The hearing gene *Prestin* reunites echolocating bats

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The remarkable high-frequency sensitivity and selectivity of the mammalian auditory system has been attributed to the evolution of mechanical amplification, in which sound waves are amplified by outer hair cells in the cochlea. This process is driven by the recently discovered protein prestin, encoded by the gene *Prestin*. Echolocating bats use ultrasound for orientation and hunting and possess the highest frequency hearing of all mammals. To test for the involvement of *Prestin* in the evolution of bat echolocation, we sequenced the coding region in echolocating and nonecholocating species. The resulting putative gene tree showed strong support for a monophyletic assemblage of echolocating species, conflicting with the species phylogeny in which echolocators are paraphyletic. We reject the possibilities that this conflict arises from either gene duplication and loss or relaxed selection in nonecholocating fruit bats. Instead, we hypothesize that the putative gene tree reflects convergence at stretches of functional importance. Convergence is supported by the recovery of the species tree from alignments of hydrophobic transmembrane domains, and the putative gene tree from the intra- and extracellular domains. We also found evidence that *Prestin* has undergone Darwinian selection associated with the evolution of specialized constant-frequency echolocation, which is characterized by sharp auditory tuning. Our study of a hearing gene in bats strongly implicates *Prestin* in the evolution of echolocation, and suggests independent evolution of high-frequency hearing in bats. These results highlight the potential problems of extracting phylogenetic signals from functional genes that may be prone to convergence.

evolution | phylogenetics | convergence | cochlea | mammals

Acute and sensitive hearing is important in communication, prey detection, and predator avoidance (1). In mammals, remarkable high-frequency sensitivity and selectivity have been conferred by the evolution of a mechanical sound amplification system involving specialized outer hair cells (OHC) located in the organ of Corti in the cochlea (2, 3). Each OHC is characterized by a bundle of stereocilia, which when stimulated by incoming sound waves triggers a change in the cellular membrane potential that influences cell length by means of contraction and elongation (4–7). This so-called electromotility generates mechanical energy, and the resulting increase in the amplitude of the vibration patterns in the organ of Corti can enhance hearing sensitivity by >100-fold (by 40 dB) (refs 8 and 9, but see ref. 10). The membrane motor protein that drives the somatic amplification of OHCs was recently identified and named prestin (3, 11), a member of the *SLC26* superfamily of anion transporters that is encoded by the gene *Prestin*. The prestin protein comprises 10–12 transmembrane domains linked by intra- and extracellular loops and flanked by cytoplasmic N and C termini (12). Studies of *Prestin*-knockout mice (8, 13) and humans with nonsyndromic deafness (ref. 14, but see ref. 15) have confirmed the importance of *Prestin* for cochlea function and hearing. Yet despite its pivotal role in mammalian auditory amplification, orthologues of *Prestin* have been sequenced in very few species. A phylogeny of *SLC26* genes showed positive selection during the evolution of the *Prestin* gene on the branch leading to the mammals, but it suggested strong purifying selection among the four placental mammal species surveyed (16). This observation suggests that the origin of prestin was a key innovation during the evolution of auditory sensitivity in mammals, and that *Prestin* gene sequence has been largely conserved during the adaptive radiation of mammals.

Among all mammals, sensitivity to the highest frequencies occurs in echolocating cetaceans and bats (17), which use sound for orientation and often for the detection, localization, and classification of prey (18, 19). The processing of echolocation signals begins at the hair cells in the organ of Corti, continues along the auditory nerve, and terminates in the auditory cortex in the brain (1). Prestin seems to be of major importance for hearing high frequencies and for selective hearing, and both of these processes are vital for echolocation. Bats, in particular, show a tremendous diversity in signal design, with calls being shaped not only by phylogeny but also by perceptual constraints imposed by their habitat (20, 21). Bat echolocation calls range in dominant frequency from 11 kHz to 212 kHz (22), although most species emit ultrasonic calls dominated by frequencies between 20 and 60 kHz (23). Recent molecular phylogenies have placed important new perspectives on the evolution of bat echolocation (24, 25). Contrary to earlier views that grouped all bats that produce and transmit echolocation calls in the larynx (i.e., "laryngeal echolocators"), a wealth of recent molecular evidence has shown that laryngeal echolocators are paraphyletic. The resulting new arrangement, which we term the "species tree," indicates that bats are classified as comprising two major clades, the Yangochiroptera and Yinpterochiroptera, the latter of which includes some laryngeal echolocators and the nonecholocating Old World fruit bats (25, 26). Thus laryngeal echolocation and associated high-frequency hearing has either evolved at least twice during bat evolution or has been lost in fruit bats. These competing scenarios remain contentious and unresolved; whereas a synthesis of molecular and fossil data supports a loss (24, 25), some workers favor multiple origins (27), and others still dispute the paraphyly of echolocating bats (28).

Echolocation calls show both similarities and differences in structure and function among bats in the two main clades. Most members of the Yangochiroptera use relatively brief (<20 ms) signals, the faint echoes of which are processed in the time window before the next call is emitted (e.g., see ref. 29). In contrast, horseshoe bats (Rhinolophidae) and leaf-nosed bats (Hipposideridae) within the Yinpterochiroptera emit relatively long calls dominated by a constant-frequency (CF) component, which are adapted to detect and classify the wing beats of insects (30). These bats are characterized by exceptional frequency selectivity, with enhanced sensitivity to the frequency they emit while resting and reduced sensitivity to frequencies around this (31, 32). This heightened tuning arises from an overrepresentation of the narrow frequency band (auditory fovea) in the cochlea (33) and specializations in the auditory centers in the brain (31). By lowering their

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call frequency in relation to flight speed, these bats compensate for Doppler shifts induced by their motion (34) and thus ensure that the echoes of their calls always return at the frequency of the acoustic fovea. The acoustic fovea appears to be more sharply tuned in horseshoe bats than in leaf-nosed bats (33).

The convergence of call types (and of echolocation itself) makes bats excellent subjects for studying genes associated with sensory performance. We previously showed that the *FoxP2*, a gene implicated in orofacial coordination, has undergone accelerated evolution in echolocating bats compared with all other vertebrates (35). Yet given the auditory specializations of bats, including their use of high frequencies, extreme auditory sensitivity, call diversity, and specialized audiograms, there are especially good *a priori* reasons to suspect that key genes underpinning the evolution and development of echolocation are expressed in the auditory system, and, in particular, the cochlea. To address this possibility, we studied the phylogenetic history and molecular evolution of *Prestin* in bats and other mammals, given that *Prestin* is fundamental to frequency sensitivity and selectivity in the mammalian auditory system.

Results

Our phylogenetic trees based on the *Prestin* coding sequence showed a clade that grouped together members of the superorder Euarchontoglires (rodents, rabbits, and humans) and a separate clade that grouped members of the superorder Laurasiatheria (carnivores, artiodactyls, perrisodactyls, and bats). However, arrangements within the bat clade did not conform to expectations on the basis of multigene phylogenies. Both the maximum-likelihood (ML) and Bayesian analyses recovered a tree (which we refer to as the putative gene tree) in which all laryngeal echolocators group together in a strongly supported clade [bootstrap values 73–98% and Bayesian posterior probability (BPP) of 99%], to the exclusion of nonlaryngeal echolocating fruit bats (Fig. 1). We assessed the relative confidence of the putative gene tree vs. the species tree by using the approximately unbiased test. We found that the putative gene tree, in which we forced monophyly of the laryngeal echolocators, was not significantly worse than the species tree with Yinpterochiroptera monophyly ($P = 0.848$ and $P = 0.168$, respectively) and was identical to the unconstrained tree ($P = 0.848$). Therefore, there was no greater support for the species tree over the observed putative *Prestin* tree, or vice versa. The results of both the split-network and spectral analyses also suggest a mixed phylogenetic signal, with considerable support for both the putative gene tree and species tree topologies [see supporting information (SI) Fig. S1]. Our reconciled tree analysis found that a single gene duplication in the ancestor of the Chiroptera, followed by independent losses in ancestral branches of the Yangochiroptera, fruit bats, and laryngeal echolocating Yinpterochiroptera would be needed to explain the evolution of the gene tree in terms of birth and death of gene copies. It is therefore unlikely that the observed *Prestin* tree (Fig. 1) arose as a consequence of gene duplication events.

Repeated analyses of phylogenetic reconstruction based on nucleotides corresponding to variable amino acid sites only (414 bp), thus reflecting areas of nonsynonymous change, also recovered the putative gene tree topology with strong support for the clade of laryngeal echolocators (62% ML bootstrap and 97% BPP). Conversely, analyses of the remaining nucleotides (1,800 bp) recovered the species tree, albeit with reduced support for the Yinpterochiroptera clade (<50% ML bootstrap, 47% BPP). Therefore, the monophyly of laryngeal echolocators is supported when only those parts of the gene that lead to amino acid changes are analyzed, but this arrangement is lost and laryngeal echolocators become paraphyletic (as suggested by recent molecular phylogenies) when areas of the gene that do not result in amino acid changes are analyzed.

Sliding window analyses were used to highlight where rates of nonsynonymous substitution (d_N) exceeded rates of synonymous substitution (d_S) along specific branches, and hence to identity

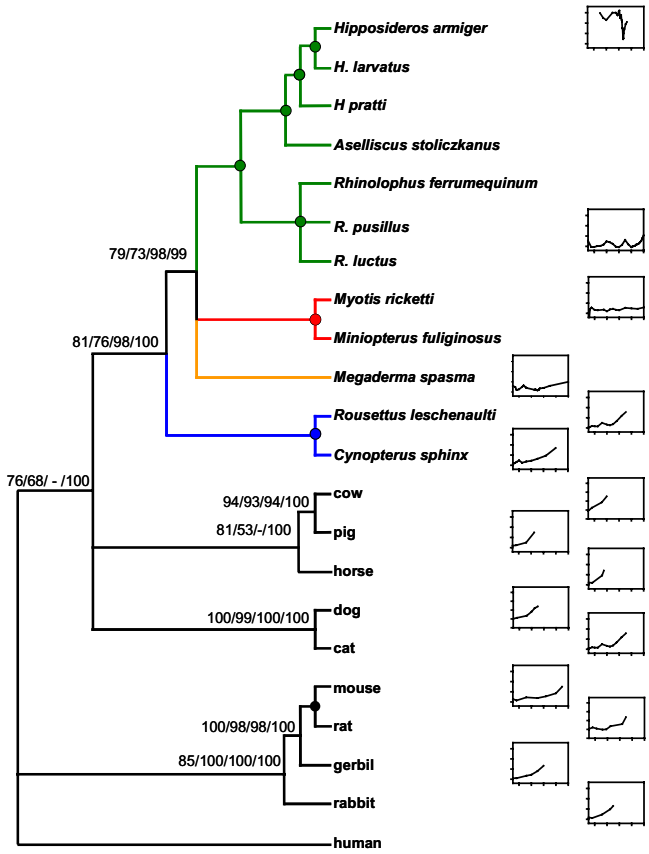

Fig. 1. Maximum-likelihood putative gene tree based on the complete *Prestin* coding sequence of 22 mammals. Values indicate statistical support (maximum likelihood, maximum parsimony, neighbor-joining, and posterior probability, respectively). A filled circle indicates 100% support across methods and a negative sign (−) indicates lack of support for a specific method. Audiograms are given on the basis of published data (for references see *SI Materials and Methods*). For each plot, the *x*-axis ranges from 10 to 180 kHz, with ticks at 50, 100, 150, and 200, and the *y*-axis ranges from −20 to 100 dB with increments of 20 dB. The Yinpterochiroptera comprise the CF bats (green), *Megaderma* (orange), and the Old World fruit bats (blue), and both Yangochiroptera species are red.

positive selection. Higher values of d_N/d_S were found along the branch ancestral to CF bats that use Doppler-shift compensation (DSC). Moreover, d_N/d_S values were relatively higher for exposed domains of the protein (extracellular loops and termini) than for transmembrane regions in all three branches (Fig. 2). Similar results were also obtained when this analysis was repeated using the putative gene tree topology (data not shown). After removal of the 10 putative transmembrane domains, we repeated the phylogenetic analysis on the basis of an alignment (1,539 bp) of concatenated loops and termini. This analysis also showed support for the clade of laryngeal echolocators (59% ML and 98% BPP), whereas the transmembrane domain alignment (681 bp) again recovered the species tree with limited support for the Yinpterochiroptera (<50% ML and 64% BPP). Site-wise likelihood values showed that sites supporting the species tree were concentrated in transmembrane and α-helix domains, whereas the putative gene tree topology was supported by the cytoplasmic and extracellular loops, and, more specifically, the coil domains and the sulfate transporters and antisigma factor antagonists (STAS) domain (see ref. 12) (Table 1 and Fig. S2). Again, these results suggest that differential support for the topology of the putative gene tree *vs*. the species tree arises from changes in key areas of the protein that are of functional importance. Our detailed comparisons of *Prestin* isoforms obtained

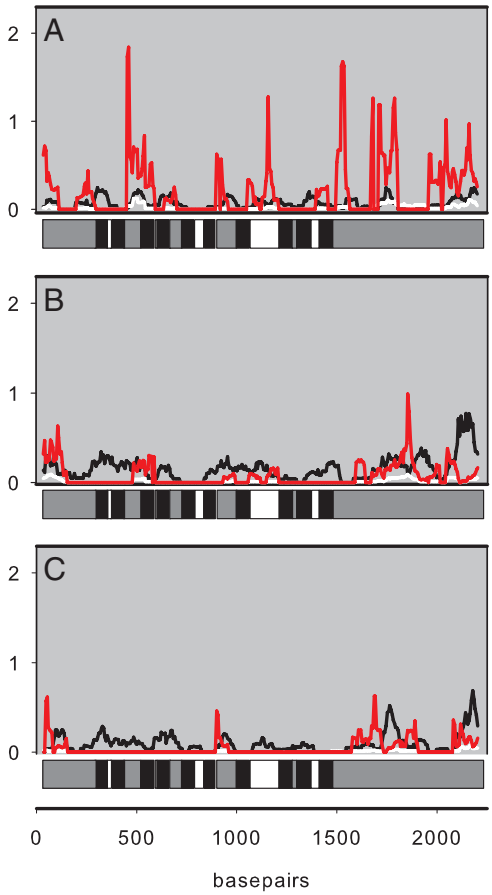

Fig. 2. Sliding windows of Nei–Gojobori estimates of d_S (black), d_N (white), and d_N/d_S (red) along the ancestral branches of CF bats (*A*), Yangochiroptera (*B*), and Old World fruit bats (*C*). Each plot is compared to a gene schematic showing the transmembrane (black), extracellular (white), and intracellular (gray) domains.

from cochlea and brain tissue also revealed that such functional regions were preserved in nearly all cases.

A free ratio test in which d_N/d_S was allowed to vary among branches showed a significantly better fit to the data than a model where d_N/d_S was fixed across the tree (one-ratio model) [log-likelihood ratio test (LRT) = 156.3, df = 39, $P < 0.001$]. The free ratio test also revealed that the values of d_N/d_S on the CF and Yangochiroptera ancestral branches (0.66 and 0.26, respectively) were an order of magnitude greater than the nonbat branches (see Fig. 3). This result, which is consistent with bursts of positive selection along these branches, was also obtained when the analysis was repeated with the putative gene tree topology.

Table 1. Relative support for gene tree and species tree topologies

Domain	Length of domain	Sum of log-likelihood difference	Mean of log-likelihood difference
N terminus	363	2.1285	0.0059
Transmembrane	750	−0.5306	−0.0007
Extracellular and cytoplasmic loops	474	1.5960	0.0034
α-Helix	186	−1.6305	−0.0088
C terminus	741	6.2428	0.0084
STAS	453	0.9662	0.0021
Charge clusters	33	0.7908	0.0247
Coil regions	120	3.1374	0.0261
All nontransmembrane	1578	9.9674	0.0063

Numbers are the sums and means of site-wise negative log-likelihood scores for the species tree minus the sum of site-wise negative log-likelihood scores for the gene tree, calculated for different domains of the *Prestin* gene. Positive values indicate greater support for the gene tree than the species tree.

Branch-specific tests of positive selection undertaken separately for the ancestral branches of the Old World fruit bats, the Yangochiroptera, and CF bats (indicated in Fig. 3) found evidence of positive selection in the CF branch only (see Table S1). For this branch, the null hypothesis, in which ù was fixed at 1 (neutral evolution) gave a log-likelihood value of −11417.23, whereas the equivalent value for the alternative hypothesis in which the ù could exceed 1 (positive selection) was −11411.51. (LRT = 11.44, df = 1, $P < 0.001$). This finding supports the hypothesis that positive selection on the *Prestin* gene occurred around the time of origin of these CF bats. Bayes empirical-Bayes (BEB) analysis suggested that of 33 amino acid replacements in the CF ancestral branch, 23 sites were identified as positive selection sites at $P > 0.5$, 14 at $P > 0.8$, and 3 at $P > 0.90$. Repeated branch-site tests in which ancestral sequences were reconstructed from the species topology gave similar results, with positive selection detected only in the ancestral branch of the CF bats, and the same amino acid sites identified as under positive selection (data not shown).

Multivariate analysis of protein polymorphism (MAPP) scores of physicochemical impact were estimated for 33 amino acid variants among CF bats and predicted that 20 of these would have had a large functional effect [mean MAPP score = 17.7 ± 2.1 (SE), range 9.2–37.6] and 13 a small effect (4.4 ± 0.4, range 2.0–7.00). Ranks of MAPP scores and BEB values were significantly correlated (Pearson coefficient = 0.43, $P = 0.042$) with two of the three sites with highest BEB values (A585I and A720E) also assigned the highest MAPP scores (37.2 and 37.6).

Discussion

Our phylogenetic reconstruction based on the *Prestin* coding sequence was congruent with the superordinal arrangement of mammals of recent molecular phylogenies, recovering both the Euarchontoglires and Laurasiatheria clades (36, 37). However, arrangements within the bat clade did not conform to expectations on the basis of multigene phylogenies. Instead, phylogenies of bats based on the *Prestin* gene revealed consistent conflict with the established species tree derived from large-scale analyses of both mitochondrial and nuclear genes (24–26). All three methods of phylogenetic reconstruction that we used recovered trees in which the species with laryngeal echolocation formed a monophyletic clade to the exclusion of the nonecholocating Old World fruit bats. This arrangement is particularly intriguing because it resembles the earlier classification of two suborders, Microchiroptera and Megachiroptera, a view that has been largely rejected in recent years in light of overwhelming molecular evidence (25–27, 38).

Conflicts between species and gene trees can arise for several reasons. The possibility that the *Prestin* gene has undergone a past duplication with secondary loss, leading to shared paralogous sequences among echolocators, seems extremely unlikely given the requisite number of independent gene losses. Moreover, a gene duplication event would be expected to result in a single dominant phylogenetic signal in the data with a bimodal distribution of pairwise sequence distances, neither of which were supported by our results. We also found no evidence of gene duplicates from searches of published mammal genome databases, or from amplification using universal primers. Instead, spectral analysis revealed that the data contained two phylogenetic signals, corresponding to the putative gene and species trees, and we were unable to reject either of these as the most likely hypothesis.

An alternative explanation is that the clustering of the echolocating bats is a consequence of accelerated evolution along the fruit bat lineage, resulting from either relaxed or positive selection.

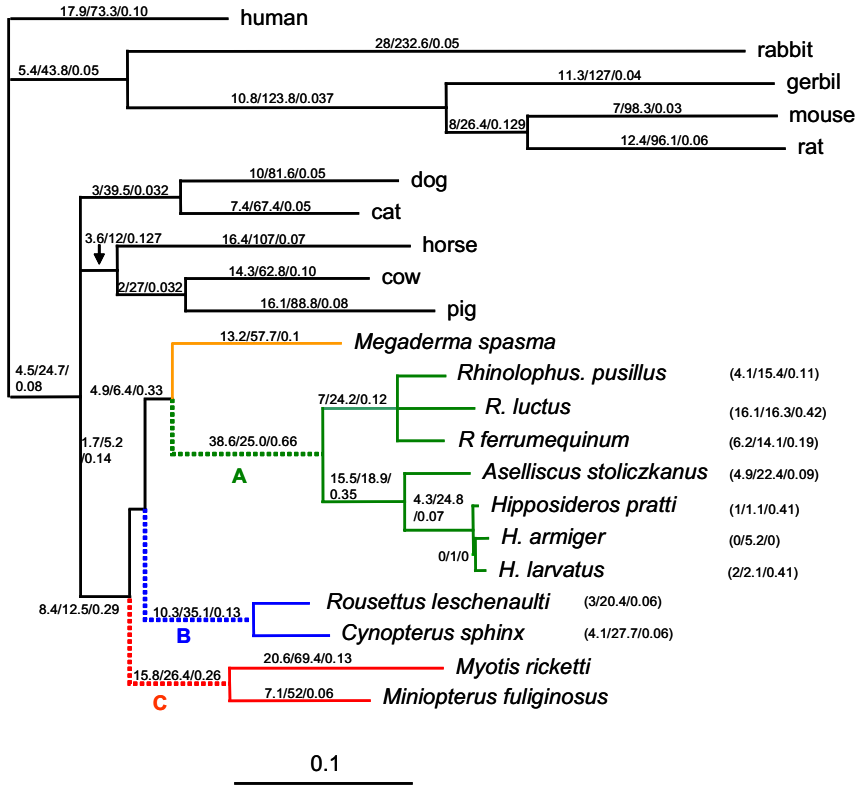

Fig. 3. Species tree topology based on refs. 24–27. Values given on the branches, or in parentheses, are the maximum-likelihood free-ratio estimates of the non-synonymous substitution rate (d_N), the synonymous substitution rate (d_S) and omega (d_N/d_S), respectively. Branch lengths are scaled by the number of nucleotide substitutions per codon. The Yinpterochiroptera comprise the CF bats (green), *Megaderma* (orange), and the Old World fruit bats (blue), and both Yangochiroptera species are red. The dashed lines *A*, *B*, and *C* show the ancestral branches of the three groups studied.

Although relaxation in *Prestin* would resonate with the theory that fruit bats have lost the ability to echolocate, the branch lengths of the fruit bat clade were not unusually long. Indeed, we found evidence for strong purifying selection in the *Prestin* gene along this branch.

Instead, we suggest that the most likely explanation is that the *Prestin* gene tree reflects convergent evolution at the molecular level, associated with high-frequency hearing among echolocating species. Indeed, removal of synonymous changes resulted in a reversion to the species topology; whereas a tree based solely on amino acid differences gave a well-supported putative gene tree. These findings thus appear to add credence to the view that the high-frequency hearing associated with echolocation has evolved more than once. However, it is important to emphasize that echolocation is likely to involve many hundreds of genes, and thus additional evidence is needed to confirm whether echolocation itself has evolved on more than one occasion.

The adaptive evolution of the *Prestin* gene in bats is also suggested by the results of our selection tests. We found evidence of significant positive selection along the ancestral branch of horseshoe and leaf-nosed bats. Therefore, contrary to a previous study that suggested that *Prestin* is under purifying selection within mammals (16), we show that this gene has continued to undergo adaptive evolution since mammals diversified, and, in particular, that this appears to be related to the origin of the specialized form of constant-frequency (CF) echolocation that characterizes these bats.

The distribution of codons under selection and site-wise likelihood values are also highly informative. Over 80% of sites under positive selection occurred in regions thought to be functionally important, including the extracellular loops (4 sites), the transmembrane domains (3 sites), and the N and C termini (17 sites), both of which are known to be important for normal voltage sensing (12, 39). The N terminus is also thought to mediate prestin–prestin interactions (12), while the C terminus controls prestin's cellular localization and function (40). It is interesting that 9 sites found to be under selection are located in the C-terminal region known as the sulfate transporters and STAS domain, which is common to all members of the *SLC26A* family and was previously reported to be a hotspot of adaptive change in *Prestin* associated with the origin of electromotility in mammals (16). Our own results suggest that changes in the STAS domain, mutations in which are known to compromise both protein function and targeting (41), might also be key to the hearing of some or all echolocating bats. An examination of the site-wise likelihood values confirm that specific sites in the STAS domain and neighboring coil region contribute much of the support for the putative gene tree topology, suggesting that *Prestin*'s role in high-frequency hearing is associated with convergence in these regions in both lineages of echolocating bats.

Detection of positive selection in the CF bats, but not along the branch ancestral to the other clade of echolocating bats (Yangochiroptera), either could point to a specific role of prestin in CF echolocation or could reflect the fact that CF bats often emit calls of higher frequencies than other echolocating bats of equivalent body mass (42), in part because most of the signal energy is focused into the second harmonic. Indeed the recovery of the putative gene tree from a nucleotide alignment is based on concatenated intra-/extracellular loops and termini, yet the species tree from the concatenated transmembrane sections does suggest that areas under positive selection in CF bats are also of adaptive significance in the other echolocating bat species. This suggestion is also reflected in the broadly concordant distribution of sites with high BEB posterior probabilities in the echolocating bats from both major clades.

Although the extent to which the cochlear amplifier can tune to high frequencies has been questioned (43, 44), studies on guinea pigs have shown that isolated outer hair cells (OHCs) can react to frequencies of up to 100 kHz at 20°C (45), and that these frequencies induce correspondingly rapid basilar membrane oscillations *in vivo* (46). There is also mounting evidence to show that, in addition to somatic electromotility, cochlear amplification is augmented by rapid force generation by the stereocilia bundles themselves (47), a

process that also appears to involve prestin (48). Moreover, the OHCs of echolocating bats from both the Yinpterochiroptera (49, 50) and Yangochiroptera (51) show structural characteristics that might be adaptations to high-frequency reception, including cell shape, angle, and stiffness. In particular, OHCs and stereocilia are shorter in bats than in all other mammals (52, 53). Thus while there is no evidence to date that bats use a specialized form of cochlear amplifier (54), our results do support a link between *Prestin* and ultrasonic stimuli. Studies are now needed to determine whether the residue changes in *Prestin* reported in this study are responsible for the morphological characteristics of bat outer hair cells and so modify their electromotility.

Alternatively, positive selection in *Prestin* in the CF bat lineage might relate not to the use of high frequencies *per se* but to the unique cochlear adaptations associated with this form of biosonar that enhance selectivity. The auditory fovea that characterizes CF bats contains nerve cells that are extremely sharply tuned to the dominant frequency emitted at rest (33, 55). Cochlea threshold curves based on distortion-product otoacoustic emissions (DPOAEs) show that Q_{10dB} values in CF bats that use DSC can reach 200–600 at the resting frequency, compared with values of ~20 in other mammals (56). This level of tuning at the resting frequency exceeds the capabilities of a normal cochlear amplifier, and it remains unclear how bats that use Doppler-sensitive echolocation are capable of possessing such narrow frequency filters without showing marked deterioration in temporal acuity through the resonance involved (54). Distortion measurements in the cochlea imply that specializations in cochlear mechanics are responsible for the steep variations in threshold and frequency tuning (56). Therefore, in light of our results, and the observation that *Prestin*-knockout mice lose frequency selectivity (57), it is tempting to propose that the adaptive changes in *Prestin* seen in the ancestral CF branch might relate to the evolution of their exquisite frequency selectivity.

To our knowledge, previous work on prestin in bat hearing has been limited to a single preliminary published study in which *Prestin*-like expression was confirmed in the OHCs of adult mustached bats (*Pteronotus parnelli*) (58). Our study, which sequences a hearing gene in bats, strongly implicates a role for importance and associated modification of prestin in the evolution of echolocation. Moreover, our findings support the scenario that high-frequency hearing, which is intimately linked to echolocation, has evolved more than once in bats. Finally, these results from *Prestin* highlight the dangers of using phylogenies based on sequences of putative functional genes for reconstructing the evolutionary history of taxa, where, like many morphological traits, such genes are likely to be subject to convergent evolution.

Materials and Methods

Taxonomic Coverage, Nucleic Acid Amplification, and Sequencing. We sequenced the *Prestin* coding region in 12 bat species with divergent auditory characteristics. From the Yinpterochiroptera, *Cynopterus sphinx* and *Rousettus leschenaulti* (family Pteropodidae) are Old World fruit bats without laryngeal echolocation or associated specialized high-frequency auditory sensitivity and selectivity, although *Rousettus* does echolocate in caves by tongue clicking (59). *Megaderma spasma* (Megadermatidae) produces brief multiharmonic broadband signals. The horseshoe bats *Rhinolophus ferrumequinum*, *R. luctus*, and *R. pusillus* (family Rhinolophidae) and leaf-nosed bats *Hipposideros armiger*, *H. pratti*, *H. larvatus*, and *Aselliscus stoliczkanus* (Hipposideridae) all possess an auditory fovea and emit constant-frequency calls. From the Yangochiroptera, *Myotis ricketti* (Vespertilionidae) emits brief broadband signals (60) and *Miniopterus fuliginosus* (Miniopteridae) emits brief broadband calls ending in a narrowband tail (unpublished data). Accession numbers of all taxa, including outgroups obtained from direct sequencing and from sequence databases, are given in the *SI Materials and Methods*.

We used RT-PCR to amplify genes from total RNA isolated from brain tissue, as previously described (35). See *SI Materials and Methods* for details of primers and protocols used. All PCR products were isolated from a 1% agarose gel and cloned using the pGEM-T-easy vector (Promega). Positive clones were cycle sequenced in both directions by using Big Dye Terminator kits (Applied Biosystems) on an ABI 3730 automated DNA sequencer. To check for the existence of cochlea-specific *Prestin* isoforms, we also repeated this method for cochlea tissue obtained from single individuals of *Hipposideros armiger*, *Rousettus leschenaulti*, *Miniopterus fuliginosus*, and *Myotis ricketti*. Our results based on 220 sequenced clones revealed the existence of several cochlea splice variants; however, the most common transcripts were identical to those obtained from brain tissue (see Table S2).

Phylogenetic Reconstruction. Nucleotide sequences (2,232 bp) of 22 species were aligned in the software ClustalX (61). After alignments of indels by eye with reference to amino acids translated in MEGA 3.1 (62), the alignment comprised 744 amino acid sites, of which 141 amino acids (~19%) were variable in eutherian mammals (Fig. S3). Maximum-likelihood and Bayesian phylogenies were constructed and bootstrap support for particular relationships was estimated. We also estimated phylogenies constraining monophyly of the Yinpterochiroptera (the species tree) and laryngeal echolocating bats (the putative gene tree) and compared support for these topologies by using the approximately unbiased test (63). See *SI Materials and Methods* for details of these analyses. To explore conflicts in the phylogenetic signal, tree reconstruction was repeated separately for synonymous and nonsynonymous sites only and also for different parts of the protein (external loops and termini vs. transmembrane domains), which are considered to differ in functional importance. We determined the number of duplications and losses that would be required to reconcile gene and species topologies by using GENETREE (64) and also assessed whether topological ambiguities arose from multiple phylogenetic signals by using two additional methods. First we undertook a neighbor-net analysis in SPLITSTREE4 (65, 66) to generate a distance-based (uncorrected-p) split network. Second, we converted the sequence alignment to a spectrum of splits and visualized the support and conflict for the major splits by means of a Lento-Plot in SPECTRONET (67). We also identified the relative support for the two topologies for individual sites and domains along the gene by using site-wise likelihood scores.

Molecular Evolution. To characterize variation in the rate of molecular evolution over the *Prestin* gene, we performed sliding-window analyses (window = 20 codons, step = 2 codons) of Nei–Gojobori estimates of synonymous and nonsynonymous substitutions per site (d_S and d_N, respectively) and the d_N/d_S (omega, ù) in SWAAP1.0.2. These analyses were performed on the branches from the common ancestor of all bats to the ancestral nodes of the Old World fruit bats, Yangochiroptera, and CF bats, using ancestral sequences inferred by using the empirical Bayes (EB) method implemented in PAML 3.15 (68). Sliding-window analyses were repeated for both species and putative gene tree topologies. We also tested for positive selection by using PAML. Briefly, we obtained maximum-likelihood estimates of d_S and d_N under a free ratio model in which the d_N/d_S ratio (ù) can vary among branches (69). In addition, given our *a priori* hypothesis that *Prestin* has undergone accelerated evolution associated with the evolution of high-frequency sensitivity and selectivity, we tested for positive selection along the same three ancestral branches as in the sliding-window analysis (labeled in Fig. 3). In each case, we applied Test 2 of the branch-site model (70), which compares the likelihood when the focal branch is fixed at ù = 1 (neutral evolution) to when it can exceed one (ù > 1) and is thus under positive selection. (See ref. 35 for a full description of this approach.) Given ambiguities in the phylogenetic signal, we also repeated the selection tests based on both the species and putative gene tree topologies, and, to confirm the robustness of our results, we repeated the analyses multiple times with different initial ù values. Where tests indicated positive selection, we recorded sites under selection according to high posterior probabilities following Bayes empirical-Bayes (BEB) prediction. To estimate the possible physicochemical impact of amino acid replacements along branches identified as under selection, we used the multivariate analysis of protein polymorphism implemented in MAPP (71). Physicochemical constraints for each site were estimated from an alignment of *Prestin* orthologues of all other mammals and corrected for phylogenetic similarity (see ref. 35).

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