artifactually close, but apparently there is no algorithm that works its way in from the distant pairs.


Many of Linnaeus’ identifications have survived down to the present day. It would be very interesting if he is eventually proved right about flying primates. It was the German anatomist, Winge, who provided new evidence from microbats, whose bizarre features were clearly not shared with megabats and led to the removal of all bats from primates, rather than the more appropriate excision, one might think, of microbats. Leche debated with Winge over the clear differences between the two kinds of bats, but in those early days of evolutionary theory, the battle lines were not as clearly drawn as they are today, so Leche and Linnaeus did not prevail. The late John Hill was curator of mammals at the Natural History Museum in London. Like his equivalent across the Atlantic, the late Karl Koopman, curator of mammals at the American Museum of Natural History, Hill had an encyclopedic knowledge of all bats. He told me that I was right about flying primates, having come to the same view many years before and expressed it quietly in his book. On the other hand, he told me that this view was not going to be accepted in my lifetime, an expression of the social aspects of the phenomenon along with the intense emotion that attends phylogenetic reconstruction where different viewpoints are possible in the absence of empirical testing. James Dale Smith (“Smitty”) is a brilliant student of bats who made an incisive hypothesis concerning flying primates in 1970, using different data and reasoning from my own in 1986. Smitty was an unusual American in that he had had direct contacts with megabats in the Western Pacific, where he described new species. There are no naturally-occurring megabats so Americans can be shocked when they encounter their first megabat and it looks them in the eye and treats them as another sentient, not like a post, the usual reaction of a microbat. Smitty was driven from the field by the mafiosi and now refuses to talk about it. Based upon my own experiences, I am guessing that he suffered exclusion from conferences, journals and books, engineered in a way to make it look as if he had declined to participate. In a famous, but unrecorded, debate between Smitty and Koopman, Smitty won on scientific grounds but lost overall because he was so irascible, as a couple of witnesses told me. Both Smitty and Karl had disabilities, but Karl apparently handled his in a gentlemanly way, the audience’ opinion in favour of the human aspect over the science further emphasizing Hill’s opinion about the social dimension.


Wing temperature in flying bats measured by infrared thermography

16 b. **Thomas SP* and Suthers RA**
J. Exp. Biol. (197a), 57. 317-335 317
The physiology and energetics of bat flight

16c. **Voigt CC and Lewanzik D**
Trapped in the darkness of the night: thermal and energetic constraints of daylight flight in bats

**17. Mutational Bias, Isochores and the “Genomic Jewel Box”:**
Every day we excrete large amounts of a waste nucleotide that comes from our DNA, but one that is not generally recognized. 8-oxoguanine is an oxidized form of guanine and its massive excretion is the result of guanine’s sensitivity to oxidation, compared with the other 3 bases. 8-oxoguanine is misread by DNA repair enzymes as adenine, setting up a “mutational ratchet” of ever-increasing A+T levels (the A inserted to replace 8-oxoG is matched by T). This oxidative effect mutates all living things but becomes dramatic in homeotherms where metabolic rate and oxygen transfer increase the effects of free radicals. Nowhere is this more significant than in bats and birds, where body temperatures and oxygen consumption reach high levels in flight. We still do not have a clear picture of how evolution has dealt with this insidious, never ending accumulation of excess A+T levels, but the work of Bernardi and colleagues gives a glimpse of the genomic reorganizations that have taken place to deal with it in the form of isochores, where large tracts of the genome are segregated into regions of differing base composition, from high A+T (isochores L1 &L2) to high G+C (H1, H2, H3 each with increasing G+C level). In birds, there is a fourth H isochrome (H4) where the G+C level is so high (100% in GC3) that the DNA is too “sticky” at the usual temperatures for polymerase sequencing, so that the older chemical methods at high temperatures are needed. The more extreme form H4 isochrome in highly-metabolic birds is consistent with Bernardi’s idea that isochores have evolved to deal with increasing levels of metabolism, such as the mutational ratchet imposed by oxidation of guanine.

There are many different ways that evolution may have tried to deal with the problem. Isochores are almost certainly a reflection of that. My own personal view is pictorial and may not be borne out exactly by future research, but gives the flavour of the complex changes that we might expect to have resulted in evolution to deal with the problem.

To protect itself against the worst effects of the mutational bias, evolution may have evolved a protected site where the effects of the mutations are minimized. I call this the “genomic jewel box” where vital genes are locked for safe-keeping, away from the mutation-ridden A+T rich regions, perhaps accompanied by specialized repair mechanisms that can deal with mutations more specifically when they occur. We know that vital, “house keeping” genes are located (“locked away”) in the most G+C-rich H isochores, for example. Kathryn Pollard and colleagues have shown another striking example, from the newly-evolved genes that have appeared since we diverged from chimpanzees, such as FoxP2 (which is associated with language). They find that all of these genes have high GC3 levels and so belong to the H3 isochrome. In other words, by some unknown mechanism, all these new genes are located in the H3 isochrome. Much work will be needed to sort out the mechanism of isochrome formation and the way in which important genes have come to live there, but we know a little. For example, DNA replication takes place first for the H isochores, before the L isochores, at a time when G- and C- nucleotide precursors are at their highest concentrations. There may be many other such features like this that contribute to the phenomenon, such as the spatial organization of the genome, which may protect H isochores from free radicals by surrounding them with absorbent material like A+T rich DNA. Another phenomenon is gene conversion, where a sequence that is badly riddled with too much A+T is converted to a version with a G+C bias instead.

I feel that a clearer picture of the isochrome phenomenon in bats would be useful for a general understanding of
the phenomenon. In the meantime, the organizational changes underlying isochores in all mammals are very relevant to the flying primate controversy. Although there has been no detailed study of isochores in the genomes of bats, we know that they are massively reorganized compared to other mammals. Megabat DNA has the highest A+T content of any vertebrate, for example, no doubt a direct result of evolution during high metabolism, and should therefore have the most differentiated isochore system of any mammal on that basis. Some details of the isochore organization are bound to be shared with equally metabolically active microbats, so an important task for the future is to determine whether this is explained best by shared ancestry, or whether it is a unique example of convergence, brought about by the demands of powered flight, that impacts on every aspect of the morphology, physiology and biochemistry of bats.