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W(H)ITHER FOSSILS? STUDYING MORPHOLOGICAL CHARACTER EVOLUTION IN THE AGE OF MOLECULAR SEQUENCES[1]

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ABSTRACT

A major challenge in the post-genomics era will be to integrate molecular sequence data from extant organisms with morphological data from fossil and extant taxa into a single, coherent picture of phylogenetic relationships; only then will these phylogenetic hypotheses be effectively applied to the study of morphological character evolution. At least two analytical approaches to solving this problem have been utilized: (1) simultaneous analysis of molecular sequence and morphological data with fossil taxa included as terminals in the analysis, and (2) the molecular scaffold approach, in which morphological data are analyzed over a molecular backbone (with constraints that force extant taxa into positions suggested by sequence data). The perceived obstacles to including fossil taxa directly in simultaneous analyses of morphological and molecular sequence data with extant taxa include: (1) that fossil taxa are missing the molecular sequence portion of the character data; (2) that morphological characters might be misleading due to convergence; and (3) character weighting, specifically how and whether to weight characters in the morphological partition relative to characters in the molecular sequence data partition. The molecular scaffold has been put forward as a potential solution to at least some of these problems. Using examples of simultaneous analyses from the literature, as well as new analyses of previously published morphological and molecular sequence data matrices for extant and fossil Chiroptera (bats), we argue that the simultaneous analysis approach is superior to the molecular scaffold approach, specifically addressing the problems to which the molecular scaffold has been suggested as a solution. Finally, the application of phylogenetic hypotheses including fossil taxa (whatever their derivation) to the study of morphological character evolution is discussed, with special emphasis on scenarios in which fossil taxa are likely to be most enlightening: (1) in determining the sequence of character evolution; (2) in determining the timing of character evolution; and (3) in making inferences about the presence or absence of characteristics in fossil taxa that may not be directly observable in the fossil record.

Key words: Character mapping, Chiroptera, convergence, echolocation, fossil, homoplasy, molecular scaffold, molecular sequence data, morphological character evolution, phylogeny, simultaneous analysis, total evidence.

At one time, extinct taxa represented by fossils (hereafter, fossil taxa) were considered central to understanding the evolution of organisms through time (see, for instance, Eldredge & Cracraft, 1980; Smith, 1998). Phylogenetic hypotheses were developed by a qualified expert or experts on the basis of comparative anatomy and morphology, to which fossils were considered to contribute primitive and intermediate forms through which one could trace evolution from ancestor to descendant to the most recent members of a group. Characters considered meaningful to the development of evolutionary scenarios were entirely at the discretion of the investigator, and overall similarity as well as the appearance of advanced features (i.e., synapomorphies) were considered important in interpreting relationships. With the advent of the framework explicated by Hennig (1966) and the development of analytical methodologies and programs for tree-building (e.g., Farris, 1970; Fitch, 1971; Felsenstein, 1981), paleontology became less central to understanding evolution through geologic time (despite the early recognition of the logic and utility of the cladistic methodology by some paleontologists [e.g., Schaeffer et al., 1972]) because extant organisms could be grouped on the basis of shared derived traits—or synapomorphies—without reference to the fossil record. In fact, fossil taxa, for which many data were often missing and whose interpreta-

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tion was potentially difficult, became viewed by some as an impediment to understanding phylogenetic relationships among extant taxa (e.g., Patterson, 1981). Molecular systematics, which provides large numbers of sequence characters, has altered our understanding of the relationships among and within many groups, often without reference to fossil taxa.

However, fossil taxa provide unique types of information not available in extant organisms, and, because of this, the recognition of fossil taxa as an important component of phylogenetic studies has recently experienced a renaissance (Smith, 1998). Some of this may be due to the temporal information that fossil taxa can provide about the rate and timing of group diversification, principally in the application of temporal data associated with the occurrences of fossils as calibration points in studies of the rate of evolution of molecular sequence characters (e.g., Peterson et al., 2004; Schneider et al., 2004). Fossils are also unique repositories of data on extinct morphologies for groups both with and without representation in the extant biota. Thus, fossil taxa can provide insight into the sequence of evolution within morphological characters that are correlated in extant taxa, as well as access to suites of characters or variations within characters that would be entirely lost if not for knowledge of the vast extinct flora and fauna that once flourished on earth (Donoghue et al., 1989; Smith, 1998; Forey & Fortey, 2001).

One of the biggest challenges for paleontologists and systematists alike in the post-genomics era will be to figure out how best to incorporate paleontological data (primarily anatomical and morphological data, hereafter simply referred to as morphological data) with molecular sequence data from extant organisms to take advantage of these unique aspects of fossil taxa (e.g., see Peterson et al., 2007). The issues involved in this subject are complex, ranging from character delimitation and interpretation, the effect of missing data on analyses, and whether to combine data sets and analyze fossil taxa directly with extant taxa—referred to as simultaneous analysis (Nixon & Carpenter, 1996) or combined analysis, or the total evidence (Kluge, 1989) or supermatrix approach (see review in de Queiroz & Gatesy, 2007)—or to use more indirect methods, such as trees based on molecular backbone constraints, sometimes referred to as "molecular scaffolds" (Springer et al., 2001: 6242). Herein, we compare several methods for combining morphological and molecular sequence data for a group (Chiroptera, bats) that has attributes (e.g., a large body of molecular sequence data conflicting with traditional groupings based on morphology, several well-preserved fossil representatives) emblematic of the current problems confronting the integration of extant with fossil taxa in which molecular sequence data are involved. We will use these analyses as examples in a review of major issues surrounding tree-building and the interpretation of character evolution in joint fossil-extant taxon analyses that include a molecular sequence and morphological component. In the first part of this discussion, we argue that, because many of the issues surrounding character mapping on a phylogeny are not unique to analyses in which fossil taxa are included, the underlying problem in studying fossil taxa in a phylogenetic context is to identify the most effective way to integrate our knowledge of morphological characters with our evolving knowledge of the tree of relationships among extant organisms as suggested by molecular sequence data. In the second part, we discuss the utility and complications of mapping characters and studying character evolution in a context in which fossil taxa are included as terminals in a phylogenetic analysis, emphasizing examples from simultaneous analyses.

Background

The Cenozoic record of the placental mammal clade Chiroptera (bats) provides a good data set to explore how paleontological, morphological, and molecular sequence data interact in phylogenetic analyses, and how, in turn, these data types can inform hypotheses of the sequence and timing of morphological character evolution. A plethora of morphological and molecular sequence data has been collected about bats, and several analyses have integrated data sets in order to explore patterns of character evolution and biogeography within both extant and fossil members of the Chiroptera (Springer et al., 2001; Teeling et al., 2005). Traditionally, bats have been grouped by morphological data into two clades, Microchiroptera (microbats), with laryngeal echolocation (a biological form of sonar to hunt prey), and Megachiroptera (Pteropodidae; flying foxes and Old World fruit bats), lacking laryngeal echolocation (Simmons, 2005a). Recently, analyses of molecular sequence data have challenged this traditional view of bat evolution (see reviews in Simmons, 2005a; Jones & Teeling, 2006); these data suggest that Pteropodidae (flying foxes and Old World fruit bats) and an echolocating microbat group called Rhinolophoidea (horseshoe bats) are more closely related to one another than either is to the remaining microbats. The clade including Pteropodidae and Rhinolophoidea is known as Yinpterochiroptera, while the clade including other echolocating bats has been referred to as Yangochiroptera (Springer et al., 2001). While it was once thought that laryngeal echolocation—which has a complex morphological basis (Arita

& Fenton, 1997)—evolved only once in bats, the new view of bat phylogeny based on molecular sequence data raises the possibility that laryngeal echolocation either evolved twice independently, or evolved once, but was lost in Pteropodidae (Springer et al., 2001; Jones & Teeling, 2006).

To date, direct simultaneous analyses (Nixon & Carpenter, 1996) of combined morphological and molecular sequence data sets from extant families across the order Chiroptera (with or without fossil taxa) are lacking, despite the potential that these combined data may have for further clarifying the relationships of bats (Simmons, 2005a) and the evolution of echolocation. Here, we explore whether performing such an analysis will support the new molecular view of bat phylogeny, as predicted by Simmons (2005a: 167). Prior research in this area has been undertaken only indirectly: Springer et al. (2001) and Teeling et al. (2005) used a molecular scaffold approach (e.g., used backbone constraints) to place fossil taxa within a phylogenetic context.

Materials and Methods

combined data matrix

The combined morphological and molecular sequence data set analyzed here was constructed from two previously published data matrices. The NEXUS data file representing the morphological matrix published by Gunnell and Simmons (2005) was downloaded from the American Museum of Natural History FTP site linked directly from Nancy Simmons' homepage (http://research.amnh.org/mammalogy/personnel/simmons.php). This morphological matrix features a total of 35 terminal taxa, six of which are fossil taxa—*Archaeonycteris* Revilliod, *Hassianycteris* Smith & Storch, *Icaronycteris* Jepsen, *Palaeochiropteryx* Revilliod, *Tanzanycteris* Gunnell et al., and an undescribed genus from the Eocene Green River Formation of Wyoming (Gunnell & Simmons, 2005)—and five of which are extant outgroup taxa (*Cynocephalus* Boddaert, flying lemur; *Erinaceus* L., hedgehog; *Felis* L., cat; *Sus* L., pig; and *Tupaia* Raffles, tree shrew). There are 204 morphological characters, 94 of which are soft tissue and 110 of which are osteological characters; 165 have nonadditive (unordered) transformations and 39 have additive (ordered) transformations. All extant ingroup terminals (n = 24) in this morphological matrix are extant familial or subfamilial bat taxa. Fossil taxa are scored at the genus level.

The aligned molecular sequence data matrix, the basis for the study by Teeling et al. (2005), was kindly provided to the authors (specifically, JRH) by Dr. Teeling on June 9, 2006. The complete molecular sequence data matrix includes 13,792 aligned sequence characters representing "nuclear sequence data from portions of 17 nuclear genes" (Teeling et al., 2005: 581) from 30 bat genera (representing all families of Chiroptera; sequence data for some ingroup terminal genera are composites from multiple infrageneric species) and four outgroup terminals represented by composite sequence data gathered from two or more genera each. Details of this matrix, including GenBank accession numbers, were provided by Teeling et al. (2005; supplementary table S6).

Three options for reconciling overlapping taxa between the two matrices presented themselves at the beginning of this study: (1) culling taxa from the molecular sequence data set, leaving one generic-level terminal to combine with the corresponding family or subfamily terminal in the morphological data set; (2) fusing terminals in the molecular sequence data set so that all molecular sequence variability for each family or subfamily was encompassed in one corresponding terminal in the morphological matrix; or (3) duplicating morphological terminals in order that each terminal represented by a molecular sequence data set also had a morphological data set, some of which would be identical for members of the same family or subfamily. Each option has potential pitfalls. The first would discard the most data, the second would result in an increase of polymorphisms in the molecular sequence data set, and the third would result in multiple terminals sharing the same set of morphological characters. For a more generalized discussion of the problem of terminal mismatch in combining data matrices, see Nixon and Carpenter (1996).

We decided to use option three (duplication of morphological terminals), because at least some studies have suggested that phylogenetic accuracy increases with greater taxon sampling (e.g., Zwickl & Hillis, 2002), and we did not wish to discard information; further, we did not want to add to the ambiguity of the combined data set (which already includes many cells coded as missing) by fusing molecular sequence terminals. This option allowed us to keep all terminals represented in the molecular sequence data set except Perissodactyla (odd-toed hoofed mammals), for which we could find no reasonable combination with a terminal in the morphological data set (see further discussion below). The number of terminals represented by molecular sequence data that share the same duplicated morphological data in the combined data set range from zero (17 taxa, including three of the outgroups) to two (two groups of two taxa), three (one group of three taxa), or four (two groups of four taxa). For genera with identical morphological data sets, the morphological data obviously supply no information on intrafamilial

relationships; in the simultaneous and molecular scaffold analyses, these are completely structured by the molecular sequence data.

One potentially problematic aspect of the morphological data set is that, where polymorphisms existed in the earlier Simmons and Geisler (1998) morphological matrix, of which the Gunnell and Simmons (2005) matrix is a modification, Gunnell and Simmons (2005) replaced them either with an inferred ancestral state (IAS) for the family or subfamily (IAS coding) or with the most common state in the family or subfamily, or used ambiguity coding for that character (see Simmons & Geisler, 2002). Simmons and Geisler (1998) listed the number and percent of polymorphisms within each terminal in the previous version of this matrix, thus giving some indication of how many cells may have been converted from polymorphic to single state for each terminal in Gunnell and Simmons (2005). This means that, in cases in which polymorphisms may occur intrafamilially or intrasubfamilially, the inferred plesiomorphic state within the higher-level terminal may be substituted for a polymorphism (Simmons & Geisler, 2002), and this state may or may not occur in the genus matched with the higher-level terminal. Hence, the analyses could be improved in the future by coding the states present in individual genera, rather than in families or subfamilies. As a corollary, not all characters coded for each higher-level terminal may have been observed in each genus included in the molecular sequence matrix, so some extrapolation of character states within genera may be occurring (see Nixon & Carpenter [1996] concerning extrapolation). According to Simmons (2005c: 527), extant bats are classified into 18 families, another six families are known from fossils, and "biologists have long agreed that these groups represent distinct evolutionary lineages," although "there has been no consensus concerning relationships among them." Because the monophyly of the families within Chiroptera is apparently not in question and is further supported by the molecular sequence data set employed here (Teeling et al., 2005), we do not anticipate error caused by incorrectly assigning some genera to the wrong family. According to Simmons and Geisler (1998), monophyly of all bat taxa (families and subfamilies) included as terminals in the analysis is well established, except perhaps for Vespertilioninae; only one genus, *Rhogeessa* H. Allen, is assigned to Vespertilioninae in this study.

Combining these two matrices required renaming the terminal extant bat taxa in the morphological matrix (suprageneric) with the generic terminal names in the molecular sequence data matrix. The classification of Simmons (2005b) was used to match each extant bat genus with the suprageneric taxon to which it belongs (see Table 1). For the outgroups, the composite terminal *Felis*/*Panthera* Oken in the molecular sequence data set was matched to the morphological data set for *Felis* and named *Felis*; the composite terminal *Condylura* Illiger/*Talpa* L./*Scalopus* Desmarest (moles) was matched with *Erinaceus* (hedgehog), and these were renamed Eulipotyphla, a monophyletic clade composed of some former members of the Insectivora, including hedgehogs, shrews, and moles (e.g., Murphy et al., 2001). Finally, the composite molecular sequence terminal *Tragelaphus* Blainville/*Bos* L. (bovines) were combined with the morphological data for *Sus* (pigs) to form the terminal Cetartiodactyla, a clade supported by molecular sequence data (e.g., Montgelard et al., 1997; Murphy et al., 2001; Boisserie et al., 2005) that also includes additional ruminants, whales, and hippopotami. The Perissodactyla outgroup, composed of sequence data from *Equus* L. (horses) and *Ceratotherium* Gray (rhinoceroses), could not be combined with a morphological terminal, as no perissodactyls are outgroups in the morphological matrix. Because it lacks morphological data, the Perissodactyla outgroup can then act as a "wild card" (Nixon & Wheeler, 1992: 134; see discussion below), interacting with fossil bat taxa that group between the outgroup taxa and extant ingroup bats, since Perissodactyla and the fossil bat taxa are coded for mutually exclusive data sets. Perissodactyla was thus removed from the combined matrix. Four extant bat subfamilies and two outgroup terminals represented in the Gunnell and Simmons (2005) matrix that are not represented at all in the Teeling et al. (2005) data set were allowed to remain in the combined matrix, since they were all coded for the morphological characters. These taxa were thus similar to (but more complete than) the fossil taxa. The total combined morphological and molecular sequence data set (hereafter referred to as the combined data set) included 45 terminal taxa (five outgroups) and 13,996 characters (39 additive). All characters were weighted equally.

CLADISTIC ANALYSES

Terminals were duplicated and renamed in WordPad (Microsoft Corporation, Redmond, Washington), matrix dimensions were modified (where necessary), and the files were opened in WinClada (Nixon, 1999–2002). Matrices were combined in WinClada, with terminals matched as detailed in Table 1. The combined data set was saved in .ss format before it was opened in the software program TNT (Goloboff et al., 2003a), where it was resaved in TNT format. Tree searches were performed under the parsimony criterion using TNT (Goloboff et al., 2003a). For each

Table 1. Morphological (family and subfamily) data set matched to each molecular sequence (genus) terminal. Assignments of genera to higher taxa follow Simmons (2005b). Abbreviations in the molecular sequence data column correspond to the GenBank molecular sequence accession information in supplementary table S6 of Teeling et al. (2005); the first letter corresponds to the genus name, the next two letters to the molecular source species name, and the number in the bracket refers to composite terminal numbers in table S6 of Teeling et al. (2005). Four extant terminal taxa possess morphological data (from Gunnell & Simmons, 2005) but lack corresponding molecular sequence data; these include Tomopeatinae Miller (Molossidae) and Miniopterinae Dobson, Murininae Miller, and Kerivoulinae Miller (Vespertilionidae).

Extant bat genus	Taxonomic level (family or subfamily) from which morphological data were coded[1]	Molecular sequence data source[2]
Antrozous H. Allen	Antrozoidae	Apa[19]
Craseonycteris Hill	Craseonycteridae	Cth[30]
Emballonura Temminck	Emballonuridae	Eat[11]
Taphozous E. Geoffroy	Emballonuridae	Tnu[12]
Rhynchonycteris Peters	Emballonuridae	Rna[13]
Furipterus Bonaparte	Furipteridae	Fho[26]
Hipposideros Gray	Hipposideridae	Hco[6]
Megaderma E. Geoffroy	Megadermatidae	Mly[7]
Macroderma Miller	Megadermatidae	Mgi[8]
Tadarida Rafinesque	Molossinae	Tbr[28]
Eumops Miller	Molossinae	Eau[29]
Pteronotus Gray	Mormoopidae	Ppa[23]
Myotis Kaup	Myotinae	Mda[21]
Mystacina Gray	Mystacinidae	Mtu[25]
Myzopoda Milne-Edwards & A. Grandidier	Myzopodidae	Mau[22]
Natalus Gray	Natalidae	Nst[27]
Noctilio L.	Noctilionidae	Noctal[18]
Nycteris G. Cuvier & E. Geoffroy	Nycteridae	Ngr[9]
Tonatia Gray	Phyllostomidae	Tsi[14]
Artibeus Leach	Phyllostomidae	Aja[15]
Desmodus Wied-Neuwied	Phyllostomidae	Dro[16]
Anoura Gray	Phyllostomidae	Age[17]
Pteropus Erxleben	Pteropodidae	Pgi[1]
Cynopterus F. Cuvier	Pteropodidae	Cbr[2]
Rousettus Gray	Pteropodidae	Rla[3]
Nyctimene Borkhausen	Pteropodidae	Nal[4]
Rhinolophus Lacépède	Rhinolophidae	Rcr[5]
Rhinopoma E. Geoffroy	Rhinopomatidae	Rha[10]
Thyroptera Spix	Thyropteridae	Ttr[24]
Rhogeessa H. Allen	Vespertilioninae	Rtu[20]

[1] From Gunnell and Simmons, 2005.
[2] From Teeling et al., 2005.

analysis, the collapsing rule (determining which nodes will be collapsed from dichotomous to polytomous in most parsimonious trees [MPTs]) was set to rule 3, which only collapses nodes with no character support (max length = 0). For each analysis, a heuristic search was employed using the following parameters: starting Wagner trees were calculated using a random seed of 0; 1000 search replications were performed with tree bisection-reconnection branch-swapping (Swofford & Olsen, 1990), saving up to 10 shortest trees per search replication. Trees were collapsed after the search (in other words, branches with a maximum length of 0 were collapsed into polytomies rather than being displayed as dichotomies). The ensemble consistency index (CI; Kluge & Farris, 1969) and ensemble retention index (RI; Farris, 1989) were calculated based on the total minimum and maximum number of steps for each matrix as calculated by WinClada.

For analysis 1a, the modified morphological data partition was analyzed without constraints. This analysis was performed to confirm the results of the original Gunnell and Simmons (2005) analysis with a matrix including the cloned terminals. For analysis 1b, bat genera represented in the molecular sequence data matrix (Table 1) were constrained to conform

with the Yinpterochiroptera–Yangochiroptera groupings as shown in Teeling et al. (2005). Only two positive constraints (one for each clade) were used. Fossil taxa and supergeneric extant ingroup taxa were designated as floaters (unconstrained). This analysis was performed to determine how many additional steps would have to occur in the morphological data in order for them to support the major dichotomy in the Chiroptera suggested by the molecular sequence data set (Teeling et al., 2005).

For analysis 2a, the modified Teeling et al. (2005) matrix was analyzed without constraints. For analysis 2b, the outgroup Perissodactyla was deactivated and the modified Teeling et al. (2005) data set was analyzed. The purpose of this analysis was to insure that the Yinpterochiroptera and Yangochiroptera were still recovered as monophyletic clades with Perissodactyla removed from the molecular sequence data set. For analysis 2c, the molecular sequence data matrix minus Perissodactyla was analyzed with taxa traditionally assigned to Megachiroptera and Microchiroptera constrained to belong to those groups (two positive constraints). The purpose of this analysis was to determine how many additional steps must occur in the molecular sequence data in order for them to support the major dichotomy in the Chiroptera suggested by the morphological data (Gunnell & Simmons, 2005) and traditional classifications.

For analysis 3a, the combined data matrix was analyzed with only those taxa for which both data partitions were coded (other taxa were deactivated). The purpose of this analysis was to determine whether taxa with large amounts of missing data were significantly affecting the results of the simultaneous analysis with fossil and extant taxa. For analysis 3b, the combined data set with all taxa except Perissodactyla was analyzed. For analysis 3c, the combined data set was analyzed with Yinpterochirpotera–Yangochirptera constraints, as in analysis 1b.

For analysis 4, all nodes from the molecular tree topology found in analysis 2b were constrained, and only taxa lacking molecular sequence data were allowed to float. This analysis emulates the methods of Teeling et al. (2005) and others (see below).

CHARACTER MAPPING

The binary (presence/absence) laryngeal echolocation character was mapped onto the trees resulting from analysis 3b using Fitch optimization (Fitch, 1971).

RESULTS

Table 2 summarizes the results for each analysis, including number of MPTs, length of MPTs, and CI and RI. Topological results are discussed below as relevant (e.g., when analyses were not performed under constraints). For analysis 1a (morphological data with no constraints), the strict consensus of all MPTs was concordant with the results shown in Gunnell and Simmons (2005: fig. 1). The strict consensus of the two MPTs for analysis 2a (the molecular sequence data set without constraints) has the same overall structure as that shown in Teeling et al. (2005: fig. 1), which illustrated the maximum likelihood (ML) tree calculated under the GTR + Γ + I model of molecular sequence evolution. In both trees, Yinpterochiroptera and Yangochiroptera are monophyletic sister groups composed of the same taxa. The Pteropodidae (flying foxes and Old World fruit bats) and Rhinolophoidea (horseshoe bats) form monophyletic clades sister to one another in Yinpterochiroptera, although the internal arrangement of Pteropodidae is different in our parsimony and the ML trees of Teeling et al. (2005). Emballonuridae, Phyllostomidae, Vespertilionidae, and Molossidae form monophyletic groups within Yangochiroptera (other families are represented by only one terminal), although the internal structure of Yangochiroptera is both different from and less resolved in the strict consensus of the parsimony trees found here as compared to the ML tree (see Teeling et al., 2005: fig. 1). The results of analysis 2b (the molecular sequence data set with no constraints, Perissodactyla deactivated) also support the Yinpterochiroptera–Yangochiroptera groupings. Support values—standard bootstrap (Felsenstein, 1985), Poisson bootstrap (Farris et al. in Horovitz, 1999b), and symmetrical resampling (Goloboff et al., 2003b) with traditional search—calculated for this pruned data set suggest that removing Perissodactyla does decrease the support (as expressed as absolute frequency) for the Yinpterochiroptera clade, although the degree to which support was affected was dependent on the resampling procedure used. Support values for Yinpterochiroptera ranged from 75 (5000 replicates of standard bootstrapping) to 89 (5000 replicates of symmetrical resampling at 33% change probability), as compared to 96 as reported by Teeling et al. (2005) when Perissodactyla was included (1000 replicates of standard bootstrapping in PAUP 4.10b10 [Swofford, 2003]).

The results of analysis 3a (the unconstrained combined data set including taxa with both data partitions only) support the traditional groupings (monophyletic Microchiroptera and monophyletic Megachiroptera) among extant bats. Similarly, the results of analysis 3b (the combined data set without constraints, Perissodactyla deactivated) do not support paraphyly of Microchiroptera with respect to Megachiroptera. Two fossil bat taxa, *Icaronycteris* and the

Table 2. Results of phylogenetic analyses. Descriptions of analyses are as follows (see text for further details): analysis 1a, morphological data set; 1b, morphological data set with Yinpterochiroptera–Yangochiroptera constraints; 2a, molecular sequence data set, all outgroups; 2b, molecular sequence data set, Perissodactyla deactivated; 2c, molecular sequence data set with Megachiroptera–Microchiroptera constraints, Perissodactyla deactivated; 3a, combined data set, extant taxa only; 3b, combined data set, extant and fossil taxa; 3c, combined data set, Yinpterochiroptera–Yangochiroptera constraints; and 4, molecular scaffold analysis, all nodes constrained. MPTs = most parsimonious trees.

	Analysis								
	1a	1b	2a	2b	2c	3a	3b	3c	4
Taxa (outgroup, ingroup, fossil)	45 (5, 40, 6)	45 (5, 40, 6)	34 (4, 30, 0)	33 (3, 30, 0)	33 (3, 30, 0)	39 (5, 34, 0)	45 (5, 40, 6)	45 (5, 40, 6)	45 (5, 40, 6)
Characters (total and no. informative)	204 (202)	204 (202)	13792 (3792)	13792 (3533)	13792 (3533)	13996 (3735)	13996 (3735)	13996 (3735)	NA
MPTs	4	30	2	1	2	3	16	1	3
Morphological characters									
Length	791	827	NA	NA	NA	750, 763, 771	875 (8), 888 (8)	901	923
CI	0.363	0.347	NA	NA	NA	0.357, 0.360, 0.367	0.323 (8), 0.328 (8)	0.319	0.311
RI	0.732	0.713	NA	NA	NA	0.659, 0.664, 0.673	0.681 (8), 0.688 (8)	0.674	0.662
Molecular characters									
Length	NA	NA	15782	15238	15249	15258, 15271, 15250	15258 (8), 15271 (8)	15248	15238
CI	NA	NA	0.531	0.539	0.538	0.537, 0.538 (2)	0.537 (8), 0.538 (8)	0.538	0.539
RI	NA	NA	0.485	0.491	0.490	0.489 (2), 0.490	0.488 (8), 0.489 (8)	0.490	0.491
All characters									
Length	NA	NA	NA	NA	NA	16021	16146	16149	16161
CI	NA	NA	NA	NA	NA	0.529	0.526	0.526	0.526
RI	NA	NA	NA	NA	NA	0.506	0.512	0.512	0.511

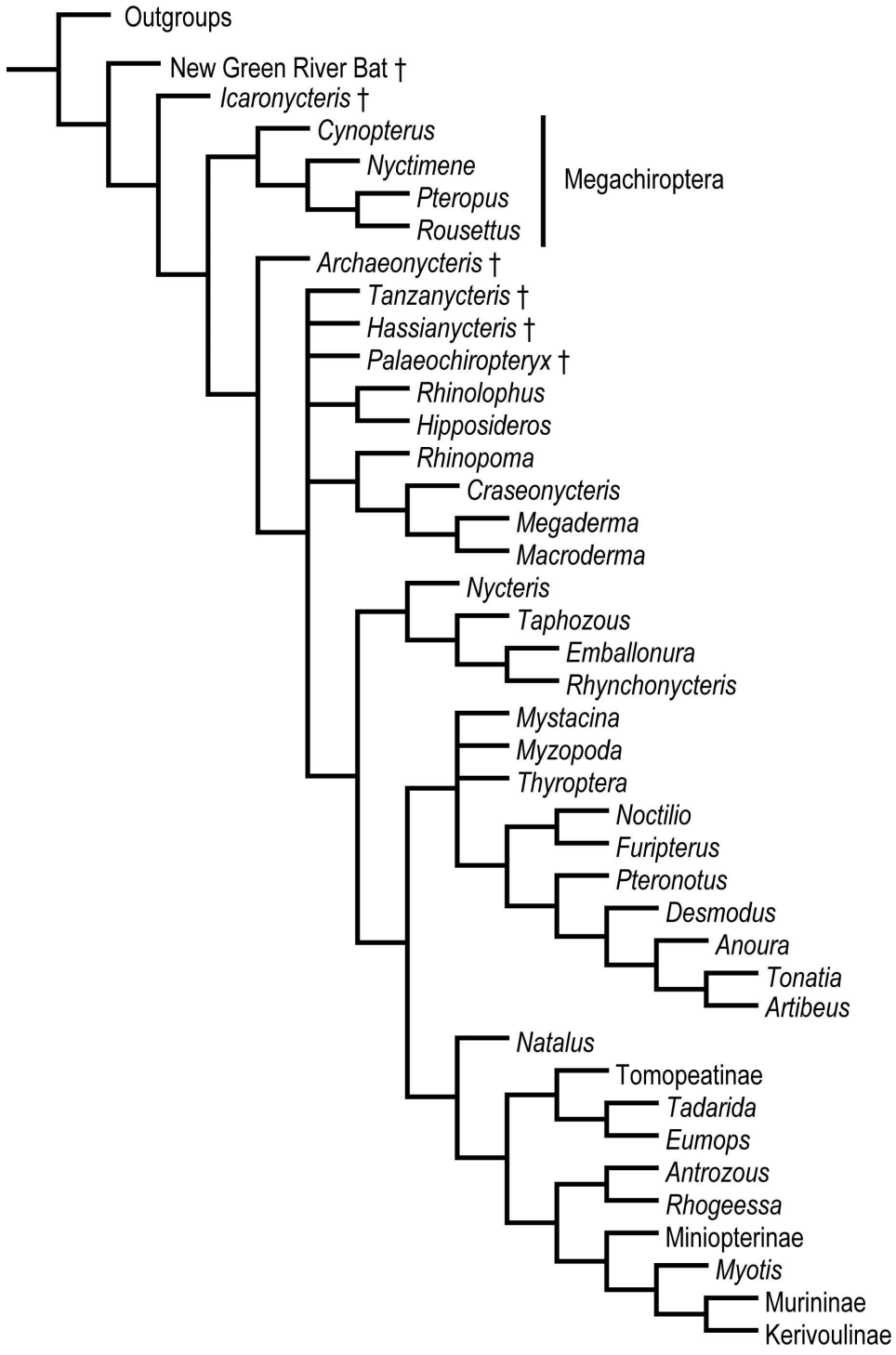

Figure 1. Strict consensus of 16 MPTs (16,146 steps; CI = 0.526; RI = 0.512) resulting from the analysis (3b) of the combined morphological and molecular sequence data set, including all extant and fossil taxa, without constraints. Fossil taxa are indicated by a dagger (†).

Green River Bat, group outside of all extant bats. The other four fossil bat taxa and extant Microchiroptera form a monophyletic group (Fig. 1). The clade that includes Rhinolophidae, Megadermatidae, Craseonycteridae, and Rhinopomatidae is partially collapsed due in part to interaction with the fossil taxa (Fig. 1).

The strict consensus of three MPTs found during analysis 4 (the molecular scaffold with all nodes constrained, Perissodactyla deactivated) is shown in Figure 2. *Tanzanycteris* is resolved sister to Rhinolophidae. In one tree, *Hassianycteris* is sister to all Yangochiroptera and in the other trees is outside of the clade including all extant bats. All other fossil taxa are on the stem lineage of extant bats in all MPTs.

Discussion

The most basic problem of studying character evolution in a phylogenetic context is a problem of methodology: how will the trees be built? Different methodologies have been employed to integrate data from fossil taxa (primarily composed of morphological data) and extant taxa (now including, or often composed entirely of molecular sequence data) in analyses that incorporate information from multiple sources. One of these is simply to suggest the position of a fossil taxon on a tree of extant taxa by reference to morphological synapomorphies mapped on a tree (e.g., Rowe, 1988, for select fossil taxa with more than 12% missing data; Boucher et al., 2003). Two others, which take a more analytical approach, are the molecular scaffold (also known as molecular backbone constraints, molecular constraints, etc.) and simultaneous analysis (also known as total evidence or combined analysis, or the supermatrix approach). The first of these, the molecular scaffold, gives greater precedence (at least to some degree, depending on the constraints used) to the topology suggested by the molecular sequence data. In this type of analysis, extant taxa are analyzed using molecular sequence data, all or some of the relationships among these taxa on the resultant tree(s) are constrained, and then a morphological matrix including fossil taxa is analyzed under the constraints (e.g., Springer et al., 2001; Sánchez-Villagra et al., 2003; Roca et al., 2004: supplementary data; Asher et al., 2005a; Teeling et al., 2005). In a total evidence approach (Kluge, 1989) or a combined or simultaneous analysis (Nixon & Carpenter, 1996), all character data are combined into a single supermatrix (see de Queiroz & Gatesy, 2007), and extant and fossil taxa are analyzed together. In the latter approach, morphological data can have a greater influence on the resultant tree topologies, and inclusion of morphological data with molecular sequence data has sometimes been shown to significantly alter the topologies recovered relative to those found when sequence data are analyzed alone (see discussion below). Generally, simultaneous analyses have been performed under equal-weights parsimony with sequence data aligned prior to analysis, although some authors (e.g., Giribet et al., 2002; Asher et al., 2003, 2004; Wheeler et al., 2004; Arango & Wheeler, 2007) have chosen to implement direct optimization of sequence data (Wheeler, 1996, 2003) during phylogeny reconstruction, in which different costs can be assigned to morphological and various types of molecular transformations.

Inclusion of fossil taxa in phylogenetic analyses increases taxon sampling and does so in a very unique way. Fossil taxa represent lineages sampled through time and, as such, can be repositories of unique morphologies that may not be represented in today's biota. Thus, direct inclusion of fossil taxa in phylogenetic analyses, rather than overlaying a morphological analysis onto a molecular scaffold, can alter tree topologies, sometimes in ways that yield different results than simply combining data partitions for extant taxa alone. In fact, the addition of fossil taxa representing extinct diversity has the potential to alter the interpretation of relationships among extant taxa any time homoplasy occurs in the data set on which a phylogenetic hypothesis is based (Nixon & Wheeler, 1992). Thus, effectively, fossil taxa that possess unique combinations of characters almost always have the potential to alter the hypothesis of phylogenetic relationships when included in an analysis and, in certain situations (e.g., cases in which large accumulations of apomorphies distinguish extant taxa; see Donoghue et al., 1989), might be expected to significantly affect the perceived relationships among extant taxa.

Perhaps the earliest illustration of this property in a combined analysis of morphological and molecular sequence data was presented by Eernisse and Kluge (1993), who studied amniote relationships. They performed analyses including morphological (Gauthier et al., 1988) and molecular sequence (Hedges et al., 1990) data sets in various combinations with and without fossil taxa. In some pairs of analyses (e.g., combined 18S rRNA sequences plus morphology), inclusion of fossil taxa was critical; without fossil taxa, birds and mammals formed a monophyletic group to the exclusion of crocodiles, turtles, and lepidosaurs (e.g., snakes, lizards, tuataras); with fossil taxa, birds and crocodiles were sister taxa in a monophyletic group with turtles and lepidosaurs to the exclusion of mammals. When all characters were considered, the results were consistent with a monophyletic bird-extant reptile clade, although the positions of turtles

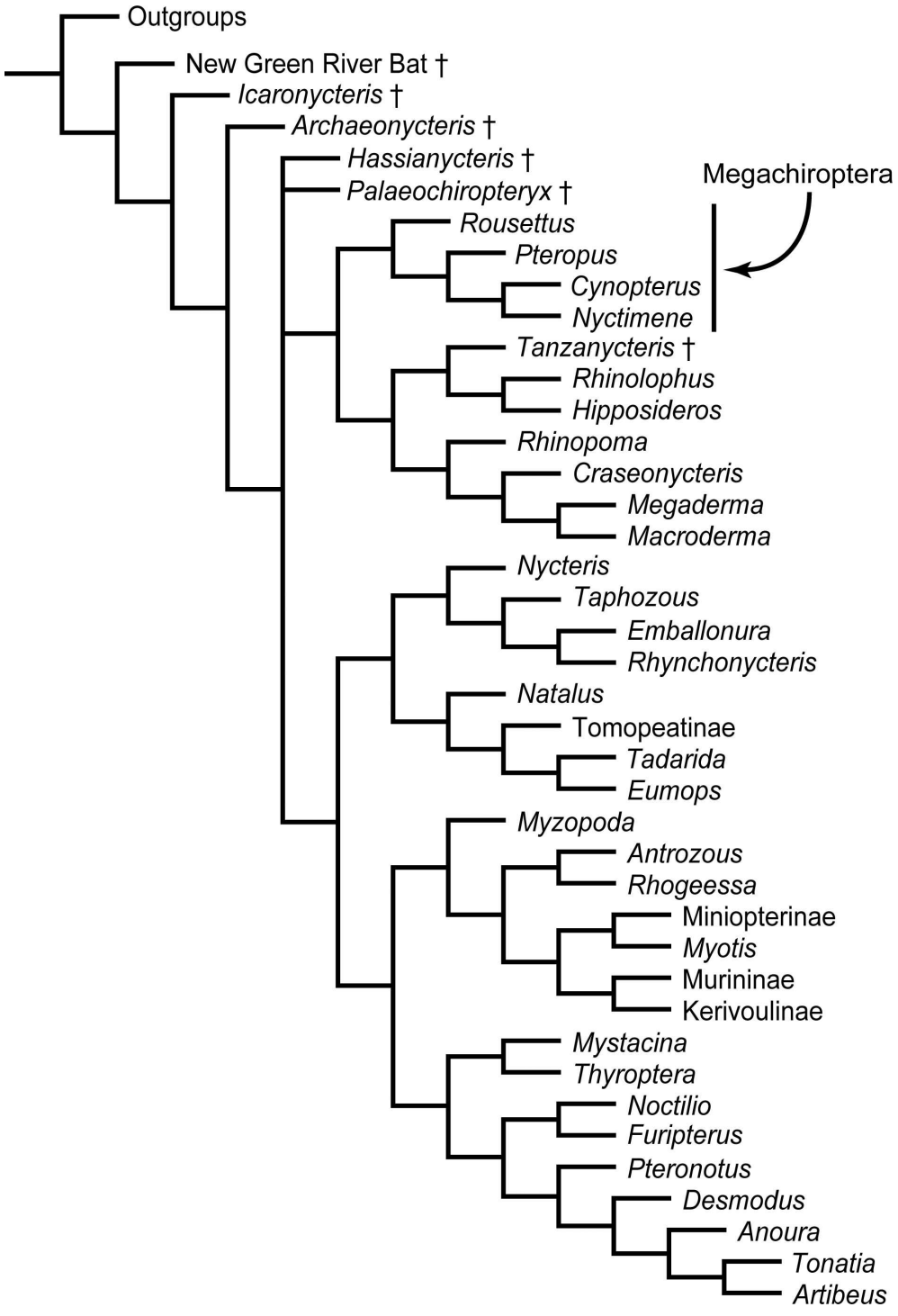

Figure 2. Strict consensus of three MPTs resulting from analysis (4) of morphological data over a molecular scaffold under full constraints (all taxa with molecular sequence data constrained). Taxa lacking the molecular sequence data partition were analyzed without constraints. Tree statistics are given in Table 2. Fossil taxa are indicated by a dagger (†).

and lepidosaurs were reversed among extant-only and fossil-extant analyses. Another demonstration of the difference that inclusion of fossil taxa can make was provided by Wheeler et al. (2004), which showed that including fossil taxa in a supermatrix of morphological and molecular sequence data for arthropods resulted in some differences in relationships among extant groups relative to an analysis with extant taxa alone. For example, the extant-only analyses always resolved Crustacea (e.g., crabs, shrimps, and barnacles) and Hexapoda (insects) as sister groups, whereas the fossil-extant analyses sometimes instead resolved Hexapoda and Myriapoda (e.g., centipedes and millipedes) as sister groups, depending on the cost ratios used to optimize the molecular data. A recent study of euphyllophytes by Rothwell and Nixon (2006) compared simultaneous analyses of sequence data (Pryer et al., 2001) with morphological data (Pryer et al., 2001, with addition of fossil taxa by Rothwell & Nixon, 2006) when fossil taxa were included and excluded. One of the notable differences in the resultant parsimony topologies was that lycophytes were a stem taxon of the euphyllophytes in the extant-only analyses (Pryer et al., 2001), whereas they were sister to the lignophytes when fossil taxa were included and the extant outgroups (bryophytes) were replaced with a fossil outgroup taxon presumably more closely related to the lignophytes (Rothwell & Nixon, 2006).

Few studies to date have directly compared the results of fossil-extant molecular scaffold and simultaneous analyses, specifically considering the effect each approach may have on the relative placements of fossil and extant taxa. Asher et al. (2005a) compared simultaneous and molecular scaffold analyses of fossil and extant placental mammals with the goal of exploring the affinities of the fossil lipotyphlan (mammalian insectivores) genus *Centetodon* Marsh. Simultaneous analyses indicated that *Centetodon* grouped with Eulipotyphla (insectivores) in a more derived position than *Solenodon* Brandt (solenodons, insectivorous mammals endemic to the Caribbean). The molecular scaffold analysis also indicated that *Centetodon* belongs within Eulioptypha but did not decisively resolve the position of *Centetodon* relative to *Solenodon*. There were many substantive differences in the inferred interrelationships of mammalian orders between the supermatrix and molecular scaffold topologies. Manos et al. (2007) compared simultaneous and molecular scaffold analyses of fossil and extant members of the angiosperm family Juglandaceae (walnut family). Both analyses recovered two clades (englehardoids and juglandoids) within the family, and the strict consensus trees resulting from each analysis were similar. The positions of the fossil taxa were also similar between analyses, the biggest difference being that *Paleooreomunnea stoneana* Dilcher, Potter & Crepet (a fruit taxon; Dilcher et al., 1976) grouped with the juglandoids in the simultaneous but not the molecular scaffold analysis. Magallón (2007) compared simultaneous and molecular scaffold analyses of fossil and extant taxa in the angiosperm family Hamamelidaceae (witch hazel family). The strict consensus of the simultaneous analysis was poorly resolved, whereas the strict consensus of the molecular scaffold analysis had greater resolution. In both analyses, the fossil taxon *Archamamelis* Endress & Friis (a floral taxon; Endress & Friis, 1991) was resolved sister to *Hamamelis* L. (witch hazel), although the relationships of the other fossil taxa were more ambiguous. The results of our study show a clear difference in the positions of fossil taxa between simultaneous and molecular scaffold analyses, with fossil taxa being divided into two stem group and four crown group taxa in the unconstrained combined analysis (3b, Fig. 1) and five stem group taxa and one crown group taxon or four stem group and two crown group taxa in the molecular scaffold analysis (4, Fig. 2). The difference in the basal dichotomy among extant bats between the two analyses—caused by addition of morphological data to the pruned molecular sequence data set in the simultaneous analysis, as demonstrated when the combined data set is analyzed with all taxa lacking the molecular partition removed (analysis 3a)—is likely affecting the inferred positions of the fossil taxa.

Comparison of the results of simultaneous and molecular scaffold analyses and simultaneous analyses with and without fossil taxa clearly demonstrates, even though examples are relatively few, that choice of methodology can affect the optimal topologies and, thus, that the type of analysis performed and/or the direct inclusion of fossil taxa does matter. The rationale for using a total evidence or simultaneous analysis approach to analyzing data (not necessarily including fossil taxa) was made by Kluge (1989), later by Nixon and Carpenter (1996), and more recently by de Queiroz and Gatesy (2007); perhaps the most persuasive argument for such an approach is that all putatively phylogenetically informative data should be used to construct phylogenetic hypotheses. Recent arguments against simultaneous analysis of morphological with molecular sequence data, against use of select morphological characters (those that are incongruent with a molecular scaffold), and/or for the molecular scaffold approach, include: (1) that the most generally accepted technique for analyzing morphological data is parsimony, whereas molecular sequence data may be better analyzed using other methods (e.g., Springer et al., 2001; Asher et al.,

2005a); (2) that molecular sequence data are not available for most fossil taxa, or the missing data argument (e.g., Springer et al., 2001; Manos et al., 2007); (3) that morphological data are subject to homoplasy due to convergence (e.g., Springer et al., 2004, 2007; Eick et al., 2005); and (4) that simultaneous analyses "fail to address the weighting problem posed by including molecular and morphological data in the same data matrix" (Springer et al., 2004: 436). The first point is certainly debatable with regard to molecular sequence data sets (e.g., Frost et al., 2001) and is becoming moot with regard to morphological data (Lewis, 2001; also noted by Springer et al., 2004). For example, recent analyses of morphological (e.g., Müller & Reisz, 2006) or combined morphological and molecular sequence data (Glenner et al., 2004; Nylander et al., 2004), sometimes including fossil taxa (Lee, 2005; Xiang et al., 2005; Asher & Hofreiter, 2006; Müller & Reisz, 2006), have used Bayesian methods, although this approach is relatively little explored for both phylogeny building and the study of character evolution using morphological data sets. The latter three arguments may be considered aspects of the topic of character evolution especially pertinent to including fossil taxa in simultaneous analyses with extant taxa. Below, we address the arguments against including fossil taxa in simultaneous analyses of morphological and molecular sequence data (primarily consisting of arguments against using morphological data in phylogenetic analyses) and go on to discuss some of the specific benefits accrued and obstacles encountered to the study of character evolution when including morphological data from fossil taxa in phylogenetic analyses.

MISSING DATA—A JUSTIFIABLE REASON FOR EXCLUDING FOSSIL TAXA FROM THE PROCESS OF PHYLOGENY RECONSTRUCTION?

One criticism that has been levied against the inclusion of fossil taxa in simultaneous analyses is that fossil taxa may be missing significant amounts of data. For example, Springer et al. (2001: 6242) in part rejected the total evidence approach because "molecular data are usually unattainable for fossils." Perhaps the most serious methodological consequence of including fossil taxa with significant amounts of missing data into an analysis is a weakening of the parsimony criterion, which is strongest when presented with maximum evidence: "the tree that is best corroborated is the tree that best explains (e.g. as homology) all character distributions among all taxa" (Nixon, 1996: 369).

One way in which this weakened test of parsimony may manifest itself is through the wild card taxon phenomenon, in which a taxon (or taxa) with large amounts of missing data may group at numerous different positions on the shortest discovered tree topologies due to its limited distribution of character states (Nixon & Wheeler, 1992). Nixon and Wheeler (1992) noted that the inclusion of wild card taxa in a matrix may result in a significant increase in the number of MPTs and deresolution of the strict consensus of all MPTs. The best solution to such a problem, when encountered following phylogenetic analysis, may be to remove such taxa, provided that the lack of resolution in the position of the problematic taxon can reasonably be attributed to missing data and not at least in part to character conflict. Kearney (2002), in fact, has suggested recognition of three different types of wild card taxa: (1) missing data wild cards, whose instability is entirely caused by missing data; (2) mixed wild cards, whose instability is due both to missing data and character incongruence; and (3) conflict wild cards, taxa whose instability is entirely due to character conflict. Missing data wild cards can be identified by taxonomic equivalent analysis (Wilkinson, 1995), in which fragmentary taxa that are identical with more complete taxa in the characters for which they are coded are removed if an initial analysis shows them to be wild cards, thus eliminating redundancy from the analysis and possibly increasing the resolution of the resultant topologies (see examples in Kearney, 2002).

A more significant barrier to including fossil taxa is the unpredictable effect(s) that missing data can have on a parsimony analysis when character incongruence is encountered, and/or when mixed or conflict wild cards are present. Due to the weakened test of character congruence and the tendency of parsimony to underestimate tree length when large amounts of missing data are present, Nixon (1996: 370) suggested "we should be suspicious when the addition of fossils with large numbers of missing data results in significantly different topologies than when they are excluded." When large amounts of extinction have occurred within a clade, however, significant rearrangements may be expected when fossil taxa are sampled (see examples from Eernisse and Kluge [1993], Wheeler et al. [2004], and Rothwell and Nixon [2006] discussed above), especially when these taxa represent much of a group's diversity. This conundrum may be insoluble, since more complete character data will often be unavailable for fossil taxa. Worse, in combined morphological–molecular sequence data sets, fossil taxa will be coded for a very small proportion of characters, as they will likely be missing the entire molecular sequence partition. In the present study, for example, *Icaronycteris* is coded (with one or more character states) for only about 2% of all

parsimony-informative characters in the combined matrix, and the Green River fossil bat taxon for about 1.3%; *Tanzanycteris*, whose position within or outside of crown group Microchirptera varies, is coded for only about 0.6% of parsimony-informative characters, the smallest proportion among all fossil bat taxa included in the matrix.

Although threshold values for excluding taxa on the basis of the proportion of missing data have been used in some studies (e.g., Rowe, 1988; Benton, 1990; Grande & Bemis, 1998), these have been rejected both logically (e.g., Kearney & Clark, 2003) and on the basis of simulation and empirical studies. Using simulations, Wiens (2003a) showed that it is not the amount of missing data that determines whether the position of a taxon will be unambiguously and correctly resolved but rather whether critical characters are coded for that taxon. This conclusion conforms to a basic principle of phylogenetic systematics: it is only critical character state transformations—synapomorphies—that provide grouping information (Hennig, 1966). Thus, the missing data problem becomes one of too few characters rather than too much missing data, since greater sampling of characters increases the chance that critical characters needed for precise and accurate resolution of a taxon's position will be included in the analysis (Wiens, 2003a). In another simulation study, Wiens (2003b: 309) predicted, "If the fossil taxa can be accurately placed in an analysis of the morphological data alone, they should be accurately placed in the combined analyses as well, regardless of their relative level of incompleteness when the molecular data are added."

Wiens' prediction has been, for the most part, borne out by studies employing pseudofossil analyses. In a pseudofossil analysis, one or more extant taxa are coded for only a subset of characters in the combined data matrix, with all molecular sequence characters and often a portion of the morphological character matrix coded as missing so that the pseudofossil(s) simulate the behavior of a fossil taxon (or taxa) during simultaneous analysis of a real data set (real fossil taxa are excluded). Jordan and Hill (1999), Jordan and Macphail (2003), and Asher and Hofreiter (2006) used an approach in which one taxon at a time was treated as a pseudofossil in combined morphological and molecular sequence data matrices in order to evaluate the behavior of these incomplete terminals in simultaneous analyses of real data sets. In each case, pseudofossils were found to place reliably to the general area of the tree suggested by analyses of the taxa with the full complement of characters, although the results of analyses with pseudofossils were not necessarily identical to analyses in which the full matrix was analyzed. Manos et al. (2007) took a different approach and created pseudofossils by duplicating one extant taxon at a time and randomly eliminating all but 25%, 50%, or 75% of the duplicate's morphological characters or by eliminating all but its organ-specific (i.e., vegetative, floral, or fruit) characters and performing simultaneous analyses. Although the randomly generated pseudofossils were seldom sister to their parent species in the resultant topologies, they did place "in the correct local clade, and neither of the two large clades (engelhardioids or juglandoids) was disrupted" (Manos et al., 2007: 422); "[r]emoval of suites of organ-specific characters did not show appreciably different results" (Manos et al., 2007: 422). In contrast, Springer et al. (2007)—in a pseudofossil study of a combined matrix of placental mammals, in which ordinal or superordinal groups were treated as pseudofossils by eliminating all but the osteological data partition for each taxon in that group—often found profound rearrangements in topology relative to a tree based on molecular sequence data alone. Although Springer et al. (2007) attributed these rearrangements to the inadequacy of the morphological data set, they did not establish whether taxon removal (i.e., taxon sampling) affected the topologies favored by the molecular sequence data partition alone. Thus, the underlying reasons for these rearrangements may have been more complex.

Studies such as those cited above suggest that, as previously noted by Kearney (2002: 370), "[t]he effects of incomplete taxa and concomitant missing character data are not general, but matrix-specific, and depend on the precise distribution of question marks and characters states across taxa"; also see Novacek (1992a). Therefore, taxa with large amounts of missing data should not be considered a priori unsuitable for inclusion in phylogenetic analyses, including simultaneous analyses of morphological and molecular sequence data. The biggest problem that missing data present, then, may be the weakened application of parsimony (however, see Kearney & Clark, 2003). This is especially the case when incongruent data are concentrated in fossil taxa, which may have undesirable effects on the results, such as deresolution of the strict consensus tree or support for misleading topologies. Novacek (1992b: 75) perhaps best summarized the problem: "The kinds of characters preserved, not just the degree of character representation, account for the potential influence of an added taxon. If among the few characters preserved are the combination of primitive and derived states that force relationships in a particular direction, then the included taxa—even when poorly represented—will play a significant role

in the outcome. Of course, the possibility that these incompletely preserved taxa force the wrong outcome, which would be apparent had the taxa been better represented, cannot be eliminated." Unfortunately, at this time, there is no definitive way to differentiate between the correct and the incorrect topology in such a situation.

The molecular scaffold approach may appear to circumvent this problem by building a topology that includes only extant taxa (for which all or most molecular sequence characters can be scored) to serve as a basis for constraints on the analysis of the morphological data, thereby isolating the ambiguity caused by missing data into unconstrained taxa in the morphological partition. Although excluding fossil taxa from the scaffold-building step thus inoculates the molecular partition from the computational and philosophical problems that can be caused by including large numbers of ambiguous cells in a data matrix, the underlying missing data problem is not truly eliminated. This is because excluding fossil taxa from direct phylogenetic analysis also reduces taxon sampling, which in general can negatively impact the accuracy of results (e.g., Zwickl & Hillis, 2002).

CONVERGENCE

According to Givnish and Sytsma (1997: 56), there are four mechanisms that can result in homoplasy: "evolutionary convergence, recurrence, transference, and character misclassification [bold emphasis removed]." Evolutionary convergence or parallelism (cf. Wiens et al., 2003; Desutter-Grandcolas et al., 2005) of form may result when similar morphological solutions to similar selective pressures are discovered. There appears to be a perceived notion among some workers that convergence of morphological form is so pervasive and misleading that it is justifiable to either (1) simply exclude morphological data from the construction of phylogenetic hypotheses or (2) regard morphological data as suspect when topological conflict with molecular sequence data occurs (Hedges & Sibley, 1994; Hedges & Maxson, 1996; Givnish & Sytsma, 1997; Eick et al., 2005). There is no denying that convergence of form is a widespread feature of evolution and that it has occurred at many scales and in many taxa through geologic time (some examples include ichthyosaurs and dolphins, rudist bivalves and reef-building corals, some placental and marsupial mammals, the mangrove habit in plants, etc.). As noted by Wiens et al. (2003: 501), "Convergence is a critical issue in systematics because it can potentially mislead phylogeny reconstruction methods, for example, causing analyses to group distantly related organisms that share similar habitats."

Of particular concern are cases in which convergence has potentially led to correlated patterns of evolution in suites of functionally related morphological characters included in an analysis (see, for instance, Wiens et al., 2003). In such cases, however, precise character definition (i.e., hypotheses of homology) and careful morphological study have reduced and will continue to reduce the occurrence of this problem (that is, the fourth class of homoplasy given by Givnish & Sytsma [1997]: character misclassification). One approach is atomization and coding of such morphological complexes, which can highlight differences in apparently convergent morphologies. Bruneau (1997), for example, contrasted the character complex of pseudo-tubular corollas, convergent among hummingbird-pollinated species of the legume *Erythrina* L. (a legume), with the atomized corolla characters detailing specific aspects of size and morphology. Consideration of pseudo-tubular corollas as a single presence-absence character would have led to a relatively uninformative hypothesis of homoplasy due to multiple origins, while breaking the complex pseudo-tubular corolla character into several atomized petal characters allowed for the detection of convergence in a single aspect of the corolla morphology (see also Luckow & Bruneau, 1997). Nixon (1996: 368) pointed out that it is especially important that homology assessments be approached carefully with fossil taxa because "poorly preserved fossils may have a higher likelihood of being misunderstood and therefore incorrectly scored for those characters that are **not** missing [boldface in original]." Thus, in some situations in which structures are poorly understood, perhaps fossil taxa should be coded using different homology assessments and the results of analyses compared or the fossil taxa should be excluded from analysis altogether. Despite this last caveat, the problem of morphological data being seriously compromised by evolutionary convergence may be overstated.

There have been demonstrated cases in which convergence of morphological characters may have produced misleading results. An interesting example was provided by a study from Gatesy et al. (2003) on the relationship of crocodylians to the extant gavial (*Gavialis gangeticus* (Gmelin)), in which results of analyses from a morphological data set (modified from Brochu, 1997), molecular sequence data sets, and a combined data set for fossil and extant crocodilians were compared. Analysis of the morphological data alone with and without fossil taxa suggested that the extant gavial was the most basally diverging lineage of the extant crocodylian groups; in contrast, molecular sequence data suggested that the gavial was in a derived position in the tree, sister to the false gavial

Tomistoma schlegelii Müller. When the data sets were combined, the strict consensus of the resultant MPTs was congruent with the molecular results for extant taxa, suggesting that the extant gavial is an atavistic taxon.

Although Gatesy et al. (2003) could have chalked up these results to the unsuitability of the morphological data, beset by convergence, to discover the proper position of the extant gavial among crocodylians, they instead tested the morphological data for secondary signals.

Secondary signals (Nixon & Carpenter, 1996) or hidden support (Gatesy et al., 1999) are patterns present in a data partition that are masked when that partition is analyzed alone—reflecting a primary signal—but which may manifest themselves as character support for clades conflicting with the primary signal for the partition when analyzed in combination with other data partitions. When data sets that independently produce competing results (i.e., have different primary signals) are combined and analyzed together, secondary phylogenetic signals common to two or more data sets may even lead to novel hypotheses of phylogeny not expressed in any individual partition (see Barrett et al., 1991; Nixon & Carpenter, 1996; Gatesy et al., 1999). In other words, during simultaneous analysis, "[t]he peculiarities of each data set are cancelled out by the unique peculiarities of the others, and the remaining common signal emerges" (Gatesy et al., 1999: 301). Gatesy et al. (2003) tested for hidden support for the derived extant gavial hypothesis in their morphological character data set using partitioned hidden branch support (PHBS; Gatesy et al., 1999). PHBS is the difference between the branch support values for a given data partition and a given node in combined and partitioned analyses (Gatesy et al., 1999); a "positive PHBS score for a particular data set indicates a secondary phylogenetic signal for the relationship of interest that emerges in simultaneous analysis" (Gatesy et al., 2003: 407). Using PHBS, Gatesy et al. (2003) found hidden morphological support for nodes linking the extant gavial to the extant false gavial and the Crocodilynae (crocodiles) to the gavial and false gavial in the combined morphological and molecular sequence topology for extant taxa. This support was increased by the addition of fossil taxa. In a more recent expanded analysis of crocodylians that included increased taxon and character sampling, Gatesy et al. (2004: 347) found "11 groups that emerged in the supermatrix analysis" that "were not implied by any combination of trees supported by separate analyses of the 17 data sets in the supermatrix," thereby revealing the secondary signals that manifested themselves during simultaneous analysis. Similarly, secondary signals must be at work in the simultaneous analysis of Chiroptera presented here, because the two major clades of bats resolved during simultaneous analysis are concordant with the primary morphological but not the primary molecular sequence signal (Fig. 1), suggesting that secondary signals in the molecular sequence partition may support the traditional Megachiropteran and Microchiropteran groupings.

The discovery of these secondary signals is only possible through simultaneous analysis and is an important advantage of this approach over the molecular scaffold approach. For instance, in the study from Asher et al. (2005a) on the extinct insectivorous mammal *Centetodon*, simultaneous analyses of molecular sequence and morphological data indicated that *Centetodon* was more derived than the Caribbean endemic insectivore *Solenodon*, whereas a molecular scaffold analysis failed to decisively resolve the position of *Centetodon* relative to *Solenodon*. Asher et al. (2005a: 919) considered this ironic since "a basal position for *Solenodon* within Holarctic lipotyphlans [mammalian insectivores] results from the molecular signal responsible for the molecular scaffold, and not from the influence of the morphological data. Analyzed alone, morphological data weakly support *Erinaceus* as basal" within the clade. Thus, the molecular scaffold actually obscured both the molecular sequence signal as well as the signal common to both data sets when analyzed together.

Furthermore, the molecular scaffold approach may tend to force more conflict into the data set that is analyzed on the scaffold, depending on the constraints used. This creates a self-fulfilling prophecy whereby the morphological data contain larger amounts of homoplasy—sometimes explained ad hoc as evidence of convergence (see also Luckow & Bruneau, 1997)—relative to the results of a morphological analysis alone, and sometimes relative to the results of a simultaneous analysis. The bat data set presented here is an interesting example of this phenomenon. The morphological data gained 132 steps relative to the MPTs for the morphological partition alone, and 35 or 48 steps relative to the MPTs for the simultaneous analysis when analyzed under a strict molecular scaffold (compare analyses 1a and 3b to analysis 4, Table 2). The overall CI and RI for the combined analysis (3b), the combined analysis with Yinpterochiroptera–Yangochiroptera constraints (3c), and the strict molecular scaffold (4) are nearly identical for the combined data set (Table 2). However, the distribution of homoplasy between partitions is shifted from the molecular sequence to the morphological characters as the data are put under constraints that are progressively more favorable to the primary

molecular sequence signal. The CI and RI become progressively lower (e.g., more homoplasious) for the morphological data and higher for the molecular sequence data (Table 2). The change in the CI and RI values for the morphological partition is also greater, reflecting the fact that, although the absolute difference in the number of steps for each partition is similar between the most and least parsimonious analyses for that partition (35 or 48 steps for morphology, 20 or 33 steps for molecular sequence), proportionally more steps are forced into the morphological partition in less parsimonious topologies for that partition than for the molecular sequence data set, because the molecular sequence data set is much larger (Table 2).

Eick et al. (2005) recently took assumptions about convergence in morphological characters to an extreme and analyzed fossil-extant bat relationships on a molecular scaffold using only morphological characters that were nonhomoplasious when mapped on a topology suggested by molecular sequence data because "the exclusion of a significant amount of homoplasious characters can potentially alter the conclusions reached" (Eick et al., 2005: 1872) and "the high level of parallel evolution when using morphological characters is problematic" (Eick et al., 2005: 1879). Interestingly, they did not remove homoplasious molecular sequence characters from their analyses, although homoplasy was present in the molecular sequence data (the ensemble RI was less than one for each data set when analyzed using parsimony). Molecular sequence data are not necessarily exempt from evolutionary convergence. Lee (1997) noted that biologists understand functional morphology well and are adept at recognizing and articulating the adaptive significance of some aspects of the morphologies of organisms. Conversely, Lee (1997) questioned whether the young discipline of functional molecular biology is yet as capable of recognizing adaptive convergence in sequence data. In fact, recent work has demonstrated the occurrence of evolutionary convergence at the molecular level (e.g., Bull et al., 1997; Zhang & Kumar, 1997; Cuevas et al., 2002; Zakon, 2002; Protas et al., 2006). While we agree with some authors (e.g., Hedges & Maxson, 1996) that molecular sequence data may be less prone to similarity caused by evolutionary convergence or parallelism, molecular sequence data are also subject to homoplasy from recurrence (random mutation leading to noisy homoplasy; see Wenzel & Siddall, 1999) and transference (horizontal gene transfer), as well as character misclassification (e.g., due to misalignment). Because of these four factors, the amount of homoplasy in sequence data may be as great as or greater than that in morphological data (e.g., Sanderson & Donoghue, 1989; Baker et al., 1998). Thus, it should be borne in mind that sequence data do not have special immunity from homoplasy—as powerfully evidenced by "the fact that different genes yield different phylogenies" (Lee, 1997: 394)—and, as in morphological data, this homoplasy is potentially misleading.

Only additional tests of a phylogenetic hypothesis through the addition of more taxa and more characters can be the ultimate arbiter of the robustness of a suggested set of relationships. As perhaps best summarized by O'Leary et al. (2003: 861), "Without insights into some yet undiscovered law of nature, there is no particular reason to think that a functional, developmental, or ecological explanation for homoplasy is a better explanation of covariation than is synapomorphy. Simply proposing such generalities does not condemn characters to being phylogenetically uninformative."

THE WEIGHTING PROBLEM

It has been argued that in a simultaneous analysis of morphological and molecular sequence data, the large amounts of molecular sequence data will simply overwhelm (or swamp) the signal of the morphological data set. For example, Alvarez et al. (2000: 184) argued that the morphological and molecular sequence data sets they used to reconstruct the phylogeny of a family of sponges "are unequally sized (95 parsimony-informative molecular characters vs. 16 parsimony-informative morphological characters) so that the molecular signal, which is different, will swamp the morphological signal. Therefore, a simultaneous analysis (e.g. one including both types of data) was considered inappropriate." Some might argue that because morphological transformations might be presupposed to involve more complex underlying mechanisms than transformations among nucleic acid bases, perhaps morphological data partitions should be upweighted with respect to molecular sequence data partitions. This assumption may be analogous to that employed in some analyses of molecular sequence data sets: "Within that paradigm of probabilistic assumptions, it is often argued that transitions are more probable (i.e., observed more frequently) and should therefore deserve a lower cost...than transversions. However, no consensus has been reached for the appropriate cost ratio, and an arbitrary choice is required" (Frost et al., 2001: 354). The arbitrariness in weighting schemes may be the problem alluded to by Springer et al. (2004: 436) when they wrote, "One potential difficulty with combined analyses is that they fail to address the weighting problem posed by including

molecular and morphological data in the same data matrix."

Empirical studies of combined morphological and molecular sequence data sets do not bear out the assumption that large numbers of molecular sequence data will necessarily overwhelm the signal present in less numerous nonmolecular sequence data when all characters in a combined matrix are weighted equally (e.g., Omland, 1994; Mattern & McLennan, 2004; Wahlberg et al., 2005). This observation ties into the idea of secondary signals discussed above: when the data are combined, a common signal present in both data sets may be expressed that is not evident in either data set (or one of the data partitions) when analyzed alone (see similar argument made by Barrett et al., 1991). Obviously, the bat data set analyzed here exemplifies this: although the molecular sequence data set is much larger than the morphological data set, even when only parsimony-informative characters are considered (202 morphological, 3533 molecular sequence, or a ratio of parsimony-informative molecular sequence to morphological characters of over 17:1), the combined data set produces major groupings that are congruent with the morphological but not the molecular result, clearly showing that the morphological signal is not completely swamped by the signal from the molecular sequence data set but rather contributes to the structures of the most parsimonious topologies.

A study done by Baker et al. (1998) specifically examined the distribution of morphological and molecular sequence character support for nodes in trees generated by equal-weights parsimony applied to combined data sets. These authors compared the partitioned Bremer support (PBS; Baker & DeSalle, 1997; Lambkin et al., 2002) values from 10 previously published combined morphological–molecular sequence data sets (five additional combined data sets in their study featured non-DNA sequence molecular data) with ratios of parsimony-informative morphological to molecular sequence characters ranging from 0.08–0.33; results of the incongruence length difference (ILD) test (Farris et al., 1995a, b) for these data sets indicated that in five of 10 cases, there was significant incongruence between morphological and molecular sequence data partitions. When PBS values were summed across nodes for each of the studies, the morphological data provided proportionally slightly more support (relative to the number of parsimony-informative characters in each partition) to the topology resulting from simultaneous analysis than molecular sequence data for nine of the 10 data sets. Notably, the morphological data were also, in nine of 10 instances, less homoplasious than the molecular sequence data sets on the topology(ies) resulting from simultaneous analyses as measured using the CI of parsimony-informative characters for each partition; Baker et al. (1998) suggested that this may account for the greater impact of the morphological partitions relative to their sizes. Similarly, Lee (2005) combined morphological and molecular sequence data in analyses including extant and fossil squamates (e.g., lizards, snakes, amphisbaenians, and extinct relatives) and examined PBS values. The results of the combined analyses (under parsimony and Bayesian criteria) were mostly congruent with the result of the morphological analysis alone (under the parsimony criterion). Furthermore, "PBS values [from the parsimony analysis] revealed that the morphological signal (although weak) is still stronger than the molecular signal" (Lee, 2005: 229), even though there were more parsimony-informative molecular sequence characters than morphological characters. (See also Wortley & Scotland, 2006, for a more recent study using different methods.) Baker et al. (1998) have suggested, in fact, that the larger data partition in a combined matrix should have more influence on the results of a phylogenetic analysis—for the result to be otherwise would cast doubt on the quality of the larger data set.

Another argument against the necessity of weighting—character independence—has been effectively addressed by a number of authors (e.g., Kluge & Farris, 1969; Kluge, 1989; Nixon & Carpenter, 1996). Nixon and Carpenter (1996), who made a thorough argument for employing equal weights in a parsimony analysis a priori, addressed the issue of weighting data partitions using an appeal to character independence. Characters chosen for inclusion in a cladistic analysis are hypothesized to be independent of one another—they are logically independent, or assumed to evolve independently (Farris, 1983; Kluge & Wolf, 1993; Nixon & Carpenter, 1996). If characters are assumed to be independent from the outset, as they are in a parsimony analysis, then there is no more justification to apply weights among different data partitions than to apply differential weights to characters within a data partition. Thus, the perceived greater complexity of morphological characters need not be used as justification for upweighting them relative to molecular sequence characters.

Some have employed differential costs for morphological and molecular transformations, however, without resorting to completely arbitrary weighting schemes. In the context of simultaneous parsimony analyses including both morphological and molecular sequence data, this has been done using POY (Wheeler et al., 1996–2003), in which differential costs can be assigned to transitions, transversions, insertion and deletion events, and morphological

transformations, and direct optimization is employed during analysis (Wheeler, 1996, 2003). In empirical studies in which POY is applied to simultaneous analyses of morphological and molecular sequence data with fossil and extant taxa, measures such as ILD metrics and a topological index have been used to choose the optimal weighting scheme(s) from those applied to the data (Giribet et al., 2002; Asher et al., 2003, 2004).

Finally, the molecular scaffold approach does not overcome the weighting problem and, in fact, appears to resurrect a widely recognized weighting problem that arises when topology is used as a proxy for character matrices: although an explicit weighting scheme is not imposed, an implicit weighting scheme is employed (e.g., Barrett et al., 1991). The molecular scaffold implies differential weights for the characters used to construct the molecular scaffold topology relative to the characters in the matrix analyzed under the scaffold constraints, and these weights are node specific. The morphological characters are effectively weighted to zero for extant taxa where nodes are constrained (e.g., the nodes cannot be overturned by morphological characters), whereas the molecular sequence characters, where applicable, are effectively weighted to zero at unconstrained nodes. Thus, far from circumventing the weighting problem, the molecular scaffold generates a unique weighting problem of its own.

Whether or not one wishes to view the molecular scaffold approach under the rubric of a weighting scheme, the implicit assumptions built into the approach are contradictory. At nodes that are constrained, the molecular sequence data are considered reliable, whereas at unconstrained nodes they are not. Thus, in a strict molecular scaffold—in which all nodes suggested by the molecular sequence data are constrained (e.g., O'Leary & Geisler, 1999; Sánchez-Villagra et al., 2003; Roca et al., 2004; Asher et al., 2005a; Lee, 2005; Teeling et al., 2005; Xiang et al., 2005)—the sequence data are assumed to provide a reliable estimate of phylogeny at all nodes where they are informative, whereas the morphological data only contribute to the structure of the tree where molecular sequence data cannot be applied. In a semi-strict molecular scaffold—in which only some nodes recovered by the molecular sequence data are constrained (e.g., Springer et al., 2001; Lee, 2005; Kay & Cozzuol, 2006; Magallón, 2007; Manos et al., 2007)—the sequence data are only allowed to contribute to the tree topology at nodes that meet a given criterion (for instance, have certain support values; cut-off values for nodes in semi-strict molecular scaffolds have ranged from 70% [Lee, 2005] to 90% [Springer et al., 2001] in previous studies), whereas the morphological data contribute structure only at nodes that do not meet the threshold criterion or where sequence data do not apply. Thus, an implicit and contradictory judgment about the value and reliability of each data partition for phylogeny reconstruction is built into the molecular scaffold approach—molecular sequence data are generally favored, whereas morphological data have value only when evidence from sequence data is poor or nonexistent.

CHARACTER MAPPING AND THE STUDY OF MORPHOLOGICAL CHARACTER EVOLUTION

Regardless of their effect on tree topology or the method used to include them in an analysis, fossil taxa are undoubtedly very significant in the application of phylogenetic hypotheses, specifically interpreting morphological evolution within and across monophyletic groups. In fact, it seems safe to suggest that the study of character evolution across major organismal groups such as the seed plants and amniotes must incorporate fossil taxa to be relevant. This is because so much of the diversity that links extant end points—or even stands on its own as a major component of the evolutionary history of these groups—is missing in analyses that incorporate only extant taxa. In such cases, the hierarchical nature of evolution coupled with the removal of diversity through extinction may have the power to obscure relationships and morphological character evolution in a way that can only be addressed through reference to the fossil record (e.g., Wheeler, 1992; Cobbett et al., 2007).

Before discussing the potential benefits and difficulties of studying character evolution using a phylogenetic hypothesis that includes fossil taxa, it is necessary to address an old argument that continues to persist in the literature: that it is circular to map a character on a phylogeny when that same character is incorporated into the matrix on which the phylogeny is based. Thus, the argument goes, if one wishes to explore morphological character evolution, molecular sequence data should be used to construct a phylogeny, and morphological data should be mapped on the phylogeny after the fact, wherein they can be used to trace the evolution of morphological traits (Armbruster, 1992; Hedges & Maxson, 1996, 1997); or, more generally, characters to be mapped should be removed from the matrix when building a phylogeny so that that phylogeny is independent of the mapped characters (e.g., Springer et al., 2001). If inclusion of morphological characters in matrices and subsequent mapping of those same traits on the resultant trees were circular, this would pose a grave problem for paleontologists. How best to study morphological

character evolution in a phylogenetic context when morphological characters are usually the only source of information about fossil taxa that can be harnessed to build phylogenies including those taxa?

The most fundamental assumption of the independence or circularity argument appears to be that the study of character evolution should be independent of tree building, or that the character(s) under consideration in proposing a specific evolutionary hypothesis must be independent of the construction of the tree used to test that evolutionary hypothesis (Coddington, 1988; Brooks & McLennan, 1991). However, there is no logical reason that a phylogeny should be independent of the characters to be mapped on it. If the characters to be mapped are assumed to be among the class of characters that are suitable for phylogeny reconstruction, they should not be independent of phylogeny, and there is no circularity in mapping those same characters onto the phylogenetic topology or topologies on which they have been optimized during the tree search (e.g., Deleporte, 1993; Kluge & Wolf, 1993). This is because all characters coded for a terminal coexist within the same taxon and are hypothesized to be the result of the evolutionary history of that taxon and informative with respect to phylogeny. Thus, independence of a phylogeny from a character to be mapped on that phylogeny is not really achieved by removing a phylogenetically informative character from the analysis: although the removed character is independent of the mechanics of building the phylogeny, it is not independent of the true phylogeny or evolutionary history of the taxa under consideration in the analysis, which we presume to exist independently of our ability to correctly recover it. In fact, the unintended consequence of character removal in order to avoid circularity may be to make phylogenetic hypotheses themselves less robust, because evidence pertinent to evolutionary history is being ignored (see Kluge, 1989; Nixon & Carpenter, 1996), which can impact the tree topologies recovered.

That argument addressed, inclusion of fossil taxa among the terminals in a phylogenetic analysis has great potential for elucidating the evolutionary histories of individual aspects of whole organisms. Applications include: (1) determination of the sequence of character transformation; (2) insight into the timing of evolutionary events; and (3) inference of unknown aspects of the morphology (e.g., due to incomplete preservation) or behavior of extinct organisms and especially testing of evolutionary hypotheses.

SEQUENCE OF CHARACTER EVOLUTION

Inclusion of fossil taxa as terminals on trees can help to bridge gaps in our understanding of morphological character evolution as viewed solely from the present. Fossil taxa provide a broader sampling not only of morphologically but also of temporally diverse taxa, and thus are actual data points representing samples taken from across time at different stages in the evolutionary history of a group (Cobbett et al., 2007). As such, their inclusion as terminals in phylogenetic analyses may alter the perceived sequence of character state transformations in a group regardless of whether their inclusion significantly alters tree topology.

A simple situation in which a fossil taxon or fossil taxa can impact understanding of character evolution is when a fossil taxon or fossil taxa are intercalated among the synapomorphies along an internode that would be unbroken if only extant taxa were considered. Intercalation of such taxa may indicate the sequential evolutionary order of synapomorphies that otherwise cluster at the same node when only extant taxa are considered. For instance, in a simultaneous analysis of morphological and molecular sequence data for extant and fossil taxa within the angiosperm order Saxifragales by Hermsen et al. (2006), inclusion of the Cretaceous flower and fruit taxon *Microaltingia* Zhou, Crepet & Nixon indicated that the evolution of unisexuality and loss of the corolla preceded the evolution of winged seeds and decurrent stigmas in the stem group leading to the clade including extant *Cercidiphyllum* Siebold & Zucc. (katsura) and Altingiaceae (sweet gum family). Thus, inclusion of *Microaltingia* broke up a series of synapomorphies that would otherwise have occurred together at the node subtending the extant clade, assuming the same topology and character optimizations if *Microaltingia* were removed (Hermsen et al., 2006). Horovitz (1999a) compared the results of simultaneous analyses of morphological (primarily skeletal) data and molecular sequence data from platyrrhines (New World monkeys) in which the relative relationships among the extant taxa were the same with and without inclusion of the fossil taxa. In at least three specific instances, inclusion of the fossil taxa broke up clusters of synapomorphies, introducing "a stepwise appearance of different characters along the phylogeny, that would seem to appear in larger clusters of synapomorphies in the phylogeny composed of Recent taxa only" (Horovitz, 1999a: 24). One instance involved the subfamily Callitrichinae (marmosets and tamarins), which was supported by eight synapomorphies in the extant-only analysis; these character transformations were spread among four separate nodes when fossil taxa were included. Horovitz (1999a: 26) suggested that the breakup of the synapomorphy cluster by the fossil taxa "shed some light on the process that was presumably a consequence of reduction in body size."

Inclusion of fossil taxa may also change the perceived utility of characters to provide grouping information. Character states that are autapomorphic (parsimony-uninformative) when only extant taxa are considered may turn out to be synapomorphies when fossil taxa are included (Donoghue et al., 1989). Horovitz (1999a) provided a good example of this in her comparison of topologies recovered from simultaneous analyses for extant platyrrhines with and without fossil taxa. In that example, *Callimico goeldii* Thomas (Goeldi's marmoset) is offset by four autapomorphies in the tree including only extant taxa; two of these autapomorphies became synapomorphies for *Callimico* and two fossil taxa (*Patasola magdalenae* Kay & Meldrum and *Carlocebus carmenensis* Fleagle) when the fossil taxa were included in the analysis (Horovitz, 1999a). Alternatively, character transformations that appear to be unambiguous synapomorphies when only extant taxa are considered may become homoplasious when more diversity is sampled (Donoghue et al., 1989). The Gatesy et al. (2003) simultaneous analyses of crocodylians with and without fossil taxa provide examples of this. As they noted, "A more complete sampling of taxa [inclusion of fossil taxa] uncovered additional homoplasy, revealed uncertainties in character optimizations, and ultimately overturned hypotheses of homology that were based solely on the extant biota" (Gatesy et al., 2003: 412). As a specific example, among extant taxa alone, presence of "rectangular dorsal midline osteoderms" was an unequivocal synapomorphy for *Tomistoma schlegelii* (false gavial) and *Gavialis gangeticus* (gavial), whereas "[t]he combined evidence topology [including fossil taxa] implied that square/equant osteoderms instead were independently derived from rectangular osteoderms within Alligatoroidea and Crocodylinae" (Gatesy et al., 2003: 412).

Fossil taxa may also help to establish the polarity within characters present in a clade but inapplicable in the most closely related extant clades; this may be especially true for groups offset by many characters not represented in their nearest living relatives (Donoghue et al., 1989). Illustrative of this phenomenon in the context of simultaneous analysis of morphological with molecular sequence data is that of the relationship of the gavial to the remainder of extant crocodilians (Gatesy et al., 2003, introduced above). Simultaneous analysis of morphological and molecular sequence data including both extant and fossil crocodilians resolved some fossil taxa along the stem lineage leading to extant crocodilians, their presence clarifying and, in some cases, overturning the polarity of character states within characters for the ingroup when compared to the analysis of data from extant taxa alone. For instance, when only extant crocodilians were included in the simultaneous analysis of morphological with molecular sequence data, character state polarity could not be established in the occlusal pattern of dentary teeth in crocodilians because the character was inapplicable in the outgroup (Aves, birds); however, when fossil taxa representing more closely related outgroups were included, a clear polarity was established, suggesting Alligatoroidea (alligators) have the plesiomorphic condition. This occurred because the nearest outgroup (Aves, birds) for the extant crocodilians alone was coded as inapplicable for 42% of the morphological characters present within the extant crocodilians, and thus the fossil taxa proved more relevant in analyzing character evolution within the least inclusive clade including all extant crocodilians (Gatesy et al., 2003).

Fossil taxa may also help to establish a single preferred optimization sequence of character states that have multiple equally parsimonious optimizations when only extant taxa are considered, reducing ambiguity byincreasing taxon sampling density. For an example from crocodylians of how a suite of characters was differentially optimized (in terms of gains and losses) when fossil taxa were included or excluded from phylogenetic analysis, see Gatesy et al. (2003).

Finally, fossil taxa may add entirely novel characters or variations within characters to an analysis, providing information that we would have been completely ignorant of were they not included. For instance, inclusion of fossil penguins in an analysis of morphological and molecular sequence data for all extant penguins by Clarke et al. (2007) demonstrated that the ancestral beak morphology for penguins is "[a]n elongate, powerfully constructed beak unknown in extant penguins" (Clarke et al., 2007: 11550).

TIMING OF CHARACTER EVOLUTION

Another, perhaps presently underexploited, aspect of character mapping in the context of a phylogeny is to bracket the timing of character transformations. While determining a maximum limit on the time of appearance of a given synapomorphy by reference to the fossil record is controversial—as it is wrapped up with determining ancestry (Hermsen & Hendricks, 2007) and is impeded by the fact that the true time of the first appearance of a fossil taxon (and thus the characters it bears) cannot be confirmed simply through a literal reading of the fossil record—determining the minimum age of a synapomorphy is straightforward. It has long been recognized that the minimum age of a clade can be estimated by reference to the oldest of the descendants of the most recent common ancestor of that clade (e.g., Hennig, 1966);

this technique for estimating the minimum age of a clade has been exploited using various permutations of the same basic idea, such as comparison of the age of a clade to that of its sister taxon or ghost lineage analysis (Norell, 1992, 1993), or minimum age node mapping (Crepet et al., 2004; also see Hermsen & Hendricks, 2006). The minimum age of the most recent common ancestor of a clade is also the minimum age of the synapomorphies that define that clade, unless one or more of the synapomorphies is demonstrably older. Thus, mapping of characters onto a phylogeny and consideration of the minimum ages of the nodes on a cladogram can allow one to place minimum ages on synapomorphies as well. The bat phylogeny does not provide a particularly compelling example of this, since some of the characters of most interest that are not preserved in the fossil taxa (e.g., laryngeal echolocation) cannot be mapped unambiguously on the resultant trees, or too many topologies exist to make accurate age interpretations; however, in some of the trees from the unconstrained simultaneous analysis (analysis 3b, Fig. 1) and from the molecular scaffold analysis (analysis 4, Fig. 2), at least one Eocene fossil bat taxon is nested within a clade of bats characterized in part by laryngeal echolocation. Therefore, minimum age node mapping performed concurrently with character mapping on these trees suggests that laryngeal echolocation had evolved by the Eocene, an inference that has also been made through structural analysis of the fossils (e.g., Simmons & Geisler, 1998).

Hermsen et al. (2006) provided another example of minimum age node mapping overlain on character mapping in their simultaneous analysis of the angiosperm clade Saxifragales (introduced above in the section "Sequence of Character Evolution"). In that example, which occurs only in two of eight MPTs, minimum and maximum ages for the timing of evolution of dorsifixed anthers in the clade including the extant families Pterostemonaceae and Iteaceae (Virginia willow family) were inferred on the basis of traits present in the fossil flower and fruit taxon *Divisestylus* Hermsen, Gandolfo, Nixon & Crepet (two species of which were included as terminals in the analysis) as well as fossil pollen that was not directly included as a terminal in the analysis. The synapomorphy of dorsifixed anthers was mapped on the branch immediately descendant to the node where the species of *Divisestylus* attached. Because one species, *D. brevistamineus* Hermsen, Gandolfo, Nixon & Crepet, had been documented to have had basifixed anthers (Hermsen et al., 2003)—suggesting that the dorsifixed condition had not yet evolved—it was used to supply a maximum age of about 90 million years ago (Ma) on the appearance of dorsifixed anthers. Dispersed diporate pollen, a synapomorphy for Iteaceae (*Choristylis* Harv. and *Itea* L.), mapped at the node descendant to the node supported by dorsifixed anthers, provided a minimum age of about 50 Ma, the approximate time of first appearance of this pollen in the fossil record (Moss et al., 2005). Thus, it was suggested that a transition from basifixed to dorsifixed anthers occurred somewhere between about 90 and 50 Ma.

Hermsen and Hendricks (2007) formalized a methodology for applying minimum and maximum ages (relative or numerical) on the appearance of synapomorphies that are mapped on a cladogram that includes fossil taxa as terminals. While methods for inferring the timing of first appearance of synapomorphies with reference to fossil taxa included directly in cladistic analyses have not often been explicitly employed by overlaying minimum age node mapping and character mapping on a cladogram, the timing of first appearance of a trait has been inferred using similar logic. For instance, inference of the existence of a given trait at a given time might be made when optimization suggests that trait was present in a fossil taxon in which it cannot be directly observed (see discussion below on inference, ambiguity, and hypothesis testing), thus providing a minimum age for the appearance of that synapomorphy.

INFERENCE, AMBIGUITY, AND HYPOTHESIS TESTING

One great promise of phylogenetics as applied to the fossil record is the ability to test structural, functional, or behavioral hypotheses by inferring the presence or absence of particular structures, behaviors, or functional features in fossil taxa through optimization of these attributes on phylogenetic trees; this is especially important when these attributes are unlikely to be preserved or are unpreservable in the fossil record. As pointed out by Bryant and Russell (1992) and Witmer (1995), a phylogenetic bracket (to use Witmer's term) is necessary in order to unambiguously infer the presence or absence of traits in a fossil taxon in which these traits cannot be observed; in other words, taxa that are unambiguously known to lack or possess a feature of interest must flank the taxon in which the trait is unknown in order to unambiguously infer that trait's absence or presence in that taxon (e.g., to unambiguously optimize the character state transformation at the node where the fossil taxon attaches to the tree; see also discussion in O'Leary, 2001). O'Leary (2001), for instance, was able to infer whether basal Cetacea (whales) had hair (extant cetaceans do not have hair; O'Leary, 2001) using a simultaneous analysis of morphological and molecular sequence data for cetaceans and artiodac-

tyls (even-toed hoofed mammals). Mapping of the character for hair (with presence/absence states) onto two of 33 MPTs unambiguously indicated that the fossil taxa *Ambulocetus* Thewissen, Hussain & Arif and *Pakicetus* Gingrich & Russell (extinct basal whales) were hairless, since they were bracketed by extant taxa, the hippopotamids and extant cetaceans, which lack hair (O'Leary, 2001: figs. 6b, 6c). The extinct mammal group Mesonychia was reconstructed as either hairless or hairy depending on where the group was resolved in the MPTs.

Phylogenetics may be limited as a tool for inferring behavioral or functional (or even unpreserved structural) features of extinct taxa, however. O'Leary (2001) provides several examples of this in her total evidence analysis of cetaceans and artiodactyls. Structural evidence has been used to hypothesize that, for instance, basal whales were capable of terrestrial quadrupedal locomotion and of processing sound under water (see citations in O'Leary, 2001). However, O'Leary (2001) was unable to unambiguously infer whether basal whales were capable of these behaviors through optimization of behavioral characters on phylogenetic trees. She suggested that direct fossil evidence—when available—can provide the ultimate corroboration (or refutation) of a functional or behavioral hypothesis, and, where direct evidence is unavailable, indirect evidence (for instance, interpretation of an organism's habitat) could be utilized to address some questions that cannot be addressed successfully through character optimization. For instance, the hypothesis that early whales were quadrupedal was suggested by observation of the breadth of the sacro-iliac joint in the extinct whale *Ambulocetus* and also inference about the breadth of the sacro-iliac joint in *Pakicetus* through character optimization on phylogenetic trees (O'Leary, 2001: fig. 6). The behavior of quadrupedal locomotion was, however, ambiguously optimized for these taxa; thus, the hypothesis of terrestrial quadrapedal locomotion in early whales cannot be corroborated through character optimization. In this case, direct fossil evidence could theoretically provide a solution: "if footprints of a pakicetid or ambulocetid were found" (O'Leary, 2001: 501), the behavioral hypothesis of quadrupedal locomotion in these early whales could be corroborated. In the case of adaptation to hearing under water—where presence of a pachyostotic bulla in the ears of extinct basal whales suggests they were capable of hearing underwater—optimization is ambiguous and direct fossil evidence about hearing capabilities does not (and likely cannot) exist. Thus, "we are left with character correlation argument only or an inference from design or environment based on the characteristics of organisms that happen to be alive" (O'Leary, 2001: 501).

For bats, extrapolation from structural features has been used to suggest that the extinct taxa *Archaeonycteris*, *Hassianycteris*, *Palaeochiropteryx*, and *Tanzanycteris* were capable of laryngeal echolocation (e.g., Simmons & Geisler, 1998; Gunnell et al., 2003). While mapping of the echolocation character on the topologies based on unconstrained combined data is equivocal for presence or absence of this behavior in these taxa in most instances (except *Tanzanycteris*, which can be inferred as capable of the behavior in eight of 16 trees; Fig. 3), hypothesizing that they were capable of laryngeal echolocation on the basis of structural features does not add extra steps to the MPTs. Such an interpretation instead imposes a preferred approach to mapping the echolocation character such that its presence would support the node immediately descendant to the node at which Megachiroptera attaches to the tree, although presence of this behavior is not directly testable using the fossil record. Certainly, the examples from bats and whales expose one of the great weaknesses of phylogenetics for addressing aspects of evolution that go unrecorded in the fossil record: "While questions about stem taxa to major clades are often some of the most interesting, they can expose areas where it is very difficult to test functional and behavioral inferences in fossils with cladistic character data" (O'Leary, 2001: 501) due to the lack of extant taxa that can serve as phylogenetic brackets that allow for unambiguous optimization of intangible features.

When structural (or other) evidence contravenes the optimization of a behavioral character on a phylogeny, the problem of functional and behavioral inference becomes even more complex. Simmons and Geisler (1998), for instance, have argued that structural evidence suggests that *Icaronycteris* was capable of laryngeal echolocation (see similar discussion of echolocation in *Icaronycteris* by Novacek [1987], Gunnell & Simmons [2005], and Simmons [2005a]), although mapping of the laryngeal echolocation character on the more recent most parsimonious topologies from morphological data (Gunnell & Simmons, 2005) and the tree resulting from the combined analysis here (Fig. 3) suggest that it was not. When structural features and character mapping disagree, one must choose which is preferable, or whether the evidence is equivocal. In the bat example, therefore, one must decide whether *Icaronycteris* should be coded as missing (?) for the presence of laryngeal echolocation (as it is in the matrix of Gunnell and Simmons, 2005), or whether it should be assumed to be an echolocator a priori on the basis of other evidence. The latter extrapolatory approach

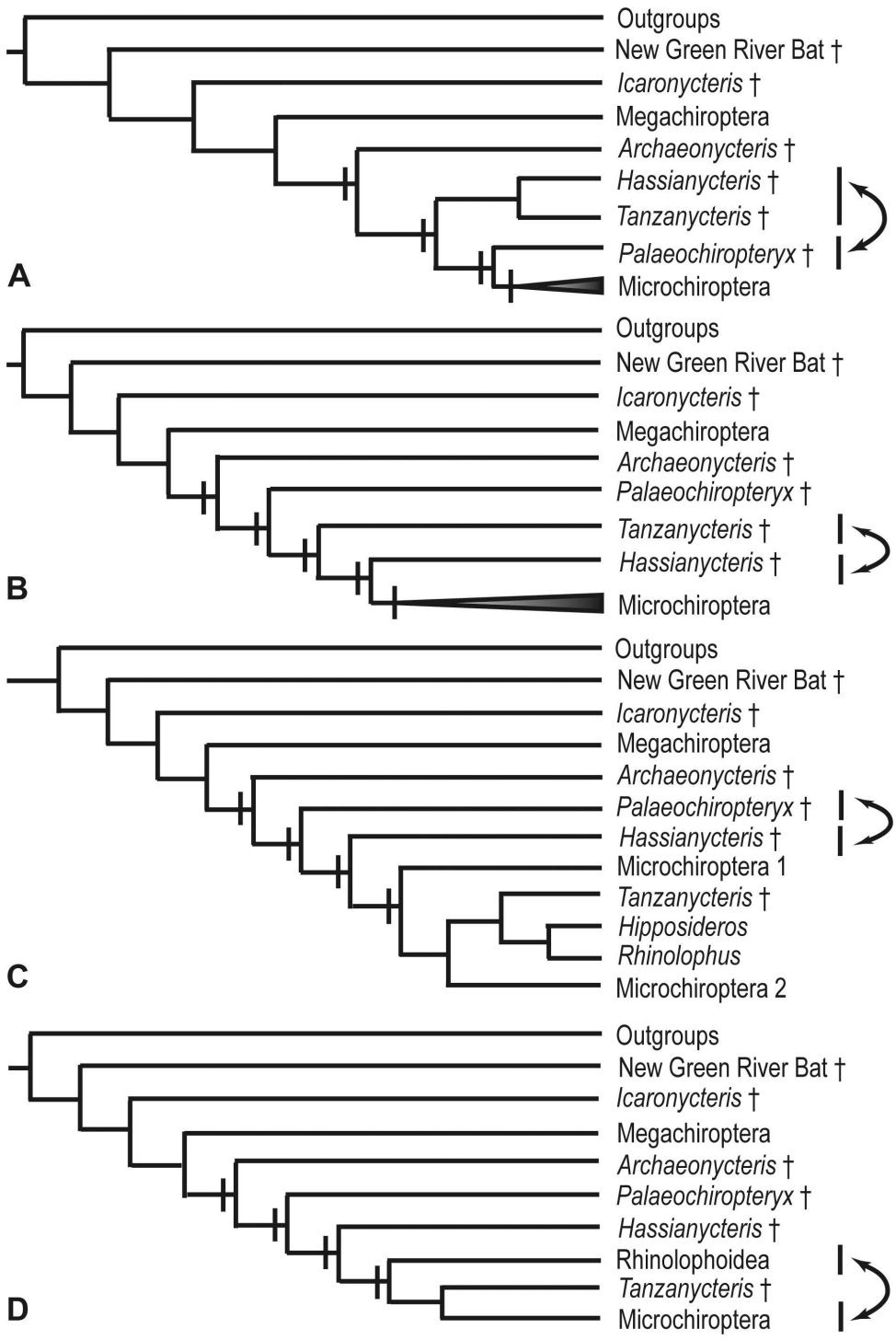

Figure 3. Four scenarios (each representing four MPTs) of character evolution based on 16 MPTs resulting from analysis of combined morphological and molecular sequence data sets without constraints (analysis 3b; see Fig. 1). In each case, arrows indicate a reversal of the positions of taxa in two of four MPTs. Fossil taxa are indicated by daggers (†). Equally optimal positions for a transition from absence to presence of laryngeal echolocation are indicated by hash marks. Each scenario supports one origination of laryngeal echolocation with no losses. Mapping of this character does not unambiguously affirm the hypothesis that *Archaeonycteris*, *Palaeochiropteryx*, and *Hassianycteris* were able to echolocate. *Tanzanycteris* is

(Bryant & Russell, 1992), of course, overrides the potential utility of phylogenetics to help one infer the presence or absence of behavioral characteristics that cannot be directly observed; furthermore, the phylogenetic topology and/or interpretation of character evolution may be impacted by choosing a character state on the basis of inferred rather than observed characteristics or behaviors. Mapping of co-varying structural characters that indicate the presence of a particular behavior may be one way to circumvent this apparent conundrum (e.g., Springer et al., 2001). However, when this reasoning is employed, it renders questionable the coding of both structural and behavioral characters in the matrix on which the analysis is based—as they are in the Chiropteran but not the Cetacean data set—since doing so would violate the assumption of character independence.

Another promising avenue for hypothesis testing is the potential utility of phylogenies to provide a framework on which the sequence and timing of character evolution can be juxtaposed; this is important, for instance, for corroborating or refuting proposed correlations between the evolution of structural features and extrinsic selective forces that occurred during geologic time. Hypothetical instances of this are given by Maddison and Maddison (2000) and Hermsen and Hendricks (2007). Both examples rely on the existence of a clade of extant taxa that is characterized by a unique adaptation that has been hypothesized to have arisen due to a selective force (e.g., appearance of a predator, climatic shifts, etc.). The extant clade is then analyzed with fossil taxa, which are found to nest within it. These taxa are older than the selective force that was hypothesized to have favored the adaptation that is a synapomorphy for the clade, so the cause-effect hypothesis is rejected.

As in determining minimum and maximum possible ages for the appearance of synapomorphies, the utility of this approach is biased—it is much easier to reject a cause-effect relationship between an extrinsic factor and the appearance of a synapomorphy if the minimum age of that synapomorphy is older than the selective pressure that supposedly favored its establishment. Clarke et al. (2007) provided a good example of this in penguins, although it is not directly linked to one specific character. Molecular divergence dating has suggested that the origin of the Spheniscidae (the clade including all extant penguins) occurred in the Paleogene (~40 Ma), possibly "concomitant with the initiation of the circum-Antarctic current, initial onset of Cenozoic global cooling, or at the proposed extinction of giant penguins" (Clarke et al., 2007: 11549). Clarke et al. (2007) cast doubt on these correlations through simultaneous analysis of fossil and extant penguins, which suggested instead that Speniscidae arose in the Neogene, based on the stratigraphic occurrences of the fossil penguins included in their analysis. They noted that accommodating a Paleogene–Spheniscidae radiation given their inferred phylogeny would require a 164.1–334.2 million year ghost lineage (estimated using MSM* [Manhattan Stratigraphic Measure], Pol & Norell, 2001). The extensive penguin fossil record known from between 40 Ma and 8 Ma includes no fossils that can be assigned to Spheniscidae. Despite this, they noted that their evidence is suggestive, not conclusive, since "stratigraphic data can only falsify a divergence date when a fossil discovery is older than estimated" (Clarke et al., 2007: 11549).

Conclusions

The challenge for biologists and paleontologists in coming years will be to more fully integrate our expanding knowledge of phylogeny as elucidated using molecular sequence data with our growing knowledge of the fossil record. Molecular sequence data have certainly helped to clarify relationships among extant taxa and will likely continue to modify and improve our understanding of the tree of life. Although fossil taxa almost always lack sequence data, they represent extinct morphologies that may be informative to the overall history of life on earth, as well as evolution within particular extant and extinct groups of organisms.

Several perceived obstacles to identifying the phylogenetic positions of fossil relative to extant taxa have been addressed above. Some of these purported problems are closely related to fossil taxa in particular (e.g., missing data), while others are related to morphological data in general (e.g., convergence and character weighting). These issues have been overemphasized as obstacles to effectively including fossil taxa in phylogenetic analyses, despite significant empirical advances in our understanding of how well the simultaneous analysis (or supermatrix) approach

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unambiguously inferred as possessing laryngeal echolocation in eight of 16 trees (C, D). In scenario C, Microchiroptera 2 includes the extant genera *Rhinopoma* E. Geoffroy, *Craseonycteris* Hill, *Megaderma* E. Geoffroy, and *Macroderma* Miller and Microchiroptera 1 contains all other ingroup microbat taxa. In scenario D, Rhinolophoidea includes *Rhinolophus* Lacépède, *Hipposideros* Gray, *Rhinopoma*, *Craseonycteris*, *Megaderma* and *Macroderma*, and Microchiroptera represents the remainder of the microbats.

often works for elucidating the phylogenetic context of fossil taxa relative to the extant biota. A priori arguments that fossil taxa should not be included in simultaneous analyses are insupportable—the suitability of fossil taxa for inclusion in these data sets needs to be tested empirically in the context of each data matrix and their behavior evaluated on a case-by-case basis (for instance, using pseudofossil analyses). The molecular scaffold approach has several weaknesses as a method for integrating morphological and molecular sequence data in order to include fossil taxa in phylogenetic hypotheses. Perhaps the largest of these is that the methodology obscures secondary signals that may manifest themselves during simultaneous analysis, that it minimizes the impact of fossil taxa on tree topology, and, when a semi-strict molecular scaffold is employed, it provides a contradictory perspective on the reliability of molecular sequence and morphological data.

A final note should be added in reference to paleobotany in particular, since the subject of this symposium is paleobotany in the post-genomics era. Most examples of simultaneous (and scaffold) analyses including fossil taxa in this paper come from studies of vertebrates (in addition to studies cited above, see, for instance, Brochu, 1997; Shaffer et al., 1997; O'Leary, 1999; Gao & Shubin, 2001; O'Leary et al., 2004; Asher et al., 2005b; Démére et al., 2005; Geisler & Uhen, 2005; Horovitz et al., 2006; Ksepka et al., 2006). This is because vertebrate paleontologists and zoologists have been leaders in the field, exploring methods to integrate fossil and extant taxa in greater numbers than those who study plants (in addition to the studies cited above, see Sun et al., 2002; Hermsen et al., 2003; Gandolfo et al., 2004; Crepet et al., 2005; Xiang et al., 2005) or invertebrates (in addition to the studies cited above, see Littlewood & Smith, 1995; Smith et al., 1995; Arango & Wheeler, 2007; Cardinal & Packer, 2007). Some of this may be the result of the large number of osteological characters that can be scored for vertebrates, perhaps making them more amenable to such analyses than other groups. Paleontologists and biologists who study vertebrates are also leading the way in the study of character evolution on phylogenies including extant and fossil taxa (see examples discussed above). While botanists have certainly kept pace in the arena of collecting sequence data and building phylogenies of extant organisms from those data, more work needs to be done to integrate our evolving view of the relationships of extant groups to one another with our knowledge of morphology, development, and the fossil record. Only integration of these disparate data types will provide us with a holistic view of plant evolution.

Addendum

Following the completion of the analyses documented in this paper, the "Green River bat" was formally published as *Onychonycteris finneyi* Simmons, Seymour, Habersetzer & Gunnell, 2008. Along with the formal description of this bat, Simmons et al. (2008) published new morphological and molecular scaffold phylogenetic analyses. These analyses were based on an updated morphological matrix and a molecular scaffold derived from a more recent molecular analysis than the Teeling et al. (2005) data used in our study. Notably, structural evidence from the newly published taxon is interpreted as confirming our inference that *Onychonycteris* was incapable of echolocation.

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