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Report

Phylogenomic Analyses Elucidate the Evolutionary Relationships of Bats

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Summary

Molecular phylogenetics has rapidly established the evolutionary positions of most major mammal groups [1, 2], yet analyses have repeatedly failed to agree on that of bats (order Chiroptera) [3–6]. Moreover, the relationship among the major bat lineages has proven equally contentious, with ongoing disagreements about whether echolocating bats are paraphyletic [7–9] or a true group [10] having profound implications for whether echolocation evolved once or possibly multiple times. By generating new bat genome data and applying model-based phylogenomic analyses designed to accommodate heterogeneous evolutionary processes [4, 11], we show that—contrary to recent suggestions—bats are not closely related to odd-toed ungulates but instead have a more ancient origin as sister group to a large clade of carnivores, ungulates, and cetaceans. Additionally, we provide the first genome-scale support showing that laryngeal echolocating bats are not a true group and that this paraphyly is robust to their position within mammals. We suggest that earlier disagreements in the literature may reflect model misspecification, long-branch artifacts, poor taxonomic coverage, and differences in the phylogenetic markers used. These findings are a timely reminder of the relevance of experimental design and careful statistical analysis as we move into the phylogenomic era.

Results and Discussion

Phylogenetic Reconstruction of Position of Bats

Bats are an ancient and diverse group that originated ~65 million years ago in the late Cretaceous/early Paleocene and underwent a rapid radiation in the Eocene [9]. However, the exact evolutionary relationship of bats to their closest mammalian relatives is poorly understood due to their unique morphological features associated with flight, a lack of intermediate forms, and a poor fossil record. Our phylogenomic analyses of 2,320 coding DNA sequence (CDS) alignments, each containing 14 to 22 species and including four new bats, allowed us to determine the position of bats within the superorder Laurasiatheria with high confidence. Maximum likelihood (ML) reconstructions undertaken separately for concatenated CDS alignments of nucleotides and amino acids (2.4 Mb and ~7.9×10^5 residues, respectively) both yielded congruent and highly resolved trees (Figure 1). In both the nucleotide and amino acid trees, the bats formed a well-supported sister group with a clade of ungulates, cetaceans, and carnivores. Within this clade (sometimes called Fereuungulata), we find strong support for placing the order Cetartiodactyla in a monophyletic clade with the Perissodactyla as a sister group to the Carnivora. We also obtain very strong support for many of the proposed clades within Laurasiatheria (Table 1) as well as for the deepest relationships within the mammals (Figure 1).

It is now widely accepted that bats belong to the superorder Laurasiatheria [1, 2, 6]; however, the relationships within this group have been strongly contended, with different studies proposing that bats are a sister group to a clade comprising carnivores and odd-toed ungulates [12–14] or to the Fereuungulata [1, 4, 6, 13, 15, 16]. The former hypothesis, termed Pegasoferae, was supported by the results of recent phylogenomic analyses that included four bat species [5], as well as by analyses of retroposon insertions [17] and conserved noncoding elements [13]. Our new results contradict this arrangement and instead support the latter hypothesis, in agreement with earlier findings [1, 6].

Conflicting results might reflect noisy phylogenetic signal, differences in taxonomic sampling, and/or the extent to which studies have controlled for heterogeneous sequence evolution inherent in large data sets of concatenated gene sequences. To account for such potential heterogeneity in our analyses, we partitioned our data set by CDS, estimated model parameters independently for each partition, and, in the case of nucleotide data, also partitioned by codon position. When we repeated our ML phylogenetic reconstructions for the full data set without model partitioning, in line with the recent genome-scale analysis [5], we recovered Pegasoferae based on the nucleotide data set, but not the amino acid data set (see Supplemental Experimental Procedures and Figure S1 available online). In both cases, the model fit was significantly worse than that of the respective partitioned model (see Supplemental Experimental Procedures). These results thus support previous studies showing that partitioning can outperform standard models of sequence evolution when phylogenetic reconstruction is based on concatenated data [11, 18, 19]. To further determine the likely cause of differences between our results, we reduced our data set by removing CDSs from taxa that were not sampled in the previous study; however, repeating the analyses still failed to robustly recover the clade Pegasoferae (Table S3).

Uncertainty surrounding the interordinal relationships within Laurasiatheria [1, 4–6, 10, 13, 17, 20–22] may also be largely attributable to rapid diversification of the main lineages (illustrated by the very short branches connecting these in the phylogenetic tree) and associated incomplete lineage sorting [4, 6, 13, 17, 21]. To account for incomplete lineage sorting and other sources of tree discordance among loci, we analyzed our data using coalescent methods of species tree reconstruction [4]. Again, coalescent trees containing all taxa were highly consistent with those inferred from the partitioned methods, both strongly supporting a sister relationship between bats

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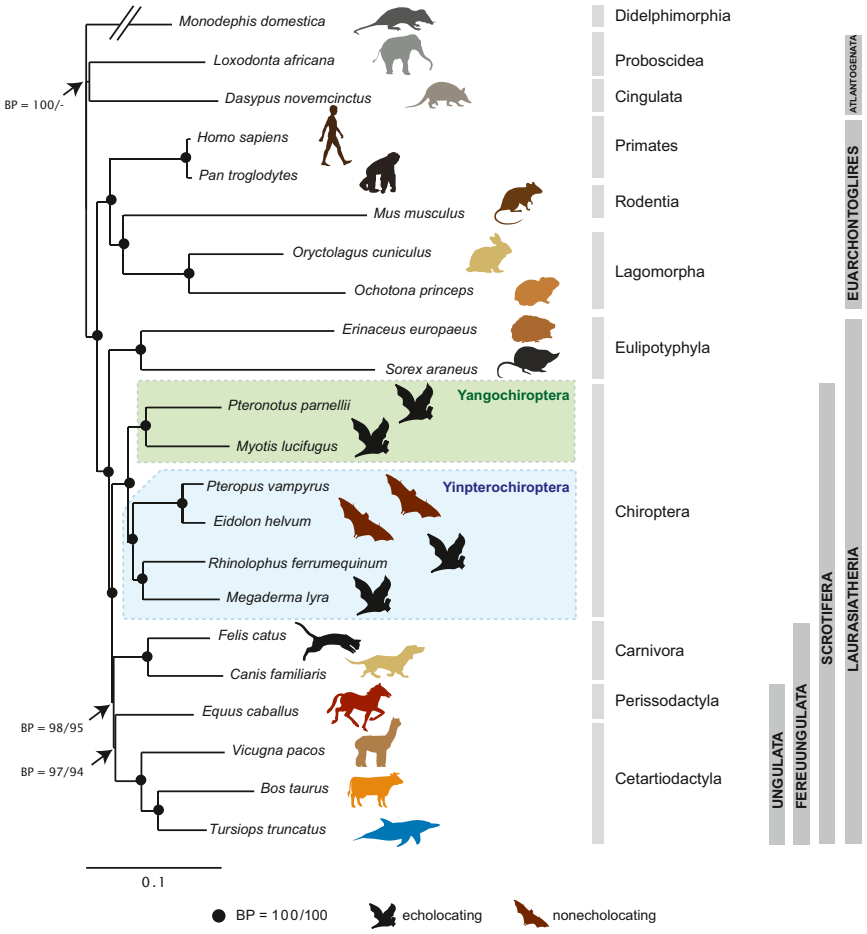

Figure 1. Evolutionary Relationships of Bats

RAxML tree inferred from the maximum likelihood (ML) analysis of the phylogenomic data set in nucleotides (2,394,810 sites) under the GTR + Γ_4 + I model of sequence evolution, partitioned by coding DNA sequence (CDS) data set (n = 2,320) and by first, second, and third codon position. ML analyses of amino acid data sets (collectively containing 787,713 sites) yielded an identical topology. Node values represent RAxML bootstrap percentages (BPs) obtained for the nucleotide and amino acid data sets, respectively. The same analyses without model partitioning recovered the clade Pegasoferae based on the nucleotide data set (see Figure S1), but not the amino acid data set.

Analyses of Bat Subordinal Relationships

Our phylogenomic reconstructions provide an equally clear and statistically robust picture of the evolutionary relationship between echolocating and nonecholocating bats, providing unequivocal support for the reciprocal monophyly of the proposed suborders Yinpterochiroptera and Yangochiroptera (bootstrap percentages [BP] = 100%; see Figure 1). Contrary to this result, a recent large-scale analysis that combined 27 genes plus morphological characters recovered the traditional subordinal split of bats into Microchiroptera, members of which are all capable of laryngeal echolocation, and Megachiroptera (Old World fruit bats), members of which are usually larger, do not possess laryngeal echolocation, and instead exhibit well-developed visual systems [23] (Figure 3B). By including bat species encompassing both proposed systems of subordinal systematics in our analyses (see Experimental Procedures), we were able to firmly reject this traditional systematic division of Microchiroptera and Megachiroptera, and this finding was also strongly corroborated by coalescent analyses (Figure 2). Within the Yinpterochiroptera, we also recovered full support for both the clades of Old World fruit bats, represented by the taxa *Eidolon helvum* and *Pteropus vampyrus*, and the laryngeal echolocators, represented by *Rhinolophus ferrumequinum* and *Megaderma lyra* (BP = 100% in both cases).

Our data therefore support most other genetic analyses that have suggested some echolocating bats are more closely related to nonecholocating Old World fruit bats than to the remaining echolocating bats [2, 7–9]. By moving from a few tens of loci to over 2,000 loci, our findings prove without doubt that the evolution of laryngeal echolocation in bats has involved either multiple acquisitions or an evolutionary loss in Old World fruit bats [7–9, 24–27].

Locus-wise Phylogenetic Support and Selection

To dissect the phylogenetic signal in our data, we assessed the relative support of each locus for the two competing hypotheses of bat subordinal systematics, each in the context of eight different recently described phylogenetic proposals and the Fereuungulata within the Scrotifera (Figure 2). Finally, to ensure that our findings were robust to potential within-locus recombination, we repeated these coalescent-based analyses using individual exons, and we recovered the same results. Our results therefore suggest that disagreements in the literature may reflect model misspecification, long branches (e.g., *Equus*), and/or poor taxon sampling.

Table 1. Recognized Orders and Proposed Higher Clades within Laurasiatheria

Grouping	Common Names of Representative Member Taxa
Laurasiatherian Orders	
Chiroptera	bats*
Eulipotyphla	shrews*, hedgehogs*, solenodons
Pholidota	Pangolins
Carnivora	cats*, dogs*, hyenas, bears, weasels
Cetartiodactyla	cows, pigs, vicunas*, deer, dolphins*, whales
Perissodactyla	horses*, tapirs, rhinoceros
Proposed Higher Clades within Laurasiatheria	
Euungulata	Perissodactyla + Cetartiodactyla
Fereuungulata	Carnivores + Euungulata (±Pholidota)
Scrotifera	Chiroptera + Carnivores + Perissodactyla + Cetartiodactyla (±Pholidota)
Pegasoferae	Chiroptera + Perissodactyla (±Carnivores)

Recognized orders and proposed higher clades within Laurasiatheria, together with the common names of representative member taxa (representatives included in our phylogenomic data set are indicated by asterisks). The first three higher clades are recovered by our study (Figures 1 and S1).

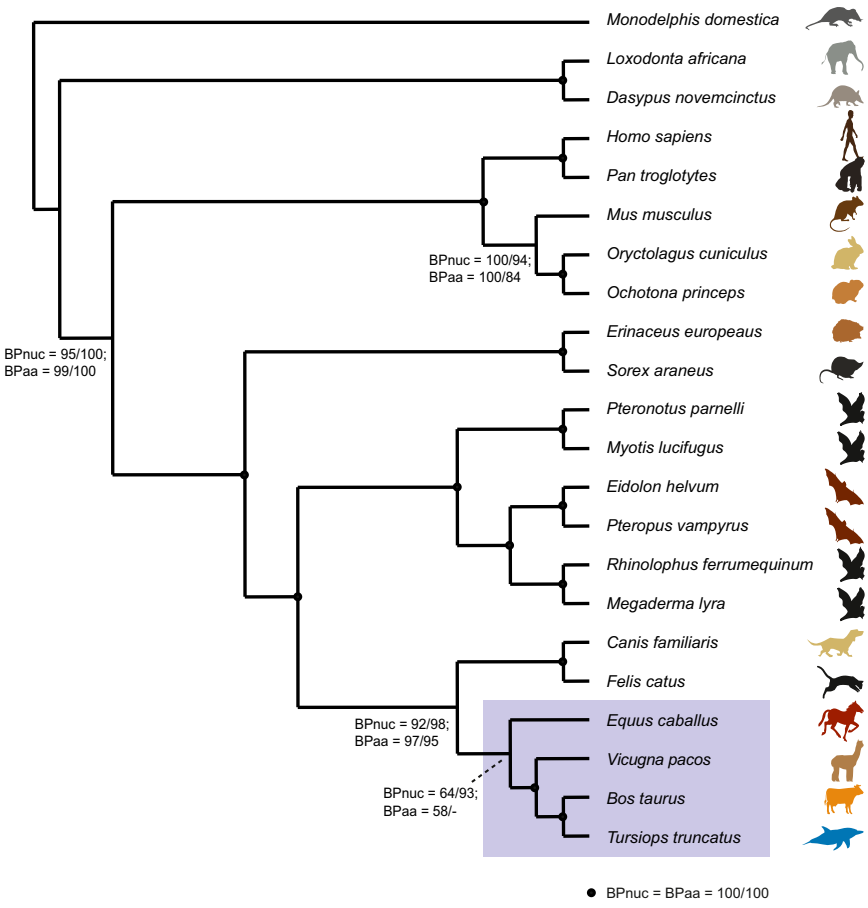

Figure 2. Evolutionary Relationships of Bats Inferred from Coalescent Model Analyses

The % bootstrap values at nodes are based on 100 bootstrap replicates of the CDS nucleotide (nuc) and amino acid (aa) data sets, shown for STAR and MP-EST methods, respectively (see Experimental Procedures). Black filled circles indicate maximal statistical support (BPnuc = 100/100; BPaa = 100/100). All four analyses yielded identical topologies with respect to the sister group relationship between Chiroptera and Fereuungulata, as well as the intraordinal subdivision of the bats into the suborders Yinpterochiroptera and Yangochiroptera. The remaining taxa relationships were also identical, with the exception of the MP-EST tree based on amino acid trees, in which Perissodactyla (represented here by *Equus caballus*) was more closely related to Carnivora than to Cetartiodactyla (not shown).

that vary in the position of the bats within Laurasiatheria (Figure 3; Table S4). Comparisons of 16 candidate topologies across 2,320 loci revealed clear and consistently greater genomic signal favoring the paraphyly of echolocating bats regardless of the relationship of bats with respect to other laurasiatherian clades (Figure 3; Table S4). Nonetheless, inspection of individual loci revealed remarkably few cases of unambiguous signal: based on approximately unbiased tests of amino acid tree selection, just 121 loci (5%) significantly rejected the Microchiroptera-Megachiroptera hypothesis in favor of the Yinpterochiroptera-Yangochiroptera division (Table S5C), whereas 19 loci (<1%) showed the opposite signature and thus favored the traditional taxonomy (Table S5B). However, these analyses did not clearly distinguish among alternative hypotheses for the placement of bats within mammals. This result highlights the common difficulties of using individual genes for recovering the species phylogeny, in this case likely due in part to the rapid early emergence of the main lineages [9], molecular convergence [28], and other factors contributing to the mixed signal in the data.

We assessed whether differential support among loci for the alternative subordinal divisions is likely due to selection by using codon-based models of molecular evolution to test for heterogeneous selection regimes in these groups (see Supplemental Experimental Procedures). These models estimate the ratio of the rate of nucleotide substitutions that result in amino acid replacements (dN) to the rate of synonymous substitutions (dS), where separate dN/dS ratios can be separately inferred for different clades in the phylogeny. Among loci supporting the Microchiroptera-Megachiroptera hypothesis, we identified three genes showing evidence of divergent selection in fruit bats: *LEF1* (lymphoid enhancer-binding factor 1), *BECN1* (beclin 1, autophagy related), and *RPE65* (retinal pigment epithelium-specific protein 65 kDa) (Table S5B). Similarly, among 161 loci supporting the Yinpterochiroptera-Yangochiroptera hypothesis, several loci (*TMED8*, *SPOK2*, *SMC3*, *KLHDC10*, *PARP6*, *ACVRL1*, *P14KB*, *LIN7C*, *BUB3*, *CCDC64B*, *C19orf55*, and *LINGO1*) showed divergent selection in these bats (Table S5C), but neither set of loci showed evidence for different selection pressure between bat lineages, suggesting that selection was not responsible for the support for these groupings (see Supplemental Experimental Procedures).

Our results show that although phylogenetic signals from single loci fail to determine bat phylogenetic affinities among laurasiatherian groups, aggregating information across loci provides compelling evidence for the phylogenetic relationship of bats to some other groups of mammals. Moreover, by providing robust statistical support for the paraphyly of laryngeal echolocating bats, this study provides the most concrete evidence to date toward resolving the long-standing debate regarding bat evolutionary history. Our results further emphasize the extraordinary phenotypic convergence seen across echolocating members of the two suborders, including the possible independent origin of laryngeal echolocation itself, a hypothesis supported by several studies of molecular evolution of sensory genes (e.g., [28–30]).

Experimental Procedures

Sequencing and De Novo Assembly of New Sequence Data

We first obtained new genome sequence data from four bat species (Tables S1A–S1C; see Supplemental Experimental Procedures). From the proposed suborder Yangochiroptera, we sequenced the echolocating species *Pteronotus parnellii* (Parnell's mustached bat), and from the proposed suborder Yinpterochiroptera, we sequenced the two echolocating species *Megaderma lyra* (greater false vampire bat) and *Rhinolophus ferrumequinum* (greater horseshoe bat) and the nonecholocating Old World fruit bat *Eidolon helvum* (straw-colored fruit bat). We combined

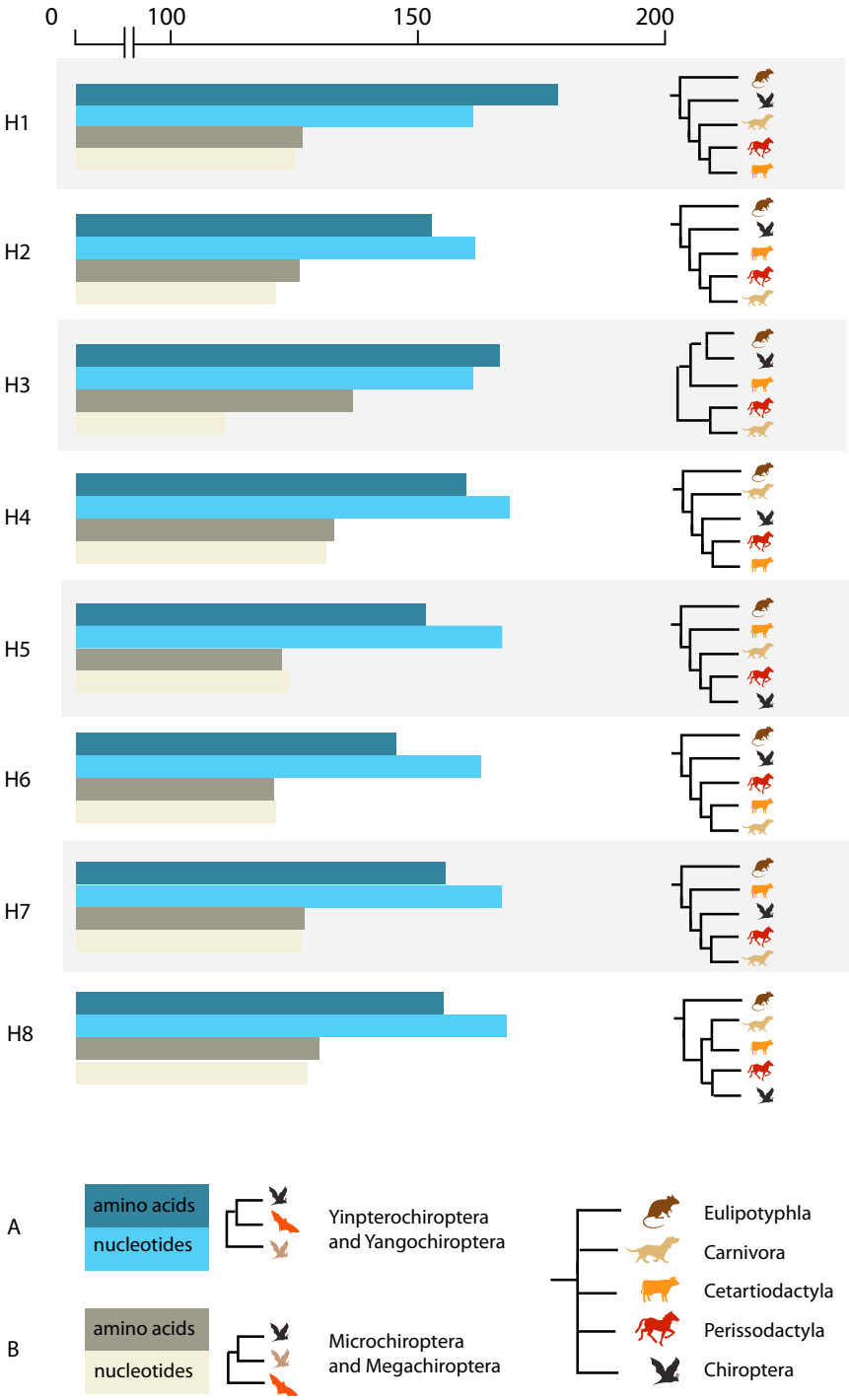

Figure 3. Relative Per-Locus Support Scores Based on Amino Acids and Nucleotides for Sixteen Alternative Species Tree Hypotheses

These hypotheses comprise eight alternative Laurasiatheria phylogenies H1–H8 that differ in the placement of bats [1, 4–6, 10, 13, 17, 20–22], and for each of the eight, the two bat subordinal hypotheses of (A) Yinpterochiroptera-Yangochiroptera (blue bars) and (B) Microchiroptera-Megachiroptera (yellow-brown bars). Details of these hypotheses are provided in Table S4. Phylogenomic analyses in RAxML based on both the concatenation and coalescent methods recovered H1. In contrast, recent genome-wide analyses based on six mammals recovered H5 [5], whereas a large-scale analysis combining morphological and molecular data recovered H4 with strong support for the Microchiroptera-Megachiroptera hypothesis [10]. The number of loci supporting a given hypothesis is the sum of weighted proportions: each proportion ranges from 1, where a single hypothesis could not be rejected, to 0.0625, where none of the 16 was rejected. Details of genes supporting the two bat subordinal hypotheses are given in Tables S5B and S5C.

these with existing sequences from a second Old World fruit bat, *Pteropus vampyrus* (large flying fox), and the echolocating species *Myotis lucifugus* (little brown bat), from the Yinpterochiroptera and Yangochiroptera, respectively. Our final data set of six bats thus contained echolocating species from both the Yangochiroptera and Yinpterochiroptera, all formerly placed in the Microchiroptera (sensu [23]), as well as two Old World fruit bats from the Yinpterochiroptera that were formerly placed in the Megachiroptera [23].

Sequencing yielded ~370 to 390 million high-quality paired-end short reads per species (Table S1C; see Supplemental Experimental Procedures). For each of the four new bats, we built contigs and scaffolds using standard methods and filled gaps with the tool GapCloser [31] (Table S1C; see Supplemental Experimental Procedures).

Phylogenomic Analyses of the Concatenated Data Set

Using new and published genome data, we built a core alignment of 12 taxa (all six bats plus dog, horse, cow, bottlenose dolphin, mouse, and human) and up to ten other mammals (see Supplemental Experimental Procedures). We conducted maximum likelihood (ML) phylogenomic analyses of codon-aligned nucleotides (n = 2,394,810) and amino acids (n = 797,713) in RAxML v7.2.8 [32] under the substitution models GTR + Γ_4 + I and JTTF + Γ_4 + I, respectively. We repeated these partitioned by CDS, and also within each CDS by codon position. In partitioned analyses, empirical base frequencies and evolutionary rates were estimated independently per partition and bootstrap support was assessing using 100 replicates (see Supplemental Experimental Procedures).

Phylogenetic Analyses of Each Locus

For each locus, we calculated log-likelihood support for nucleotide and amino acid data sets under eight proposed species tree topologies that differ in the position of bats within mammals [1, 4–6, 10, 13, 14, 17, 20–22], with bats divided into (1) Yinpterochiroptera-Yangochiroptera and (2) Microchiroptera-Megachiroptera (Figure 3; see also Table S4). For all 2,320 CDS data sets, ML optimization for each tree hypothesis was conducted using the GTR + Γ_4 + I model. For each amino acid alignment, we selected the best-fit model of substitution, using the script in RAxML as above. We then computed the approximately unbiased (AU) test statistic to compare alternative topologies [33]. For each of the 16 total species trees, we obtained a cumulative score of support by counting the number of loci supporting each phylogeny based on the AU p values for a critical value $\alpha = 0.05$, counts being weighted by the number of nonrejected tree topologies retrieved per data set (Table S4).

Phylogenetic Analyses Using Coalescent Methods

To account for incomplete lineage sorting and other potential sources of tree discordance among loci, we inferred the species tree using two coalescent methods: species tree estimation using average ranks of coalescence

(STAR) [34] and maximum pseudo-likelihood for estimating species trees (MP-EST) [35]. Both were performed with multilocus bootstraps [36] to estimate statistical support. For each CDS and amino acid alignment, ML bootstrap trees were generated using RAxML under the same model of sequence evolution used in the original heuristics and for 100 bootstrap pseudoreplicates. To account for potential within-locus recombination, we repeated these analyses using trees inferred from exon-only data, excluding exons < 450 bp (n = 632). All coalescent analyses were conducted in STRAW [37]. For details, see Supplemental Experimental Procedures.

Molecular Evolution Analyses

Molecular evolution analyses using ML codon models were implemented in PAML v4.4 [38]. For each CDS, we fitted the clade model C [39, 40] that assumes three classes of sites, which differ in their selection pressure, as measured by the nonsynonymous-to-synonymous substitution rate ratio (dN/dS, termed ω). In the first two site classes, ω was constrained to be negatively selected ($0 < \omega_0 \leq 1$) or neutral ($\omega_1 = 1$), while in the third class, ω was estimated separately in foreground (ω_2) and background (ω_3) branches without constraint. We compared the fit of clade model C to that of the nearly neutral (M1a) model and performed likelihood ratio tests (LRTs) (df = 3) to assess significance, correcting for the false discovery rate [41]. For CDSs with significant LRTs, we considered sites with a Bayes empirical Bayes posterior probability > 0.80 as being under positive selection. For details, see Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes one figure, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.09.014.

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