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Base-compositional biases and the bat problem. I. DNA-hybridization melting curves based on AT- and GC-enriched tracers

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We explored the interordinal relationships of mammals using DNA–DNA hybridization, with particular reference to the much-debated problem of whether the megabats and microbats are more closely related to each other than the megabats are to primates. To try to improve resolution when taxa are distantly related and the melting points of hybrids are low and difficult to distinguish, we increased the GC content of DNA by a fractionation method that used the same melting-point apparatus used in the hybridization studies. When we used GC-rich DNA as the tracer to make hybrids, the melting point of the self-hybrid shifted to a higher temperature as expected, but the behaviour of heterologous hybrids varied with the taxa being compared. When the melting point of the heterologous hybrid also shifted to a higher temperature so that the two compared taxa maintained the same or proportional distance, we called this 'following behaviour', because the heterologous hybrid made with GC-rich tracer 'followed' the GC-rich self-hybrid to higher temperatures. We also commonly saw anomalous behaviour, where the melting point of the heterologous hybrid shifted to a lower temperature when compared with an AT-rich hybrid. In these anomalous cases, the distance measured between the taxa increased markedly as a result of GC-enrichment, indicating that an underestimate of distance may have resulted from AT bias in DNA. This inference was supported by the finding that it was rare to observe a decrease in measured distance between taxa using GC-rich DNA, but very common to find an increase as would be expected from the generally higher AT contents of eutherian DNAs. Moreover, the most extreme cases, where distances changed most using GC-rich DNA, were usually those involving comparisons between taxa known to have the most extreme AT-biases among mammals, such as the megabats and rhinolophoid (including megadermatid) microbats. Our results show consistent underestimates of measured differences between eutherian taxa with extreme AT-biases.

Keywords: base-compositional bias; bat phylogeny; bat monophyly; molecular evolution; Megochiroptera; Chiroptera

1. INTRODUCTION

(a) *Bats: monophyletic or polyphyletic?*

Of the many unsolved problems in the interordinal phylogeny of mammals, the relationship of bats has special interest. To explain the origins of bats, clear phylogenetic alternatives have been offered that have strikingly different implications for the evolution of the nervous system or the musculoskeletal flight apparatus, depending on which alternative is finally accepted (see Baker *et al.* (1991), Pettigrew (1991*a*,*b*) and Simmons *et al.* (1991) for an introduction to these differing viewpoints). Did the megabats and microbats have a common flying ancestor (in which case the brains of megabats and primates have converged to a remarkable degree)? Or did megabats share a more recent common ancestor with primates that they did not share with microbats (in which case many flight-associated musculoskeletal characters have been derived independently in the two kinds of bats)?

One might object that the problem is not an interordinal one, because the megabats and microbats are both currently placed in the single order Chiroptera. On the other hand, if one of the alternative hypotheses (that bats are diphyletic) proves correct, then the megabats will have to be placed in, or with, a separate order. Moreover, it has been repeatedly observed that divergences within the tree of microbats are so deep that studying some microbat families is for all practical purposes like studying mammalian orders (Pierson 1986; Simmons 1998), with all the usual problems of resolving deep branches (Swofford & Olsen 1990). For these reasons, it is as well to keep in mind the possibility that we may be dealing with an interordinal problem in determining the origins of bats.

Although it is generally considered that megabats and microbats form a monophyletic clade, there is much room for doubt. In the current ferment that surrounds mammalian phylogeny, the notion that bats are polyphyletic is hardly more controversial than proposals that the 'Cetungulata' (a monophyletic clade containing carnivores, ungulates and cetaceans) is the sister group to primates to the exclusion of rodents (Janke *et al.* 1994), that tarsiers are not a valid sister group to anthropoid primates (Rosa *et al.* 1996), that the guinea-pig is not a rodent (D'Erchia *et al.* 1996) or that marsupials and monotremes are sister taxa

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to the exclusion of eutherians (Janke *et al*. 1996). Simmons (1994) avers, in a recent review of the evidence, that the problem of bat monophyly is a settled issue, adducing much data from comparative anatomy. However, no convincing solution has been (or can be expected to be) forthcoming on the basis of classic morphological data. This is because, as Pettigrew (1986, 1991*a*,*b*, 1995) has pointed out repeatedly, many of the characters cited in support of monophyly are highly correlated with flight and hence with each other. As an underlying assumption of parsimony analysis is that characters are (logically, functionally and mathematically) independent, the high consistency of trees based on anatomical features (and their uniform indication of bat monophyly) reflects only this same correlation and violation of independence rather than phylogenetic concordance.

A more decisive result might be expected from biochemical data, but to date only six genes have been sequenced in just a few bats (e.g. Porter *et al*. 1996), adding a possible algorithmic artefact (the attraction of long, undivided branches due to poor taxonomic sampling; Swofford & Olsen 1990); significantly, Simmons (1994) declined to place much weight on the limited sequence data.

(b) *Inconsistencies among molecular data*

Two recent DNA-hybridization studies, by Kilpatrick & Nuñez (1993) and Kirsch *et al*. (1995), also purport to support bat monophyly. If the rationale of DNA hybridization—that it averages differences over the entire single-copy genome—is viable, these studies are at least immune to the limitations of scanty or non-independent data; but, in common with other molecular studies, taxonomic sampling is still quite poor in the investigations using hybridization. In addition, there is reason to doubt the hybridization results based on the degree of self-consistency among the data.

DNA-hybridization data are distances, which can only be analysed by ultrametric clustering or best-fit algorithms. The latter programs (FITCH, neighbour-joining, etc.) require only that the data be additive: that the segments of any pathlength between taxa on the calculated tree sum to values equal to the measured pairwise distances, at least within experimental error. However, a little-appreciated fact about minimum-evolution algorithms is that any departures from additivity are (must be) forced away from terminal branches on to internal segments (Fitch & Smith 1982); the tree-fitted distances between terminal sister-species must then equal the measured values, although pathlengths joining other, non-sister taxa may be different to the corresponding measured distances. Biologically, there is no *a priori* reason to believe that departures from additivity should preferentially accumulate on deeper internodes (although saturation can contribute to this effect among lineages that separated at very early stages of cladogenesis). In fact, as we discuss below, there is some reason to think that most homoplasy among bats might actually accrue to interbat (more terminal) distances, especially between megabats and rhinolophoid microbats. Although algorithms do exist that otherwise distribute ill-fitting distances among tree-branches including the terminal segments (e.g. Wagner distance (Farris 1972)), these methods have not yet been applied to DNA-hybridization data.

In a small data matrix, a single distance shorter than the others may therefore have a determining effect on the overall shape of, and distribution of distances on, a best-fit tree. It may be helpful to give an example that illustrates the potential problem of such short distances between microbats and megabats. Figure 1 shows an abstraction from the data of Kirsch *et al*. (1995, table 6), corrected for asymmetry. To simplify the example, only four taxa are considered—an edentate (*Chaetophractus*), a primate (*Perodicticus*), a megabat (*Rousettus*) and a microbat (*Phyllostomus*)—providing six averaged, unweighted measurements relating the quartet of taxa. The distance of 43.6 separating the two bats is clearly the reason they are paired in a FITCH tree (figure 1*a*); however, the data are not additive (i.e. the unexplained sums-of-squares is 2.81 or 0.01% of the total sum-of-squares for the 4×4 distance matrix). Would a different interbat distance produce a 'better' (more additive) tree?

To test this possibility, we deleted the microbat–megabat distance and used the additive method of Landry *et al*. (1996) to estimate a new value, which was 58.9, much closer to the other five intertaxon distances. A FITCH tree from the matrix incorporating this estimated value (figure 1*b*) provides a perfect fit of the data (i.e. a sum-of-squares of 0; note, however, that the distances have been rounded to one decimal place in figure 1). This is expected, by definition; there are just four taxa, and we estimated the only 'missing' pairwise distance to be additive. However, the new tree pairs the microbat with the edentate, and the megabat with the primate.

Landry *et al*.'s (1996) method is not guaranteed to produce the correct topology when the estimated pairwise distance may involve a terminal sister-group relationship (see the discussion in Lapointe & Kirsch (1995)), but the point is that modification of a single distance out of six produces not only a different tree, but one that is (necessarily) a better representation (fit) of the data. Despite the potential circularity of this argument, we may at least be justified in wondering if the measured microbat–megabat distance is anomalously short; and if so, in seeking reasons why this might be so.

Further clues that published megabat–microbat hybridization distances are artefactually shortened, rather than representing true values, are provided by the following facts.

(1) There is considerable variation among microbat families with regard to their apparent distances from megabats (Kirsch *et al*. 1995, table 6; Pettigrew & Kirsch 1995; Hutcheon *et al*. 1998, table 1). Some microbat taxa give relatively much larger values than that in the above example when their distances from megabats are measured. Even allowing some irregularity in the molecular clock, such variations are striking. Notable examples include *Noctilio* and *Myotis* (see figure 6 in Pettigrew & Kirsch (1995)).

(2) Those microbat taxa giving very short distances to megabats also have a high AT content in their DNAs. For example, the rhinolophoid *Rhinolophus* has one of the greatest AT biases measured among microbats (68%) and also shows the shortest distance to the

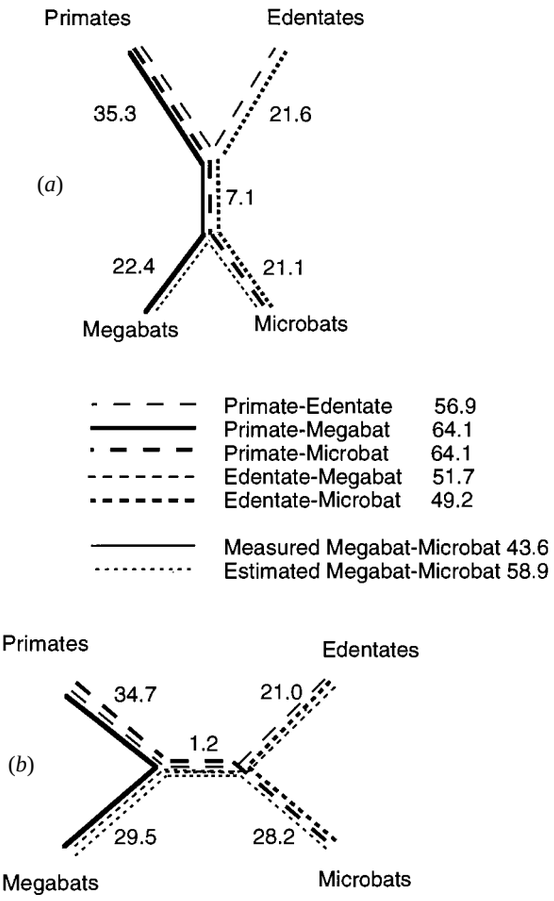

Figure 1. Inconsistencies among six averaged, unweighted ΔNPH distances (rounded to the first decimal place) relating four taxa representing Edentata, Primates, Microchiroptera, and Megachiroptera. Data after Kirsch *et al.* (1995, table 6), following symmetrization. (*a*) Undirected FITCH network based on all six distances. Sum-of-squares is 2.81 or 0.01% of the total sum-of-squares for the matrix, but all discrepancies of fitted distances from measured distances are concentrated on the internal segment. (*b*) Network based on matrix with microbat–megabat distance estimated by method of Landry *et al.* (1996). No distances are distorted; the sum-of-squares is 0. This result suggests that the major source of the discrepancies is an anomalously short microbat–megabat distance.

similarly biased (over 70% AT) megachiropteran *Pteropus* (Pettigrew & Kirsch (1995); in contrast, the mammalian average is about 60%).

(c) *AT-bias as a source of inconsistencies*

For these reasons, we thought it important to examine the apparently short DNA-hybridization distances measured between microbats and megabats in light of the expectation that a similar base-compositional bias in both megabats and (some) microbats might lead to coincidental similarities in DNA that would artificially reduce their separation. In fact, base-composition bias is a difficulty that potentially may beset any phylogenetic study using DNA, irrespective of technique; in sequence-based trees purporting to confirm bat monophyly, the sites that show matches supporting bat monophyly display a fourfold preponderance of As and Ts over Gs and Cs (Pettigrew 1994, 1995). Moreover, it is documented among other, non-bat taxa that two unrelated groups that independently share a mutational bias towards high AT will show a shortened distance inconsistent with their true relationship, if DNA is used as the basis for the comparison, because of coincidental A and T substitutions (Loomis & Smith 1992; Pettigrew 1994). Indeed, it is hard to imagine that such a bias could fail to be reflected in a topology essentially based on the numbers of shared bases among species, as in the sequence-based studies, or even in a tree based on comparisons of whole single-copy genomes, as in the hybridization tests.

(d) *A test of the AT-bias effect*

In the present study, we used DNA–DNA hybridization of fractionated DNA samples to try to tackle both the interordinal deep-branch problem and the question of bat monophyly. Our rationale was based on the presence of base-compositional biases in the DNA of some mammalian taxa, notably bats and some shrews (Arrighi *et al.* 1972; Sabeur *et al.* 1993), and on the fact that base composition alters the melting point of DNA hybrids in a defined way. By preparing tracer DNAs with a high GC content, we aimed to shift the melting curves of all comparisons to higher temperatures, thereby helping to improve discrimination at the low temperatures where melting curves for interordinal comparisons based on whole-genome tracers tend to group these curves in a way that hinders discrimination. In addition, we anticipated that DNA of high GC content would deal with the problem of AT biases. By comparing the melting curves obtained with the same driver DNA when hybridized either with high-GC tracer or high-AT tracer, we hoped to be able both to recognize, and to correct for, AT bias. We developed a simple method involving melting points to fractionate DNA into GC-rich and AT-rich fractions, using the same apparatus on which we later measured the melting points of hybrids. This fractionation does not provide sufficient discrimination to correct completely for the most extreme AT biases, such as those found in rhinolophoid and some phyllostomid microbats (Pettigrew & Kirsch 1995). However, it does successfully diagnose anomalous behaviour of DNA produced by AT bias, for which a partial correction might be introduced. The method also provides longer and more reliable distances in many 'deep' comparisons. While more data from extremely high-GC fractions will be necessary to reduce the noise level of the internodal distances at the base of the tree generated from the GC-rich hybrids, the data in hand are not inconsistent with the 'flying primate' hypothesis of separate origins for the two kinds of bats (Kirsch & Pettigrew, this issue).

In this paper we consider the characteristics and relationships of the curves obtained with our AT- and GC-enriched fractions; in the second of the two companion essays (Kirsch & Pettigrew, this issue), trees generated from matrices of distances among a few representatives of bats and possibly related taxa are discussed. In a third paper (Hutcheon *et al.* 1998), we extend our results to a matrix of whole-genome distances among a much larger suite of bat and outgroup taxa, including nine families from the diverse Microchiroptera, to test our results for algorithmic artefacts, such as those that might be due to poor choice of outgroups or long-branch attraction.

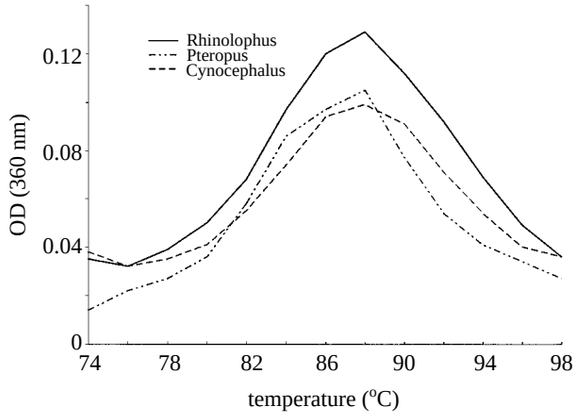

Figure 2. Melting curves of three species used to prepare enriched fractions. Amounts of DNA in each eluate (taken at 2°C increments) were determined spectrophotometrically (ODs read at 360 nm). From top to bottom: *Rhinolophus philippinensis*, *Pteropus vampyrus*, and *Cynocephalus variegatus*. Curve-fitting showed the modes of *Rhinolophus* and *Cynocephalus* to be about 88°C, but that of *Pteropus* was lower, at around 86°C, consistent with its higher AT-content.

2. METHODS

(a) *Extraction, fractionation and labelling*

Whole-genome DNA was extracted and prepared for hybridization according to protocols outlined in previous papers (Kirsch *et al.* (1990), with the modifications noted in Bleiweiss *et al.* (1994)).

For the experiments with fractionated DNA, we first melted a large quantity (1–1.5 mg) of native, sonicated DNA of each species in the thermal-elution device used for testing hybrids, collecting and assessing spectrophotometrically each fraction eluted at 2 °C intervals for DNA content. From this information, a melting curve for each species was constructed that provided the basis for defining AT- and GC-rich regions, as the major cause of the distribution of melted sequences around the T_{mode} is base composition; adenine and thymine are united by only two hydrogen bonds, while guanine and cytosine are linked by three. Thus, fractions eluted below the mode should be richer in As and Ts than those that melt above that temperature, which in all cases was about 86–88 °C (the lower temperature was typical of *Pteropus*).

Three fractions each were prepared from extracts of from five to seven species: one consisting of the combined eluates from four temperatures (2 °C increments) below the mode ('AT-rich'); a second, from the mode to and including 98 °C ('GC-rich'); and—in separate preparations—from 94–98 °C ('super-GC-rich'). Figure 2 shows the elution curves for three species. The combined eluates were dialysed for one day against several changes of deionized water and freeze-dried; the freeze-dried samples were then rehydrated in 0.48 M phosphate buffer (PB), their concentrations were determined, and repeated sequences were removed and labelled as for whole-genome DNAs. That is, the samples were boiled and allowed to reassociate to *ca.* C_0t 350 (equivalent-C_0t 2000), diluted to 0.12 M PB, and loaded onto HAP columns in a constant-temperature waterbath set at 60 °C (the criterion, or incubation temperature, for all reassociations in our experiments). Fifteen millilitres of 0.12 M PB were used to wash the uncombined, single-copy sequences from the HAP bed. These sequences were again dialysed against deionized water and freeze-dried. The resulting samples were labelled with ^{125}I (see Kirsch *et al.* 1990). Whole-genome labels were also prepared from extracts of the same species for comparison with the behaviours of enriched, labelled fractions (see Hutcheon *et al.* 1998; Kirsch & Pettigrew, this issue).

(b) *Hybridization*

These 'tracers' were then used to produce hybrid combinations, using 25 µg of 'driver' DNA at a concentration of 3 mg ml^{-1} and a tracer : driver ratio of at least 1:500 in each case. Some 1500 hybrids were produced and analysed in the following experiments (this paper; Kirsch & Pettigrew, this issue). The yields from the series of fractionation operations described above ranged from 15 to over 100 µg, and although labelling was always successful, the absolute numbers of counts were often low, leading to counting-error that sometimes produced rough and unusable curves.

(c) *Treatment of individual curves*

For the purposes of this paper, the melting profiles were indexed as T_{mode}. Modes are not usually considered apt for the distant (interordinal) comparisons attempted here, but are an easily recognized characteristic of melting curves and are generally impervious to extract or other experimental variations (Bleiweiss & Kirsch 1993). Moreover, the modes did, in the event, prove to supply satisfactory discrimination. This may be so because bats are known to have small genomes (Burton *et al.* 1989; Baker *et al.* 1992), perhaps even lacking the numerous paralogous sequences that seem to be the cause of 'false' modes in many hybrids of distantly related mammals (Fox & Schmid 1980). The often-used median melting-temperature or T_m was not a suitable index for the reason noted above, namely the raggedness of some curves, which sometimes rendered even determination of modes difficult.

As we were interested in the comparative behaviours (especially relative modal positions) of curves generated from the same species combinations but using differently enriched fractions for the tracer, several of these were plotted in pairs. The ΔT_{mode} values (differences between homologous and heterologous T_{mode} values) were also considered as the differences in GC and AT, super-GC and AT, or super-GC and GC ΔT_{mode} ('Δ–Δs'), in order to ascertain how Δs from differently enriched labels tracked with each other. In addition, regressions amongst the Δs obtained with the several kinds of labels (e.g. ΔGC versus ΔAT) were calculated to determine the equations relating distances obtained with different types of labels, and hence to assess departures from predicted values.

3. RESULTS AND DISCUSSION

(a) *Homologous melting-temperatures: fragment-length or GC-content?*

Using a roughly 50:50 fractionation around the T_{mode}, we found that we could obtain mean differences of about 3–4 °C between the homologous melting-points of the two self-hybridized fractions so obtained; for the fraction commencing at 94 °C ('super'-GC-rich), the difference from the AT-rich mode increased to 5 °C or more. Examples are shown in figure 3, based on labelled *Pteropus* DNAs, where it can be seen that a homologous hybrid made from the fractions collected from above the T_{mode}, when labelled and placed on the HAP column, melts at a modal temperature that is 4 °C higher than the melting

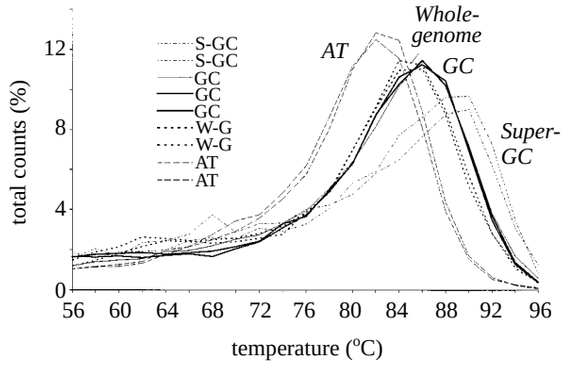

Figure 3. Examples of displacements of homologous-hybrid curves for AT- GC-, and super-GC-rich tracers made from fractions of *Pteropus vampyrus* DNA. Average T_{mode} values are 83.22, 85.48 and 88.26 °C, respectively. Mode of whole-genome homoduplex was, as expected, intermediate at 84.62 °C. In this and subsequent figures, each curve has been normalized to the per cent hybridization for that hybrid.

point of the combined fractions collected from below the T_{mode}; super-GC-rich homologues melt at a 5 °C higher temperature. This was a consistent finding over all fractionations of DNA from every taxon. We saw the same effect when we used unsonicated, slightly sheared native DNA (data not shown), although sonicated DNA was used routinely.

Tracers below 500 bp in length have the effect of lowering the melting point by a number of degrees equal to 500 divided by the tracer length (Werman *et al.* 1990). As noted, the mean difference between melting points of super-GC-rich and AT-rich fractions from the same taxon was about 5 °C. A melting point difference of around 5 °C would therefore require a fivefold ratio in fragment size if fragment size were the sole explanation for the difference. This would be easily detectable in an appropriate electrophoresis gel, but there was no obvious difference in fragment size among the fractions when they were run on a sizing gel, where the modal fragment size was always around 500 bp. Even given a range of fragment sizes, to account for the T_{mode} differences in terms of fragment length alone would require major contributions from size classes as far apart as, say, 250 and 1250 bp. Moreover, we could not have obtained the comparative results described below were the segregation based only on size. Thus, the large differences in melting points, consistent across experiments, cannot be attributed simply to differential assortment based on fragment size, with longer fragments in the higher melting-point fraction. These considerations suggest that the differences in T_{mode} for the various types of tracers are indeed attributable to base composition, as it has already been shown that the major contributor to the variation of melting points across the T_{mode} is related to this factor, with a shift of approximately 2.4 °C towards a higher temperature for a 1% increase in GC content (Mandel & Marmur 1968). Thus, the differences in homologous modes among the most extreme fractions we made represent at least a 2% difference in GC content.

By definition, then, hybrids differing only with respect to the nature of the tracer used (whole-genome, AT-enriched, or variously GC-enriched) should produce curves differing with respect to the position of the mode, AT-rich hybrids melting at a lower average temperature because of the fewer hydrogen bonds uniting As and Ts. The mean homologous T_{mode} of several labels bear out this expectation (table 1; Kirsch & Pettigrew, this issue): for seven AT-rich tracers, it is 82.99 °C; for five GC-rich tracers, it is 85.89 °C; for seven super-GC-rich tracers, it is 87.99 °C; and for seven whole-genome labels, it is 84.91 °C. These observations confirm that our simple separation scheme did enrich the fractions for sequences of differing average melting temperatures and presumably, therefore, base-composition. Table 1 lists some of the results for our more complete heterologous comparisons.

(b) *'Following behaviour' using GC-rich fractions*

If base-composition did not vary among species, one would expect that heterologous hybrids made with tracer of a higher melting-point, relatively GC-rich fraction would (like the homologues) also melt at higher temperatures than hybrids made with the same drivers but with AT-rich tracers. This expectation was fulfilled for many (but not all) comparisons, as shown in figure 4*a*, where it can be seen that the melting points of hybrids made from a GC-rich tracer are shifted to higher temperatures by an amount that is close to the difference in melting points of the two self-hybridized tracers (the homologues). We call this phenomenon 'following behaviour', because the heterologous hybrid 'follows' the GC-rich homologue to higher temperatures when made with a GC-rich instead of an AT-rich tracer. In other words, for the taxa shown in figure 4*a*, the distance between them stays nearly constant even though the melting points have shifted to the right when GC-rich tracer is used, a relation nicely captured by the notion of 'Δ–Δ' (the difference between Δs for the same driver obtained with different labels, the Δ with the higher-melting-point or more GC-rich tracer being listed first): Δ–Δ will be zero if strict following obtains. However, with increasing distance, Δ–Δ may become positive because of the expanded range of Δs obtained with the more GC-rich tracer (a negative remainder would imply that the higher melting-point hybrid not only 'followed' its homologue, but was actually 'pulled' towards it; see figure 4*b*). Generally, the increase over zero for any particular Δ–Δ is proportional to the difference in the ranges of Δs for the two labels, and the scale is 'stretched' for more GC-rich tracers (mostly at the high-temperature end). The actual Δ or Δ–Δ can be predicted from the linear regression of (for example) ΔGCs versus ΔATs (see figure 5; data from table 1): $\Delta GC = 1.16\Delta AT$ (s.d. = ±1.85; for the other pairings, $\Delta super\text{-}GC = 1.21\Delta AT$ (s.d. = ±2.69) and $\Delta super\text{-}GC = 1.04\Delta GC$ (s.d. = ±2.52)). Thus, any ΔGC more than about two standard deviations (or three, if the more conservative sequential Bonferroni correction was applied) from the predicted value must be an exception to following behaviour, either being 'pulled' towards the homologue (if less), or 'repelled' from it (if more). Some examples are examined in the next section.

(c) *Exceptions to following behaviour*

There is, of course, considerable scatter in the plot of ΔGC versus ΔAT (figure 5), or in those for other pairs of enriched fractions, some of which may be due to experimental error; but only 2 out of 51 ΔGCs–ΔATs (for

Table 1. *Tabulation of some averaged heterologous* ΔT_{mode} *values, together with 'Δ–Δs' for the three possible pairwise combinations of AT-, GC- and super-GC-rich* ΔT_{mode} *values*

(Note that very few Δ–Δs are negative, but several are strongly positive. Increases in Δ with more GC-rich tracers are due mostly to the greater range of distances (largely at the high-temperature end) obtained with such labels compared to those generated with AT-rich labels.)

comparisons	ΔAT	ΔGC	ΔSGC	ΔGC–ΔAT	ΔSGC–ΔAT	ΔSGC–ΔGC
Didelphis tracers						
×*Cynocephalus*	23.81	28.28	31.18	4.47	7.37	2.90
×*Lemur*	24.12	25.55	27.30	1.43	3.18	1.75
×*Pteropus*	24.60	24.54	31.61	−0.06	7.01	7.07
×*Rhinolophus*	23.39	29.70	28.72	6.31	5.33	−0.98
×*Noctilio*	—	—	30.09	—	—	—
×*Pteronotus*	—	—	31.84	—	—	—
×*Myotis*	—	—	29.01	—	—	—
×*Scotophilus*	24.82	26.59	—	1.77	—	—
×*Chaetophractus*	23.60	26.35	—	2.75	—	—
×*Dobsonia*	24.04	27.07	—	3.03	—	—
Cynocephalus tracers						
×*Didelphis*	19.03	25.46	28.79	6.43	9.76	3.33
×*Lemur*	19.51	23.56	24.48	4.05	4.97	0.92
×*Pteropus*	20.27	17.11	24.20	−3.16	3.93	7.09
×*Rhinolophus*	21.12	21.64	25.85	0.52	4.73	4.21
×*Noctilio*	—	—	28.28	—	—	—
×*Pteronotus*	—	—	27.59	—	—	—
×*Myotis*	—	—	27.20	—	—	—
×*Scotophilus*	—	25.88	—	—	—	—
×*Dobsonia*	22.65	—	—	—	—	—
Lemur tracers						
×*Didelphis*	26.20	—	29.95	—	3.75	—
×*Cynocephalus*	22.53	25.78	25.50	3.25	2.97	−0.28
×*Pteropus*	23.28	25.76	25.30	2.48	2.02	−0.46
×*Rhinolophus*	22.62	25.43	25.55	2.81	2.93	0.12
×*Noctilio*	—	—	27.09	—	—	—
×*Pteronotus*	—	—	27.76	—	—	—
×*Myotis*	—	—	26.39	—	—	—
×*Scotophilus*	24.12	28.19	—	4.08	—	—
×*Hipposideros*	24.39	27.95	—	3.56	—	—
×*Nycticebus*	20.89	24.80	—	3.91	—	—
×*Dobsonia*	23.04	25.89	—	2.85	—	—
Pteropus tracers						
×*Didelphis*	23.91	28.82	25.89	4.91	1.98	−2.93
×*Cynocephalus*	23.44	26.55	27.61	3.11	4.17	1.06
×*Lemur*	22.75	25.51	25.08	2.76	2.33	−0.43
×*Rhinolophus*	19.31	24.34	24.25	5.03	4.94	−0.09
×*Noctilio*	21.07	25.64	26.92	4.57	5.85	1.28
×*Pteronotus*	—	—	26.71	—	—	—
×*Myotis*	21.66	26.09	25.96	4.43	4.30	−0.13
×*Scotophilus*	22.89	26.05	—	3.16	—	—
×*Chaerephon*	21.48	25.58	—	4.10	—	—
×*Chiroderma*	21.78	24.50	—	2.73	—	—
×*Hipposideros*	17.96	27.19	—	9.23	—	—
×*Macroderma*	19.19	24.77	—	5.58	—	—
×*Nycticebus*	23.98	27.24	—	3.26	—	—
×*Chaetophractus*	24.37	26.88	—	2.51	—	—
×*Dobsonia*	6.59	6.67	—	0.08	—	—
×*Cynopterus*	6.93	7.78	—	0.85	—	—
×*Nyctimene*	5.91	6.52	—	0.61	—	—
×*Epomophorus*	6.25	6.53	—	0.28	—	—
×*Rousettus*	6.29	7.00	—	0.71	—	—
×*Macroglossus*	6.59	6.80	—	0.21	—	—
×*Pteralopex*	4.26	4.97	—	0.71	—	—

continued

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Table 1 *Continued*

Rhinolophus tracers						
×*Didelphis*	25.03	28.57	29.51	3.54	4.48	0.94
×*Cynocephalus*	21.15	25.24	26.54	4.09	5.39	1.30
×*Lemur*	21.17	24.59	26.06	3.42	4.89	1.47
×*Pteropus*	18.20	22.69	24.13	4.49	5.93	1.44
×*Noctilio*	—	—	27.84	—	—	—
×*Pteronotus*	—	—	27.73	—	—	—
×*Myotis*	20.98	26.94	26.13	5.96	5.15	−0.81
×*Scotophilus*	23.02	27.70	—	4.68	—	—
×*Chaerephon*	21.62	25.36	—	3.74	—	—
×*Chiroderma*	21.15	25.59	—	4.44	—	—
×*Hipposideros*	11.29	13.86	—	2.56	—	—
×*Macroderma*	20.15	23.54	—	3.39	—	—
×*Nycticebus*	24.29	28.23	—	3.94	—	—
×*Chaetophractus*	23.65	27.67	—	4.02	—	—
×*Dobsonia*	20.10	24.83	—	4.76	—	—
Noctilio tracers						
×*Didelphis*	26.20	—	28.67	—	2.47	—
×*Cynocephalus*	24.59	—	27.27	—	2.68	—
×*Lemur*	24.15	—	26.87	—	2.72	—
×*Pteropus*	22.83	—	25.59	—	2.76	—
×*Rhinolophus*	22.96	—	26.36	—	3.40	—
×*Pteronotus*	19.19	—	28.63	—	9.44	—
×*Myotis*	—	—	26.64	—	—	—
Pteronotus tracers						
×*Didelphis*	—	—	30.49	—	—	—
×*Cynocephalus*	—	—	27.02	—	—	—
×*Lemur*	23.16	—	24.46	—	1.30	—
×*Pteropus*	19.76	—	25.39	—	5.63	—
×*Rhinolophus*	19.28	—	25.96	—	6.68	—
×*Noctilio*	17.75	—	29.46	—	11.71	—
×*Myotis*	—	—	26.75	—	—	—
number of observations	63	52	49	51	33	22
maximum Δ or Δ–Δ	26.20	29.70	31.84	9.23	11.71	7.09
mean homologue	82.99	85.89	87.99	—	—	—
mean homologous Δ–Δ	—	—	—	2.90	5.00	2.10

example) are negative, and one of these is essentially zero (−0.06). Still, many of the scattered points do represent striking departures from following behaviour, where the differences in Δs are greater (or, more rarely, less) than expected (i.e. they depart markedly from the regression of GC- versus AT-rich Δs). Two extreme exceptions to following—the *Pteronotus–Noctilio* Δsuper-GC-rich minus ΔAT-rich comparisons (11.77 and its reciprocal, 9.44; see table 1)—are reflected in trees generated from these data (e.g. Kirsch & Pettigrew 1998, figure 2*g*), and appear to be genuine outliers. Consistently 'anomalous' behaviour of the GC-rich hybrids was, however, most marked in comparisons between the megabat, *Pteropus*, and microbats in the family Phyllostomidae (*Chiroderma* and *Mimon* in our sample) and in the superfamily Rhinolophoidea (*Rhinolophus*, *Hipposideros*, and *Macroderma*). Examples are shown in figure 6*a*,*b*, where it can be seen that the modes for the *Pteropus–Rhinolophus* and *Pteropus–Macroderma* comparisons not only do not 'follow', but are shifted to a lower temperature when GC-rich tracer DNA is used; the Δ—Δs for these pairs average over 5 °C—some 2–3 °C more than the difference between the homologous (GC-rich versus AT-rich) melting temperatures—or nearly three standard deviations from the predicted ΔGC value. This effect is even more pronounced if one takes into account the fact that the heterologous mode in each case is reduced in height (i.e. per cent hybridization is less) and therefore even more 'distant' than the values given by the ΔT_{mode}. In other words, the distance between *Pteropus* and *Rhinolophus* or *Macroderma* increases substantially when GC-rich tracer DNA is used for the comparison instead of AT-rich DNA: some differences may be more than 10 °C for the super-GC- compared to AT-rich fractions, whereas the average 'Δ–Δ' for that type of comparison is usually about 5 °C. Thus, such discrepancies are not related just to the expanded ranges obtained with GC- or super-GC-rich labels. On the other hand, very distant comparisons may have less marked Δ–Δs, e.g. with the dermopteran *Cynocephalus* in figure 6*c*. Another visualization of the relationships among Δs based on different labels is shown in figure 7, which plots ΔATs and ΔGCs for sets of comparisons with different, grouped taxa. In these plots, it can be seen that the means for groups of GC-rich Δs are shifted towards longer distances compared to AT-rich ones.

Under what circumstances might such reversals in the positions of the AT and GC melting curves occur? 'Following behaviour' is to be expected if there is no difference between the AT:GC proportions and rates of evolution of GC-rich and AT-rich DNAs. To explain the

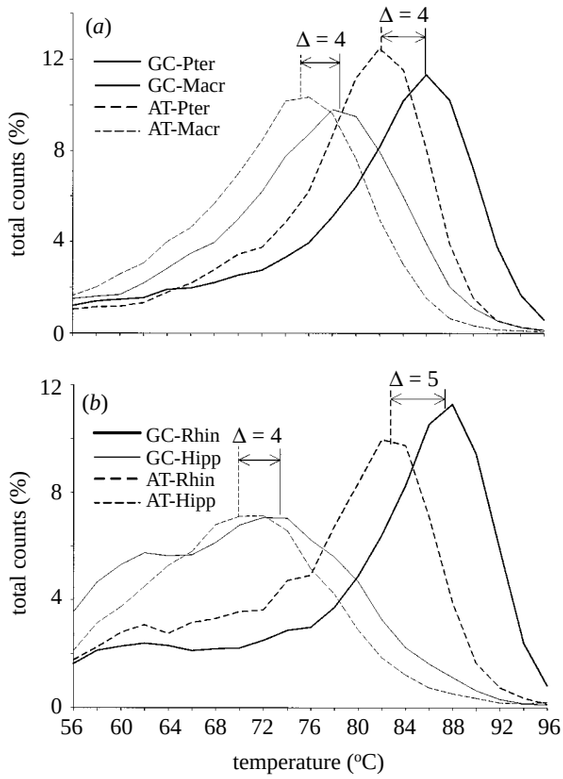

Figure 4. Examples of 'following behaviour' in heterologous hybrids made with AT- and GC-rich labels. Note that the distance measured between the comparison taxa by the ΔT_{mode} is about the same whether AT-rich or GC-rich tracer DNA is used. In other words, the increase in melting point of the self-hybrid caused by GC-enrichment is paralleled by an equal increase in the melting-point of a heterologous hybrid made with the GC-rich label. In (*a*), the ΔT_{mode} measures of the megachiropteran heterologue *Macroglossus minimus* (Macr) are about 4°C apart, equal to the difference in homologous T_{mode} for the labelled megabat, *Pteropus vampyrus* (Pter) GC- and AT-rich fractions; in (*b*), the microchiropteran heterologous curves (of *Hipposideros galeritus*; Hipp) also differ by 4°C, but the AT- and GC-rich microchiropteran homologues (*Rhinolophus philippinensis*; Rhin) are slightly further apart, at 5°C. Dotted lines denote AT-rich hybrids; solid lines denote GC-rich hybrids.

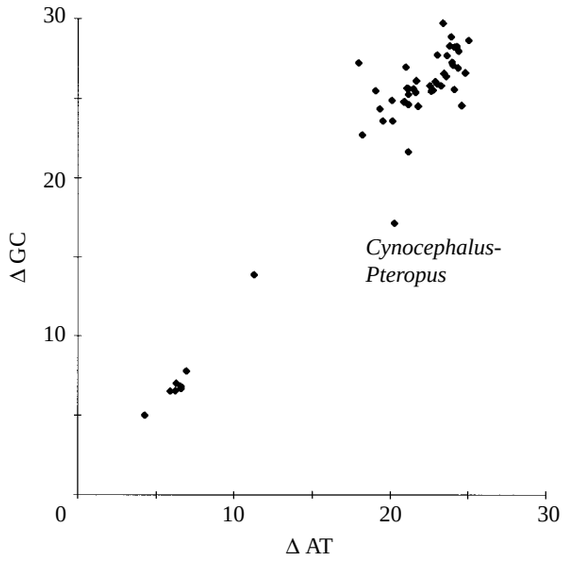

Figure 5. Plot of ΔGCs versus ΔATs (data from table 1). The regression (no intercept) indicates a linear relationship: $\Delta GC = 1.16\ \Delta AT$, with an s.d. of ±1.85. Note that there is some scatter, especially above the implied line, indicating a tendency for measured distances to increase beyond expectation when GC-rich tracer DNA is used. One outlier (labelled dermopteran, *Cynocephalus variegatus* to unlabelled megabat, *Pteropus vampyrus*) is identified.

reversals obtained, AT-rich sequences would have to have evolved relatively more slowly than GC-rich ones. This is the opposite of what might be expected on the basis of theory. Rate variation along the genome is a contentious issue, but it is generally accepted that rate-accelerations, if they occur, are greater for the AT-rich part of the genome than for the GC-rich segments, because functional (and therefore selectively constrained) genes are on the whole confined to GC-rich regions (Bernardi 1993). Yet, it may be that the AT-rich sequences are so high in adenine and thymine that they reach complete saturation in a shorter period of time than do GC-rich ones, and so mimic a retarded evolutionary rate. Thus, comparisons of AT-biased samples will seriously underestimate true genetic distances.

(d) *Is there some biochemical explanation for the anomalous behaviour of bat DNAs?*

The anomalies that we have observed indicate that the AT : GC ratio has an important bearing on estimated distances. Use of GC-rich DNA as the tracer tends, in many cases, to increase the separation between two melting curves (relative to the same hybrids compared using an AT-rich label), as well as to shift both of them to higher temperatures. But with some comparisons, the positions of AT- and GC-based curves may actually reverse. In other words, DNA that is not GC-rich tends consistently to underestimate the distances measured for such reversed curves. A base-compositional bias may be the proximate cause of these results, but what might be the reason for an AT anomaly in the first place?

As suggested in §1, we think that the explanation may lie with the sufficient causes for biases in base-composition that are known among mammalian taxa. In general, mammals have an elevated AT content in genomic DNA, with megabats having the highest known AT content of any vertebrate group, up to 15% more than the mammalian average (Sabeur *et al.* 1993; Pettigrew & Kirsch 1995). This bias in base-composition may in turn be a result of mutational biases that are more pronounced in aerobic metabolism. Mutational pressures that operate on DNA replication and repair in aerobic metabolism include the following:

1. Oxygen radicals tend preferentially to oxidize guanine rather than the other three bases, thereby possibly contributing to the lowering of the guanine content associated with aerobic metabolism.
2. Methylation of cytosine with subsequent transamination of methyl-cytosine to thymine is markedly temperature dependent and would contribute to the raised thymine levels of DNA in aerobic organisms.
3. Precursor-pool concentrations of dATP may be biased in aerobic metabolism because adenine nucleotides

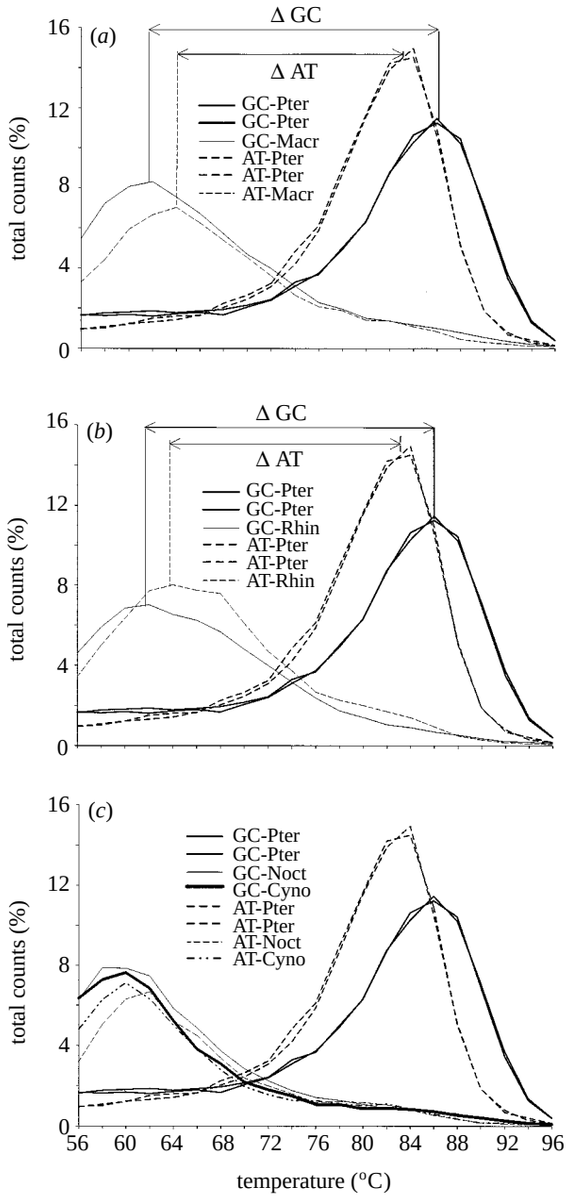

Figure 6. Examples of exceptions to 'following behaviour' in heterologous hybrids made with AT- and GC-rich tracers from fractions of the megabat *Pteropus vampyrus* (Pter) DNA. In these examples the measured distance between the taxa increased when GC-rich DNA was employed. (*a*) *Macroderma gigas* (Macr), a rhinolophoid, is the heterologous species; (*b*) *Rhinolophus philippinensis* (Rhin), another rhinolophoid, is the heterologue; (*c*) the noctilionoid *Noctilio albiventris* (Noct) and dermopteran *Cynocephalus variegatus* (Cyno) are the heterologues. In (*a*) and (*b*), instead of being displaced towards higher temperatures when the GC-rich tracer is used, the heterologues melt at a temperature about 2 °C lower than the heteroduplex made with an AT-rich label (homologues are separated by about 4 °C). In (*c*), *Noctilio* also shows some departure from following in its GC-rich curve, but *Cynocephalus* AT- and GC-rich T_{mode} values are about the same. Dotted lines denote AT-rich hybrids; solid lines denote GC-rich hybrids.

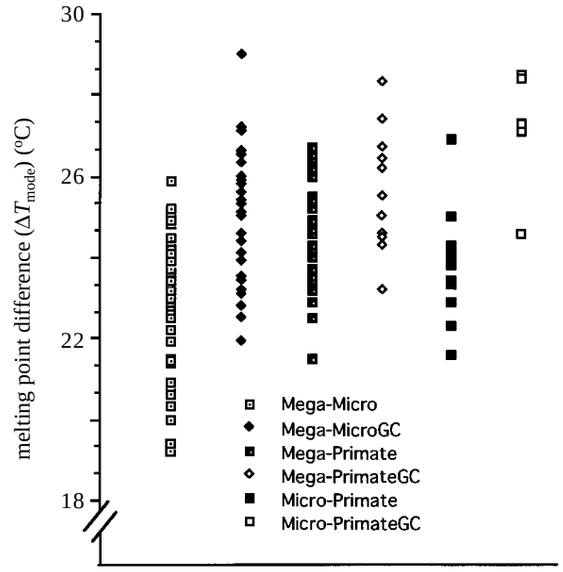

Figure 7. Raw distance data (differences in melting-points between homologous and heterologous modes: ΔT_{mode} values) plotted to show the range of variation observed for six sets of comparisons of special interest (among primates, microbats, and megabats, with AT-rich and GC-rich DNAs in each case). Note that GC-rich heterologous hybrids tend to give greater ΔT_{mode} values, particularly when the hybrid is between microbat and megabat DNA, and so the short distance between micro- and megabat DNA that is obtained with AT-rich tracers (mega–micro: square symbols with dots) is markedly increased when GC-rich DNA is used. As a result, with GC-rich labels the microbat–megabat distance is not shorter than the megabat–primate distance, even though this is the case with AT-rich DNA. Note further that the distances between these bat taxa are in the high twenties, values like those commonly found between eutherian orders. In sum, when GC-rich DNA is used, the distance between microbats and megabats is larger, is not less than the megabat–primate distance, and is comparable to distances among mammalian orders even though the two bat groups are conventionally placed in the same order. The scatter of the points makes it difficult to establish whether the megabat–primate distance is significantly shorter than the megabat–microbat distance, but it is clear that the megabat–primate distance is less than the microbat–primate one.

are in high concentration due to their role in energy metabolism, thereby raising adenine levels.

4. High metabolism is associated with smaller cell size and smaller genome size, perhaps to increase the efficiency of exchanges across membrane boundaries whose area : volume ratio has thereby been increased. This effect would exaggerate the impact of cytosolic metabolism on DNA repair and replication in the nucleus, thus leading to a magnification of the above three effects.

It is significant that these factors all point in the same direction, towards higher AT content. Of these, perhaps the most important is the extreme sensitivity of guanine to oxidation, which is much higher than that of the other three bases in DNA. Guanine is oxidized to 8-oxoguanine, which is usually misread as adenine (depending on the DNA polymerase involved), thus contributing to a mutational bias towards A and T linked to oxidative metabolism (Shibutani *et al.* 1991; Hall *et al.* 1996). It therefore seems likely that high metabolism contributes to the mutational bias towards high AT. One would then expect that a

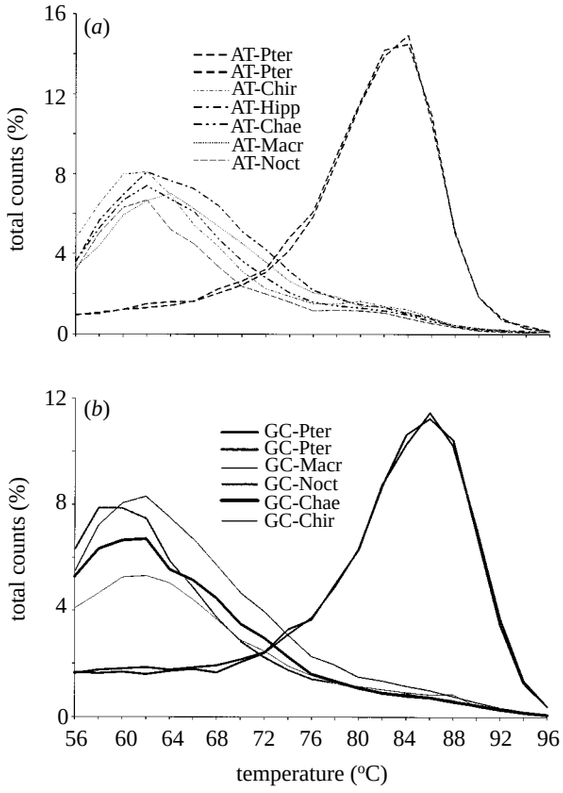

Figure 8. Examples of heterologous curves for a variety of microchiropterans using (*a*) AT-rich and (*b*) GC-rich megabat (*Pteropus vampyrus*) labels. Distances between the megabat and the various microbats vary from 22 to 24 °C when AT-rich DNA is used (*a*), but increase to 26–28 °C when GC-rich DNA is employed (*b*), these latter distances being as great as those observed for almost any similar comparison between mammalian orders. Note the 'ramp' leading toward the homologue in the AT-rich hybrid of the rhinolophoid *Hipposideros* (Hipp) in (*a*); the effect is even seen in (*b*) for the relatively GC-rich *Macroderma* (Macr; also a rhinolophoid) hybrid. Dotted lines denote AT-rich hybrids; solid lines denote GC-rich hybrids. Heterologues are generally shown using lighter-weight lines and are listed in legends from top to bottom, corresponding to the positions of these curves at the left. Abbreviations: Chae = *Chaerephon plicata* (Molossidae); Chir = *Chiroderma salvini* (Phyllostomidae); Hipp = *Hipposideros galeritus* (Hipposideridae); Macr = *Macroderma gigas* (Megadermatidae); Noct = *Noctilio albiventris* (Noctilionidae); Pter = *Pteropus vampyrus* (Pteropodidae).

shared AT bias would in turn contribute to coincidental similarities between DNAs, and that distances estimated using AT-rich DNA might therefore be disproportionately smaller than those estimated with GC-rich tracer DNA, the kind of anomaly that we have in fact observed. Indeed, the magnitude of the anomaly seems to be related to the amount of AT bias that has been noticed in the taxa under consideration. One of the most anomalous comparisons we found was between *Rhinolophus*, a rhinolophid microbat with one of the highest AT contents of all microbats (68%), and the megabat *Pteropus*, which has the highest known AT content of any mammal (74%; Pettigrew & Kirsch 1995). In all comparisons, *Rhinolophus* and *Pteropus* were much closer (both absolutely and relative to the average distances in collateral comparisons), when we used AT-rich rather than GC-rich tracer. And in all melting curves where rhinolophoids and pteropodids were compared using AT-rich DNA, there was a 'hump' or 'ramp' that extended the heterologous curve towards the other taxon, adding to the impression that there was a subcomponent of the DNA that was contributing to the greater apparent similarity using AT-rich tracer (e.g. figure 8*a* for *Hipposideros*; the ramp is even seen in a relatively GC-rich hybrid of another rhinolophoid, *Macroderma*, in figure 8*b*). This effect was also seen in a comparison between the phyllostomid *Chiroderma*, another microbat known to have a very high AT content (70%), and *Pteropus*.

There are not enough data available on AT content in microbats to test the fit between AT content and the degree of anomaly that we have observed, but we think that it is significant that the microbats with the highest AT contents (such as the phyllostomid *Chiroderma* and the rhinolophoids *Rhinolophus*, *Hipposideros* and *Macroderma*) show the greatest anomalies; and microbats that have relatively normal AT levels for eutherians, such as the vespertilionid *Myotis*, do not show as great or as consistent a departure from expectation.

4. CONCLUSION

We did not achieve our aim of improving resolution for comparisons between very distant taxa using GC-enriched tracer DNAs; the approximately 2% enrichment certainly ameliorated, but did not entirely obviate, the foreshortening and compression of distances evidently due to AT biases. The separations among modes for eutherian interordinal comparisons are roughly similar at around 23–28 °C, depending on the type of tracer (i.e. maximum Δs did not increase beyond the differences in melting temperatures of the labels), whether the pair of mammalian taxa being compared was microbat–megabat, megabat–primate, dermopteran–primate, etc. Even most interfamilial comparisons among microbats yield comparable separations, confirming the general finding that microbat families tend to be as distant from each other as are mammalian orders. On the other hand, the use of GC-enriched DNA has revealed an important feature of many comparisons that is relevant to the debate about eutherian interordinal phylogeny, namely, that some distances are consistently underestimated, particularly those involving rhinolophoid and pteropodid bats. The increased separation among these bats that we commonly observed when we used GC-rich tracer DNA to make hybrids is consistent with the generally high AT content of eutherian DNA (Bernardi 1993) and especially in these taxa. Our finding of greater megabat–microbat distances estimated using GC-rich tracer leaves open the possibility that bats are not monophyletic.

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REFERENCES

Arrighi, F. E., Lidicker, W. Z. Jr, Mandel, M. & Bergandahl, J. 1972 Heterogeneity of CsCl buoyant densities of chiropteran DNA. *Biochem. Genet.* **6**, 27–39.

Baker, R. J., Novacek, M. J. & Simmons, N. B. 1991 On the monophyly of bats. *Syst. Zool.* **40**, 216–231.

Baker, R. J., Maltbie, M., Owen, J. G., Hamilton, M. J. & Bradley, R. D. 1992 Reduced number of ribosomal sites in bats: evidence for a mechanism to contain genome size. *J. Mamm.* **73**, 847–858.

Bernardi, G. 1993 The vertebrate genome: isochores and evolution. *Molec. Biol. Evol.* **10**, 186–204.

Bleiweiss, R. & Kirsch, J. A. W. 1993 Experimental analysis of variance for DNA hybridization. II. Precision. *J. Molec. Evol.* **37**, 514–524.

Bleiweiss, R., Kirsch, J. A. W. & Matheus, J. C. 1994 DNA hybridization evidence for subfamily structure among hummingbirds. *Auk* **111**, 8–19.

Burton, D. W., Bickham, J. W. & Genoways, H. H. 1989 Flow-cytometric analyses of nuclear DNA content in four families of neotropical bats. *Evolution* **43**, 756–765.

D'Erchia, A. M., Gissi, C., Pesole, G., Saccone, C. & Arnason, U. 1996 The guinea-pig is not a rodent. *Nature* **381**, 597–600.

Farris, J. S. 1972 Estimating phylogenetic trees from distance matrices. *Am. Nat.* **106**, 645–668.

Fitch, W. & Smith, T. S. 1982 Implications of minimal length trees. *Syst. Zool.* **31**, 68–75.

Fox, G. M. & Schmid, C. W. 1980 Related single copy sequences in the human genome. *Biochim. Biophys. Acta* **609**, 349–363.

Hall, D. H., Homlin, E. & Barton, J. K. 1996 Oxidative DNA damage through long-range electron transfer. *Nature* **382**, 731–735.

Hutcheon, J. M., Kirsch, J. A. W. & Pettigrew, J. D. 1998 Base-compositional biases and the bat problem. III. The question of microchiropteran monophyly. *Phil. Trans. R. Soc. Lond.* B. **353**.

Janke, A., Feldmaier-Fuchs, T., Thomas, W. K., von Haeseler, A. & Pääbo, S. 1994 The marsupial mitochondrial genome and the evolution of placental mammals. *Genetics* **137**, 243–256.

Janke, A., Gemmell, N. J., Feldmaier-Fuchs, G., von Haeseler, A. & Pääbo, S. 1996 The mitochondrial genome of a monotreme—the platypus (*Ornithorhynchus anatinus*). *J. Molec. Evol.* **42**, 153–159.

Kilpatrick, C. W. & Nuñez, P. E. 1993 Monophyly of bats inferred from DNA–DNA hybridization. In *Abstracts sixth international theriological congress*, p. 155. Sydney: University of New South Wales.

Kirsch, J. A. W. & Pettigrew, J. D. 1998 Base compositional biases and the bat problem. II. DNA-hybridisation trees based on tracers enriched for AT- or GC-content. *Phil. Trans. R. Soc. Lond.* B. **353**.

Kirsch, J. A. W., Springer, M. S., Krajewski, C., Archer, M., Aplin, K. & Dickerman, A. W. 1990 DNA/DNA hybridization studies of carnivorous marsupials. I. The intergeneric relationships of bandicoots (Marsupialia: Perameloidea). *J. Molec. Evol.* **30**, 434–448.

Kirsch, J. A. W., Flannery, T. F., Springer, M. S. & Lapointe, F.-J. 1995 Phylogeny of the Pteropodidae (Mammalia: Chiroptera) based on DNA hybridisation, with evidence for bat monophyly. *Aust. J. Zool.* **43**, 395–428.

Landry, P.-A., Lapointe, F.-J. & Kirsch, J. A. W. 1996 Estimating phylogenies from lacunose distance matrices: additive is superior to ultrametric estimation. *Molec. Biol. Evol.* **13**, 818–823.

Lapointe, F.-J. & Kirsch, J. A. W. 1995 Estimating phylogenies from lacunose distance matrices, with special reference to DNA hybridization data. *Molec. Biol. Evol.* **12**, 266–284.

Loomis, W. F. & Smith, D. W. 1992 Molecular phylogeny of *Dictyostelium discoideum* by protein sequence comparison. *Proc. Natn. Acad. Sci. USA* **87**, 9093–9097.

Mandel, M. & Marmur, J. 1968 Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. In *Methods in enzymology*, vol. XII part B (ed. L. Grossman & K. Moldave), pp. 195–206. New York: Academic Press.

Pettigrew, J. D. 1986 Flying primates? Megabats have the advanced pathway from eye to midbrain. *Science* **231**, 1304–1306.

Pettigrew, J. D. 1991*a* Wings or brain? Convergent evolution in the origins of bats. *Syst. Zool.* **37**, 199–216.

Pettigrew, J. D. 1991*b* A fruitful, wrong hypothesis? Response to Baker, Novacek and Simmons. *Syst. Zool.* **40**, 321–239.

Pettigrew J. D. 1994 Flying DNA. *Curr. Biol.* **4**, 277–280.

Pettigrew, J. D. 1995 Flying primates: crashed, or crashed through? In *Ecology, evolution and behaviour of bats* (ed. P. A. Racey & S. M. Swift), pp. 3–26. Oxford: Clarendon Press.

Pettigrew, J. D. & Kirsch, J. A. W. 1995 Flying primates revisited: DNA hybridization with fractionated, GC-enriched DNA. *S. Afr. J. Sci.* **91**, 477–482.

Pierson, E. D. 1986 Molecular systematics of the Microchiroptera: higher taxon relationships and biogeography. Unpublished Ph.D. thesis, University of California, Berkeley.

Porter, C. A., Goodman, M. & Stanhope, M. J. 1996 Evidence on mammalian phylogeny from sequences of exon 28 of the von Willebrand factor gene. *Mol. Phylogenet. Evol.* **5**, 89–101.

Rosa, M. G. P., Pettigrew, J. D. & Cooper, H. M. 1996 Unusual pattern of retinogeniculate terminations in the controversial primate, *Tarsius*. *Brain Behav. Evol.* **48**, 121–129.

Sabeur, G., Macaya, G., Kadi, F. & Bernardi, G. 1993 The isochore patterns of mammalian genomes and their phylogenetic implications. *J. Molec. Evol.* **37**, 93–108.

Shibutani, D., Takeshita, M. & Grollman, A. P. 1991 Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. *Nature* **349**, 431–434.

Simmons, N. B. 1994 The case for chiropteran monophyly. *Am. Mus. Novitates* **3103**, 1–54.

Simmons, N. B. 1998 Higher-level relationships of microchiropteran bats. In *Bats: phylogeny, morphology, echolocation, and conservation biology* (ed. T. H. Kunz & P. A. Racey). Washington: Smithsonian Institution Press. (In the press.)

Simmons, N. B., Novacek, M. J. & Baker, R. B. 1991 Approaches, methods, and the future of the chiropteran monophyly controversy. *Syst. Zool.* **40**, 239–243.

Swofford, D. L. & Olsen, G. J. 1990 Phylogeny reconstruction. In *Molecular systematics* (ed. D.M. Hillis & C. Moritz), pp. 411–501. Sunderland, MA: Sinauer Associates, Inc.

Werman, S. D., Springer, M. S. & Britten, R. J. 1990 Nucleic acids I: DNA–DNA hybridization. In *Molecular systematics* (ed. D.M. Hillis & C. Moritz), pp. 204–249. Sunderland, MA: Sinauer Associates, Inc.

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