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Base-compositional biases and the bat problem. II. DNA-hybridization trees based on AT- and GC-enriched tracers

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We conducted a series of parallel DNA-hybridization experiments on a small group of bats (species of *Pteropus, Rhinolophus, Noctilio* and *Pteronotus*) and outgroups (*Lemur, Cynocephalus, Didelphis*), using whole-genome labels and tracers made from extracts enriched with AT and two levels of GC content. FITCH (additive phylogenetic trees) topologies were constructed from the four sets of comparisons, indexed as both ΔT_{mode} and ΔNPHs (normalized percentage of hybridization). Based on our previous work showing that the shared AT bias of pteropodids and some microchiropterans may affect the rank-ordering of taxa based on either AT- or GC-rich labels, our expectation was that the resulting trees would show differing topologies when generated from tracers made with the variously enriched DNA extracts. Whereas there was some variation among the trees, most of them grouped the bats together, and almost all paired the representative megachiropteran and rhinolophoid microchiropteran as sister-taxa in contrast to the other microchiropterans. As the pteropodid–rhinolophoid relationship is an unexpected and unlikely one, we attribute this association to an AT bias that was not obviated even by our most GC-rich labels, and suggest that such a bias may compromise the truth of some molecular trees. Accordingly, we believe the broader issue of bat monophyly remains unresolved by DNA-hybridization and probably also by gene-sequencing studies.

Keywords: bat phylogeny; Chiroptera; molecular evolution; Primates

1. INTRODUCTION

Despite a lively literature on the question of bat monophyly (see, for example, Smith & Madkour 1980; Pettigrew 1986, 1991*a*,*b*, 1995; Pettigrew *et al.* 1989; Baker *et al.* 1991; Simmons *et al.* 1991; Thewissen & Babcock 1991; Simmons 1994), the issue of relationship between micro- and megachiropterans remains unresolved, even by the emerging sequence data on a number of genes. The failure of classical anatomical data to provide a convincing case for monophyly relates to the high correlation of many features with flight: these data remain, in essence, a single functional system diametrically opposed to the indications of many independent facts from brain morphology, which argue for a special relationship of Megachiroptera (but not Microchiroptera) with Primates, and hence a polyphyletic 'Order Chiroptera.'

Gene-sequencing data (Adkins & Honeycutt 1991; Mindell *et al.* 1991; Ammerman & Hillis 1992; Bailey *et al.* 1992; Stanhope *et al.* 1993; Porter *et al.* 1996) also fail to provide a convincing case for monophyly, partly because of scanty and sometimes inappropriate taxonomic sampling of bats, but also for the specific reason that all megachiropterans and many microchiropterans share an inordinately high AT bias. As this bias is shared with some other, undoubtedly unrelated mammals such as shrews (Sabeur *et al.* 1993), AT bias by itself cannot be an unquestioned synapomorphy of (many) bats. Given that the genes examined to date show as much as a fourfold preponderance of As and Ts over Gs and Cs in the matching bases purporting to support bat monophyly (Pettigrew 1994), and that the few microchiropterans included are sometimes just those known to have high AT contents (Arrighi *et al.* 1972; Sabeur *et al.* 1993), it appears likely that the resulting topologies may be unduly influenced by this base-compositional bias. The same distorting effect may also beset the two DNA-hybridization studies that have been done on bats (Kilpatrick & Nuñez 1993; Kirsch *et al.* 1995).

These observations led us to a prediction and to a test of the effect of base-compositional bias. The prediction is that phylogenetic inferences based on methods that avoid base-composition bias (e.g. those which examine GC-rich as opposed to AT-rich sequences or fractions of the genome) might give differing results from those that do not take the bias into account, i.e. ones which use indiscriminately chosen sequences or compare entire genomes. We undertook to test this possibility by conducting DNA-hybridization experiments using tracers made from portions of the genome enriched with either AT or GC. In our previous papers (Pettigrew & Kirsch 1995, this issue) we examined the properties of the individual melting profiles obtained from enriched tracers for evidence of bias, or its lack. Were bias of no account in determining ordering among bats and other mammals, the expectation would be that the relative positions of corresponding

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curves obtained with AT- or GC-rich labels would be the same. Instead, we found that the linear order of relationships among taxa obtained with single labels varied depending on the presumptive GC content of the tracers. Such differences might also be expected to be manifest in phylogenetic trees constructed from data using differently enriched labels.

Here we present least-squares trees based on assembled data from each set of tests made by Pettigrew & Kirsch (1998) for a suite of taxa whose mutual relationships are in question, including both 'controls' based on the entire ensemble of single-copy sequences (i.e. using whole-genome labels) and hybrids employing fractions of the DNA 'enriched' for AT or two levels of GC. Whereas our results give no clear evidence against bat monophyly, neither do they uniformly support it. At the same time, a common indication of the experiments is an association of *Pteropus* (a megachiropteran) with *Rhinolophus* (a rhinolophoid microchiropteran), independently of other microbats tested, a result which superficially indicates the paraphyly of Microchiroptera. Importantly, rhinolophoids have among the highest AT contents of all microchiropterans (around 70% (Pettigrew 1995; Pettigrew & Kirsch 1995)); only some megachiropterans among other mammals exceed them in this respect. As microchiropteran paraphyly is highly unlikely to be true (or at least acceptable to most students of bats, only Sigé (1993) among recent authors having suggested such a phylogeny), and there is no obvious reason for our finding other than a bias in base-composition, our results should strike a cautionary note about the easy acceptance of trees generated from molecular data: even when those data summarize large portions of the genome, as does DNA hybridization.

2. METHODS

(a) *Laboratory protocols*

Methods for extracting, fractionating, ^{125}I-labelling, and hybridizing of DNAs were as described in previous papers (Kirsch *et al.* 1990; Bleiweiss *et al.* 1994; Pettigrew & Kirsch, this issue). Thermal elution of hybrids followed a programme of 2 °C increments from 52–96 °C inclusive, preceded by two room-temperature washes to eliminate free iodine and unhybridizable DNA fragments.

(b) *Matrices and choice of indices*

The general experimental design consisted of generating complete or nearly complete matrices comparing either five taxa (with the GC-rich tracers) or seven taxa (with all other labels), in most cases with replication of each pairwise combination including those of the homologous or self-hybrids. The four matrices represent comparisons using tracers made with: (*a*) whole single-copy genomes; (*b*) AT-rich fractions (the combined four elutions below the mode in a 'melt' of native but sonicated DNA; see Pettigrew & Kirsch, this issue); (*c*) GC-rich fractions (all elutions at and above the mode); and (*d*) 'super'-GC-rich fractions (elutions from 94 °C upward only, taken from separate melts of DNA).

The melting profiles were indexed as T_{mode} and NPHs, indices chosen because they measure completely distinct characteristics of the hybrid elution curves: in the case of the mode, this is the temperature at which most of hybridized sequences melt, determined by fitting a parabola around the highest point of the elution curve and two flanking elutions on each side; NPH is simply the amount of DNA which has reassociated under the experimental conditions, calculated here as the percentage DNA eluted from 56–96 °C compared with the total from 52–96 °C, and normalized against the percentage hybridization of the homoduplex. Modes are not generally considered apt for the most distant (interordinal) comparisons attempted here, but are an easily recognized characteristic of melting curves and are generally impervious to extract or other random experimental variations (Bleiweiss & Kirsch 1993). We did in fact find consistent discrimination among the taxa using T_{mode}, and note that bat melting curves generally lack the paralogous low-temperature peak characteristic of some mammals, which may impede detection of the mode (Fox & Schmid 1980). NPHs, on the other hand, have a very high variance but are independent of curve-shape and are apparently suitable for resolving very distant relationships (Kirsch *et al.* 1991; Bleiweiss *et al.* 1995; Lapointe & Kirsch 1995). We felt that if any signal emerged from the disparate T_{mode} or NPH measures, and particularly if that signal was similar across matrices and indices, the information provided was likely to be an accurate representation of the data.

Because the yields from our fractionation scheme were often very low, and a few tracers were made from as little as 15 μg of DNA, the concentration (and hence radioactivity) of a tracer was sometimes low and curves were correspondingly subject to counting error. For this reason, modes for some hybrids could not be determined accurately, and these were not included in matrices based on the T_{mode}; the raggedness of many curves also meant that the often-used median melting-temperature (T_{m}) was not a reliable index.

(c) *Phylogenetic analyses and tree-validation*

The eight tables (four experimental sets, each indexed as mean differences, or Δs, between homologous and heterologous T_{mode} or NPH measures) were symmetrized to obviate inter-label variation by the method of Sarich & Cronin (1976) and completed if necessary by procedures described in Lapointe & Kirsch (1995) and Landry *et al.* (1996). In all but one case, completion involved only reflection of missing cells from their known reciprocals after symmetrization; the matrix of ΔT_{mode} for the AT-rich fractions, however, lacked two pairs of reciprocals, which were estimated additively (Landry *et al.* 1996).

FITCH trees (Felsenstein 1993) were generated from each set of comparisons, using the subreplicate, global branch-swapping, and Cavalli-Sforza & Edwards options; reflected or estimated cells were conservatively considered to have been measured just once. Robustness of the trees was tested by 'bootstrapping' each matrix 1000 times (Krajewski & Dickerman (1990); note that their method assesses experimental precision, and does not resample characters), generating a consensus from trees based on the pseudoreplicate matrices; and by jackknifing on taxa using the method for weighted trees of Lapointe *et al.* (1994), doing both single- and exhaustive-deletions of taxa (that is, of all possible combinations; only single deletions were possible on the five-taxon trees generated from GC-rich tracers, however). The jackknife results were expressed in each case as a tree showing the average of pathlengths for common taxa recovered over all pseudoreplicate trees. For both the bootstraps and jackknives, each pseudoreplicate matrix was separately symmetrized and completed before FITCH analysis.

Table 1. *Matrices of* (*left*) ΔT_{mode} *values* (*number of hybrids*, $n=219$) *and* (*right*) ΔNPHs ($n=233$), *obtained using whole-genome tracers*

(Columns are tracers, designated by first four letters of genus-names and first letters of specific epithets, given in rows. First lines of cells give average Δs, except that actual mean melting temperatures (rather than zeroes) are given for homologous modes to permit comparisons of labels within and between tables. Second lines give standard deviations (s.d.s; not counting missing cells or those with only one measurement) and numbers of replicates, separated by slashes. Average table-wide s.d.s were 0.91 and 4.79 for modes and NPHs, respectively; correlations of s.d.s with distance were 0.34 and 0.46, again respectively. Third lines give values used in tree-construction after symmetrization by the method of Sarich & Cronin (1976) and completion of tables. 'Corrections' at feet of columns are initial column-multipliers (row:column ratios) used to effect symmetrization; iterations (multiplication of column values followed by recalculation of row:column ratios) were continued until the ratios reached unity. Asymmetries before correction (filled cells only) and after were 4.67 and 1.90 (modes) and 8.76 and 5.57 (NPHs). Bold-faced numbers are missing cells which were reflected from their reciprocals after symmetrization to complete the table; these reflected values were considered as measured once in tree computations. The mean homologous T_{mode} values of all labels was 84.91 °C; the average ΔT_{mode} was 24.09 °C and the maximum Δ was 26.3 °C. Abbreviation: na, not applicable.)

	DideM	*CynoV*	*LemuC*	*PterV*	*RhinP*	*NoctA*	*PterP*	*DideM*	*CynoV*	*LemuC*	*PterV*	*RhinP*	*NoctA*	*PterP*
Didelphis marsupialis	83.53 0.27/3 0	24.85 1.19/2 25.42	27.52 1.96/5 26.89	25.78 1.22/4 25.44	28.90 0.68/3 27.82	27.26 na/1 26.13	27.52 0.95/2 24.93	0 0.00/3 0	59.52 3.04/4 55.32	58.42 3.94/5 54.57	54.45 4.62/4 56.54	58.52 7.00/4 50.72	57.53 6.92/3 55.04	52.00 3.96/2 53.06
Cynocephalus variegatus	21.27 0.63/4 25.61	85.11 0.72/9 0	23.49 0.17/4 22.95	24.80 1.53/8 24.47	24.35 1.19/6 23.44	26.42 1.65/7 25.33	25.99 1.02/4 23.54	41.32 7.26/4 52.04	0 0.63/9 0	33.74 11.44/5 31.52	37.15 3.94/8 38.57	27.98 9.82/9 24.25	36.50 1.76/7 34.92	32.70 1.90/4 33.36
Lemur catta	22.42 0.76/4 27.00	21.74 0.57/5 22.24	84.76 0.34/5 0	24.59 0.97/4 24.27	24.68 0.58/5 23.76	27.12 0.88/3 26.00	25.55 0.20/2 23.14	56.22 18.08/4 70.80	31.92 1.38/5 29.67	0 0.28/5 0	30.00 7.39/4 31.15	36.58 6.91/5 31.70	36.67 4.38/3 35.08	32.45 5.30/2 33.11
Pteropus vampyrus	23.03 3.17/3 27.73	23.97 0.81/9 24.52	24.64 1.31/5 24.08	84.62 0.24/8 0	21.23 1.27/9 20.44	25.06 1.60/8 24.02	23.74 0.32/4 21.50	45.40 7.64/3 57.17	44.83 5.82/9 41.67	37.18 4.02/5 34.73	0 2.10/8 0	25.60 5.48/9 22.19	32.99 4.81/8 31.56	23.75 5.16/4 24.23
Rhinolophus philippinensis	21.36 0.93/3 25.72	23.22 1.04/7 23.75	25.01 1.52/5 24.44	21.45 1.27/8 21.17	85.84 0.83/9 0	25.19 1.19/7 24.15	24.34 0.27/3 22.05	35.45 9.59/4 44.64	27.24 4.18/8 25.32	39.56 3.56/5 36.95	24.65 2.68/8 25.60	0 0.61/9 0	19.76 3.13/8 18.91	23.15 4.57/4 23.62
Noctilio albiventris	21.28 0.22/2 25.63	23.95 0.97/4 24.50	25.48 0.51/2 24.90	24.53 0.83/4 24.21	24.28 1.44/4 23.37	84.85 0.55/4 0	22.68 0.53/2 20.54	37.75 12.94/2 47.54	38.10 5.92/4 35.41	43.50 2.97/2 40.63	29.47 1.58/4 30.60	26.47 6.77/4 22.94	0 0.00/4 0	10.40 4.53/2 10.61
Pteronotus parnellii	na **24.93**	24.38 0.09/4 24.94	na **23.14**	23.03 0.95/4 22.73	23.32 0.82/4 22.45	18.28 1.64/3 17.52	85.66 0.28/4 0	na **53.06**	29.35 1.45/4 27.28	na **33.11**	28.02 3.97/4 29.09	26.80 2.43/4 23.23	12.78 2.29/4 12.23	0 6.78/4 0
correction	1.228	1.030	0.956	0.983	0.958	0.952	0.920	1.335	0.907	0.901	1.030	0.841	0.946	1.077

3. RESULTS

(a) *Tables and figure 1*

Tables 1–4 present the results for the four sets of comparisons. In each case, the left and right parts, respectively, give the ΔT_{mode} and ΔNPH values, with raw or unsymmetrized measurements on the first lines of each cell and symmetrized (as well as reflected or estimated) values on the third. Appropriate statistics are presented on the second lines of cells (standard deviations and numbers of replicates) and in the legends for each table (total number of hybrids; average table-wide s.d.; correlation of s.d.s with distance; percentage asymmetries before and after symmetrization; and—for unsymmetrized modes—mean homologous melting temperatures across labels as well as average and maximum Δs); 'corrections' at the bottoms of tables are the column-multipliers used to ameliorate asymmetry in each case. Iterations of the Sarich & Cronin (1976) algorithm were done until the row:column ratios reached unity.

Figure 1 is a composite of the eight FITCH trees generated from the symmetrized comparisons of tables 1–4, respectively, with bootstrap numbers given for all nodes other than the root (which is fixed by definition), and discrepancies from the FITCH topologies among the jackknife average-consensus trees shown as thin lines; the residual or unexplained sum-of-squares is indicated with each tree, but note that sums-of-squares are only comparable for the same matrix analysed by two or more algorithms, not across matrices. However, for each tree we also present the percentage of the total sum-of-squares of the corresponding matrix represented by these residuals, which is a measure comparable across all trees.

(b) *Descriptions of trees*

For the whole-genome FITCH trees (figure 1*a*,*b*), the topology for modes and NPHs is the same, uniting bats in two pairs (the microchiropterans *Noctilio* and *Pteronotus* as against the microchiropteran *Rhinolophus* with the megachiropteran *Pteropus*) opposed to the paired primate *Lemur* and dermopteran *Cynocephalus*. Bootstrap support in the ΔT_{mode} tree is moderate (75%) for the chiropteran clade and high (98%) for the primate–dermopteran grouping, with the jackknives supporting all dichotomies. As expected, the less-precise ΔNPH data give poorer support: the chiropteran grouping has low bootstrap

Table 2. *Matrices of (left)* ΔT_{mode} *values (n=96) and (right)* ΔNPH*s (n=105), obtained using tracers made with AT-enriched fractions*

(Conventions as for table 1, except that pairs of missing reciprocals (bold and underlined; modes only) were estimated by the additive procedure of Landry *et al.* (1996) and considered as measured once in tree-calculations. Average table-wide s.ds were 0.75 and 3.44 for modes and NPHs, respectively; correlations of s.d.s with distance were 0.41 and 0.19, again respectively. Asymmetries before correction (filled cells only) and after were 4.16 and 1.85 (modes) and 10.58 and 4.47 (NPHs). The mean homologous T_{mode} of all labels was 82.99 °C; the average ΔT_{mode} was 22.28 °C and the maximum Δ was 26.20 °C.)

	DideA	*CynoA*	*LemuA*	*PterV*	*RhinA*	*NoctA*	*PterP*	*DideA*	*CynoA*	*LemuA*	*PterV*	*RhinA*	*NoctA*	*PterP*
Didelphis	83.22	19.03	26.20	23.91	25.03	26.20	na	0	58.00	82.25	75.30	45.88	61.00	42.43
marsupialis	0.02/2	na/1	na/1	na/1	na/1	0.37/2		0.14/2	na/1	na/1	na/1	4.81/2	4.59/3	3.18/3
	0	21.35	24.87	23.50	25.57	23.77	**21.84**	0	62.80	60.58	76.31	57.86	68.89	44.67
Cynocephalus	23.81	81.68	22.53	23.44	21.15	24.59	na	66.80	0	50.35	47.90	40.74	56.87	35.17
variegatus	0.67/2	0.16/2	0.76/2	1.14/5	1.20/3	1.51/3		4.38/2	4.53/2	7.50/2	3.20/5	5.84/3	1.74/3	3.70/3
	23.65	0	21.39	23.03	21.60	22.31	**20.38**	66.66	0	37.08	48.54	51.38	64.22	37.03
Lemur catta	24.12	19.51	82.71	22.75	21.17	24.15	23.16	62.20	36.40	0	41.18	35.48	56.25	38.73
	0.19/2	1.41/2	0.48/2	1.37/4	1.51/2	0.22/2	1.43/2	3.96/2	3.68/2	6.58/2	5.45/4	na/1	1.20/2	4.66/3
	23.96	21.89	0	22.36	21.62	21.91	22.71	62.07	39.41	0	41.73	44.74	63.52	40.78
Pteropus	24.60	20.72	23.28	82.54	18.20	22.83	19.76	62.80	50.55	55.65	0	27.43	44.87	33.07
vampyrus	0.52/2	0.33/2	0.91/2	0.44/9	1.35/3	0.43/3	1.50/2	5.94/2	3.18/2	1.56/2	0.51/9	2.47/2	0.91/3	3.27/3
	24.44	22.74	22.10	0	18.59	20.71	19.37	62.66	54.73	40.99	0	34.59	50.67	34.82
Rhinolophus	23.39	21.12	22.62	19.31	82.26	22.96	19.28	66.30	43.15	66.95	26.04	0	38.73	17.60
philippinensis	0.34/2	0.47/2	0.33/2	1.51/5	0.20/4	0.43/3	1.77/2	3.39/2	3.18/2	na/1	4.94/5	5.17/4	3.11/3	0.65/3
	23.24	23.70	21.47	18.98	0	20.83	18.90	66.16	46.72	49.31	26.39	0	43.74	18.53
Noctilio	na	na	na	21.07	na	84.63	17.75	na	na	na	50.00	na	0	24.10
albiventris				na/1		0.24/3	0.59/3				na/1		1.55/3	4.38/3
	23.77	**22.31**	**21.91**	20.71	**20.83**	0	17.40	**68.89**	**64.22**	**63.52**	50.67	**43.74**	0	25.37
Pteronotus	na	na	na	na	na	19.19	83.92	na	na	na	na	na	22.47	0
parnellii						0.84/2	0.20/3						1.25/3	2.48/3
	21.84	**20.38**	**22.71**	**19.37**	**18.90**	17.41	0	**44.67**	**37.03**	**40.78**	**34.82**	**18.53**	25.37	0
correction	0.982	1.138	0.925	0.988	1.010	0.924	1.081	1.013	1.094	0.687	1.003	1.354	1.100	0.932

Table 3. *Matrices of (left)* ΔT_{mode} *values (n=69) and (right)* ΔNPH*s (n=81), obtained using tracers made with GC-enriched fractions*

(Conventions as for table 1, but only two cells (bold-faced) were reflected from their symmetrized reciprocal and considered as measured once in tree-computations. Average table-wide s.d.s were 1.19 and 4.79 for modes and NPHs, respectively; correlations of s.d.s with distance were 0.38 and 0.57, again respectively. Asymmetries before correction (filled cells only) and after were 6.08 and 2.93 (modes) and 16.35 and 4.02 (NPHs). The mean homologous T_{mode} of all labels was 85.89 °C; the average ΔT_{mode} was 25.23 °C and the maximum Δ was 29.14 °C.)

	DideG	*CynoG*	*LemuG*	*PterG*	*RhinG*	*DideG*	*CynoG*	*LemuG*	*PterG*	*RhinG*
Didelphis	85.48	25.46	na	28.82	28.57	0	57.50	na	60.97	57.57
marsupialis	0.08/3	2.41/2		0.48/3	na/1	0.40/3	7.95/4		1.14/3	4.13/4
	0	29.62	**25.86**	25.30	28.60	0	60.65	**55.81**	67.10	60.19
Cynocephalus	28.28	84.57	25.78	26.55	25.24	46.07	0	69.05	36.64	45.70
variegatus	1.63/3	0.40/5	0.08/2	0.61/5	1.57/3	2.58/3	4.47/5	7.28/2	6.38/5	8.35/3
	28.62	0	24.92	23.30	25.26	62.71	0	40.91	40.32	47.78
Lemur catta	25.55	23.56	86.71	25.51	24.59	41.00	41.90	0	36.27	36.20
	na/1	na/1	0.49/2	1.06/4	0.62/2	11.88/2	na/1	0.42/2	4.15/4	1.70/2
	25.86	27.41	0	22.39	24.61	55.81	44.20	0	39.92	37.85
Pteropus	24.54	17.11	25.76	85.46	22.69	48.60	44.03	68.30	0	26.33
vampyrus	1.94/3	2.18/2	0.59/2	0.36/9	1.51/3	3.77/3	2.31/4	na/1	0.49/9	0.91/3
	24.83	19.91	24.90	0	22.71	66.16	46.45	40.46	0	27.53
Rhinolophus	29.70	21.64	25.43	24.34	87.24	43.35	38.35	68.45	30.26	0
philippinensis	na/1	4.52/2	1.20/2	1.98/5	0.17/3	8.95/4	8.80/4	12.80/2	6.37/5	0.20/3
	30.06	25.18	24.59	21.36	0	59.01	40.45	40.55	33.30	0
correction	1.004	1.206	0.957	0.856	1.000	1.275	1.086	0.556	1.141	1.088

Table 4. *Matrices of* (*left*) ΔT_{mode} *values* ($n=131$) *and* (*right*) ΔNPH*s* ($n=145$), *obtained using tracers made with super-GC-enriched fractions*

(Conventions as for table 1, except that no data were missing from these comparisons. Average table-wide s.d.s were 0.79 and 2.44 for modes and NPHs, respectively; correlations of s.d.s with distance were 0.31 and 0.14, again respectively. Asymmetries before and after correction were 2.77 and 1.97 (modes) and 12.96 and 5.28 (NPHs). The mean T_{mode} of all labels was 87.99 °C; the average ΔT_{mode} was 27.07 °C and the maximum Δ was 29.99 °C. Tracer and some drivers were *Noctilio leporinus* for this set of comparisons.)

	DideM	*CynoV*	*LemuC*	*PterV*	*RhinP*	*NoctL*	*PterP*	*DideM*	*CynoV*	*LemuC*	*PterV*	*RhinP*	*NoctL*	*PterP*
Didelphis	88.26	28.79	29.95	25.89	29.51	28.67	30.49	0	45.67	51.27	57.67	43.30	42.83	45.77
marsupialis	0.25/3	1.65/3	5.30/2	na/1	0.30/2	0.09/3	2.58/3	1.40/3	8.29/3	0.50/3	2.14/3	0.71/2	2.45/3	1.16/3
	0	29.71	28.84	25.84	28.71	29.63	31.69	0	50.49	39.36	51.31	44.68	59.23	52.15
Cynocephalus	31.18	87.68	25.20	27.61	26.54	27.27	27.02	51.90	0	37.50	39.37	31.50	35.67	36.80
variegatus	0.05/2	0.22/3	0.54/3	1.13/3	0.70/3	0.80/3	0.23/3	2.86/3	2.32/3	1.59/3	1.33/3	1.73/3	3.11/3	2.59/3
	30.09	0	24.56	27.56	25.82	28.19	28.08	46.72	0	28.79	35.03	32.50	49.33	41.93
Lemur catta	27.30	24.48	88.23	25.08	26.06	26.87	24.46	51.77	22.47	0	37.30	24.73	22.40	33.43
	0.49/3	0.48/3	0.43/3	0.35/3	0.15/3	0.09/3	na/1	0.67/3	0.61/3	1.36/3	1.31/3	1.02/3	4.46/2	5.11/3
	26.35	25.26	0	25.03	25.35	27.77	25.42	46.61	24.84	0	33.19	25.52	30.98	38.09
Pteropus	31.61	24.20	25.30	88.59	24.13	25.59	25.39	54.70	36.70	42.67	0	21.77	23.97	30.23
vampyrus	1.37/3	0.77/3	0.11/2	0.07/3	0.79/3	0.41/3	0.78/3	2.69/3	2.63/3	1.59/3	1.25/3	2.68/3	1.52/3	4.22/3
	30.51	24.98	24.36	0	23.48	26.45	26.39	49.25	40.57	32.76	0	22.46	33.15	34.44
Rhinolophus	28.72	25.85	25.55	24.25	87.73	26.36	25.96	54.47	25.73	38.77	25.60	0	23.33	26.50
philippinensis	2.04/2	1.15/3	0.34/3	0.32/3	0.16/3	0.34/3	0.86/3	5.80/3	0.72/3	1.67/3	2.08/3	1.71/3	0.25/3	1.97/3
	27.72	26.68	24.60	24.20	0	27.25	26.98	49.04	28.45	29.76	22.78	0	32.26	30.19
Noctilio	30.09	28.28	27.09	26.92	27.84	87.74	29.46	53.93	39.93	43.83	41.20	32.60	0	28.00
albiventris or	0.80/2	2.47/2	0.28/2	0.29/3	0.29/3	0.29/3	1.92/2	2.07/3	2.43/3	5.38/3	3.16/3	2.71/3	2.10/3	3.60/3
N. leporinus	29.04	29.19	26.09	26.87	27.09	0	30.62	48.55	44.14	33.65	36.66	33.64	0	31.90
Pteronotus	31.84	27.59	27.76	26.71	27.73	28.63	87.71	63.37	41.43	45.47	37.83	32.63	17.07	0
parnellii	na/1	0.87/2	1.46/3	0.64/3	0.31/3	0.79/3	0.40/3	3.09/3	3.82/2	2.48/3	1.18/3	1.14/3	4.95/3	3.92/3
	30.73	28.47	26.73	26.66	26.98	29.59	0	57.05	45.80	34.91	33.66	33.67	23.61	0
correction	0.959	1.037	0.957	0.998	0.968	1.039	1.046	0.868	1.098	0.740	0.879	1.042	1.449	1.185

support (55%), whereas the *Rhinolophus*–*Pteropus* pair is found in only 36% of the pseudoreplicate bootstrap trees and has no jackknife support.

Trees based on the AT-rich fractions do not differ greatly from the whole-genome topologies, except in that the FITCH ΔT_{mode} tree (figure 1*c*) separates *Lemur* and *Cynocephalus*, placing the former as sister to the bats (arranged as in figure 1*a*), with only 60% bootstrap but complete jackknife support for the lemur–bat clade. Figure 1*d*, based on ΔNPHs, is topologically identical to the corresponding whole-genome tree (figure 1*b*), but all nodes have moderate or high bootstrap support. However, bat monophyly is not supported by the jackknives, the two pairs of bats collapsing to a trichotomy with the 100%-supported *Cynocephalus*–*Lemur* pair.

The next set of experiments, based on GC-rich labelled fractions concentrated from elutions at and above the mode, included only five taxa, omitting *Noctilio* and *Pteronotus*. Even so, both FITCH trees differ markedly for common taxa from the preceding dendrograms. The ΔT_{mode} tree (figure 1*e*) does not resolve pairwise relationships among a strongly supported (bootstrap of 98%) trio including *Rhinolophus*, *Pteropus*, and *Cynocephalus* (the second and third of these are paired, but with only 38% bootstrap support); *Lemur* is of course their sister-taxon. The ΔNPH tree (figure 1*f*), on the other hand, strongly supports (at 97%) a *Rhinolophus*–*Pteropus* pair, with *Cynocephalus* weakly (58%) united with both; *Lemur* is again sister to these three. Relations in both FITCH trees are supported by the jackknives.

The two 'super-'GC-rich trees, again of seven taxa, differ markedly in one respect. The FITCH ΔT_{mode} tree (figure 1*g*) excludes the noctilionoid microbat *Noctilio* from a grouping of all other eutherian taxa, placing *Pteronotus* (another noctilionoid) alone as sister to a *Rhinolophus*–*Pteropus* duo; the latter pairing, however, is only moderately supported by the bootstrap (at 75%) as is the case for most other pairings, but is consistent with the jackknives. The ΔNPH tree (figure 1*h*), however, shows the same topology as in whole-genome and AT-rich ΔNPH and some ΔT_{mode} trees, namely, a monophyletic Chiroptera within which two pairs of taxa (*Pteronotus*–*Noctilio* and *Rhinolophus*–*Pteropus*) are contrasted with the united *Lemur* and *Cynocephalus*. Moreover, all nodes are strongly or moderately supported by bootstrapping and there are no discrepant average-consensus jackknife trees.

In summary, trees based on the four sets of labels differ among themselves, even when the same index is used. In general, however, trees based on the whole-genome and AT-rich tracers are more alike (between indices and among label-types) than are those generated from the GC-rich and super-GC-rich labels. Even so, some common features are evident overall: first, in most, but not all trees, bats are grouped together—the main exception being the ΔT_{mode} tree based on super-GC-rich tracers. Second, *Lemur* and *Cynocephalus* are frequently, but not always, paired—and

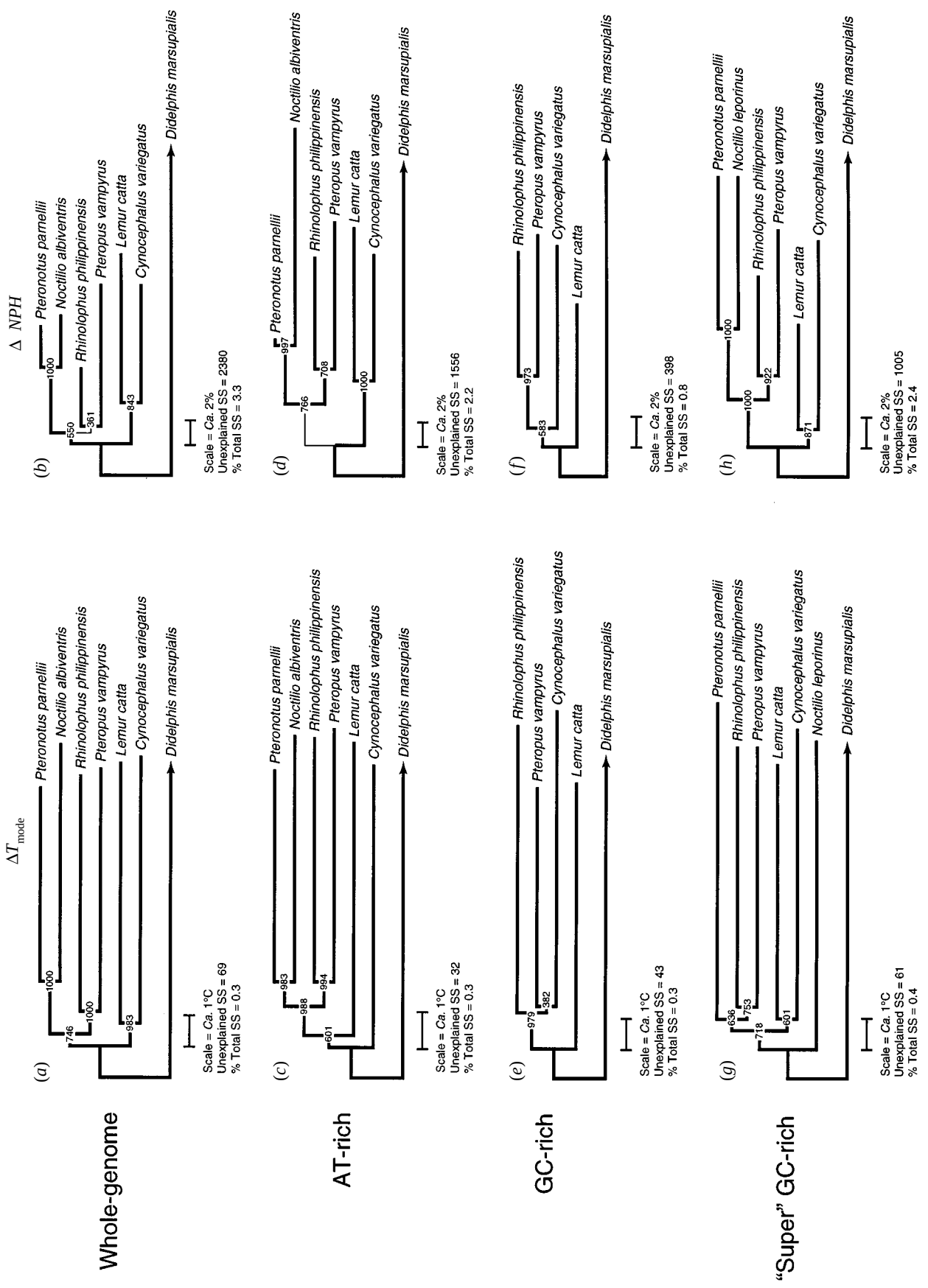

Figure 1. Results of FITCH analyses and validation tests on the symmetrized and (where relevant) completed matrices of tables 1–4; figures approximately to scale. Unexplained sum-of-squares (SS) is given for each tree; percentage of the total sum-of-squares for the corresponding data matrix is also given, which permits comparisons across all analyses. Trees in rows are based on ΔT_{mode} values (left) and ΔNPHs (right), obtained with whole-genome, AT-rich, GC-rich, and super-GC-rich tracers (top to bottom). Numbers at nodes are bootstraps (except for the root, which was fixed). Thin lines indicate pairings that were not supported in average-consensus jackknife tests (based on single- and (where relevant) exhaustive-deletions).

when they are not, it is not always clear which taxon might be nearer the bats. Third, in virtually all trees *Rhinolophus* and *Pteropus* comprise a terminal pair, and in the one where they do not (figure 1*e*), bootstrap support is low for an alternative arrangement (and no other bat but these two was included in the relevant data set). The highly distinctive ΔT_{mode} tree generated from super-GC-rich tracer data, which places *Noctilio* outside all other eutherians, may be of special significance or could possibly result from an experimental or curve-fitting artefact (see below), as the same result was not obtained with ΔNPH data from the identical labels.

4. DISCUSSION

An implication of the parallelism (or 'following behaviour') among many of the paired curves considered in Pettigrew & Kirsch (1995, this issue) is that AT : GC content may not greatly influence relative genetic distances among the species showing 'following'. It follows that trees for these taxa based on those data should be much the same, irrespective of the type of fraction. However, the longer distances obtained with GC-rich fractions might be expected to give better interordinal resolution than those generated from whole-genome or AT-rich labels. Furthermore, 'anomalous' curves—those for which the rule of 'following behaviour' was violated—might further lengthen some tree-fitted distances or even alter topologies.

In fact, the trees obtained herein were not identical, and one (based on ΔT_{mode} measures for the 'super-'GC-rich fraction) was clearly anomalous in excluding *Noctilio leporinus* from association with any other bat. The separation of *Noctilio* from *Pteronotus* in that tree (unlike in most others, where these taxa comprised a terminal pair) seems to be mostly caused by the large reciprocal ΔT_{mode} values between them (see table 4, at left). However, the underlying curves are flatter than most and are therefore recalcitrant to curve-fitting for determination of modes. Still, a tree calculated from data where those ΔT_{mode} measures were deleted and then estimated by Landry *et al.*'s (1996) method (not shown) had the same topology as figure 1*g*. Otherwise, the various trees almost always supported pairings of *Noctilio* with *Pteronotus* (when included), *Pteropus* with *Rhinolophus*, and *Lemur* with *Cynocephalus*; a larger grouping of all bats was also evident on many but not all trees. At the same time, and judging by the bootstrap percentages, resolution was not greatly improved by using the more restrictive (GC-rich) fractions, although the jackknives were more consistent when GC-rich tracers were employed.

Thus, our experiments do not clearly falsify bat monophyly, even when the most GC-rich fractions are employed; but at the same time, neither do they fully support a monophyletic Chiroptera, tending to give less support for bat monophyly when the more GC-rich tracers were used. Our suspicion is that even more restrictive fractions might well enhance the tendency of high-GC labels to dissociate the bats, because even our most GC-enriched labels only increased GC content by about 2% (Pettigrew & Kirsch, this issue)—much too little to offset the more than 10% AT bias of megachiropterans and many microbats.

Whereas the inconclusive result regarding overall bat relationships may seem to give only moderate support to the supposition of a base-composition effect on phylogenies generated by DNA hybridization, we are at a loss to explain the particular association of *Rhinolophus* with *Pteropus* in any other way. Moreover, the generally consistent pairing of these taxa in mode- and NPH-based trees (irrespective of type of label) suggests that either index of distance recovers the same 'signal', whether the pteropodid–rhinolophoid association is phylogenetically authentic or not. We note especially that the most 'extreme' trees—that based on whole-genome distances indexed by ΔT_{mode} measures and that from ΔNPHs using super-GC-rich labels—were topologically identical. Yet, the small number of species examined here raises again the spectre of an algorithmic artefact (long-branch attraction (Felsenstein 1978; Swofford & Olsen 1990), a possibility addressed by the third paper in this series (Hutcheon *et al.* 1998). An alternative explanation is that rhinolophoids and pteropodids are 'attracted' because of apparent rate-slowdowns in their lineages. In fact, in accord with the prediction that AT-biased taxa might seem to have evolved more slowly than unbiased taxa (Pettigrew & Kirsch, this issue), the branches bearing and uniting *Rhinolophus* with *Pteropus* are often among the shortest in our trees (see especially figure 1*h*). Significantly, an ultrametric KITSCH tree calculated from the super-GC-rich ΔNPH data (table 4, at right) linked the *Rhinolophus–Pteropus* pair with that of *Lemur* and *Cynocephalus*, rather than with the other two microbats.

A base-compositional bias effect on phylogeny-estimation therefore seems likely. That it may or may not affect interordinal mammalian relationships as a whole could reflect a limitation in the stringency of our fractionation scheme, but AT bias almost certainly explains in part the apparent paraphyly of Microchiroptera observed here. Experiments with even more restricted fractions are continuing and should provide additional information relevant to the question of bat monophyly and infraordinal relationships among Chiroptera.

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